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EDITORIAL

Need of righteous attitudes towards eradication of hepatitis C virus infection in Latin America

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Abstract

Over the last few years, we have expanded our knowledge on numerous facets of the hepatitis C virus (HCV). Beginning with its discovery and viral life cycle, its impact on health, the development of liver disease and currently, effective antiviral treatments. The latter point has become of great interest throughout the developed world, where the possible eradication of HCV through specific strategies to reach all HCV-infected people has been announced. However, this scenario is very different in the countries of Latin America (LA), in which < 2% of infected patients requiring treatment have access to HCV medications. It has been estimated that at least ten million Latin Americans may be infected with HCV. Despite the numbers, viral hepatitis does not seem to be considered a health problem in this region of the world. This reality poses a challenge for politicians and governments of these countries, as well as to the pharmaceutical industry, the medical practitioners, and academics in LA. In this editorial, we state the need for alterations in the attitudes of the integral players involved in this situation. A recognition shift could help to create preventive strategies of viral hepatitis and to advocate for accessibility to new HCV treatments.

Key words: Low-income; Antiviral agents; Public health; Medical societies; Drug industry

Core tip: Global eradication of hepatitis C virus (HCV) infection is causing considerable interest, especially in the developed world. However, the accessibility to the new direct-acting antiviral regimens in low- and middle- income countries is an unmet need. At least ten million HCV-infected persons in Latin America (LA) are confronted by multiple barriers to HCV treatment. Moreover, for the LA countries, paradoxically at it seems, money may not be the only issue. The health authorities, the medical community, and the pharmaceutical industry are the key players that need to alter their attitude towards the delivery of HCV treatments to all patients irrespective of their socio-

economic status.

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INTRODUCTION

Early years of hepatitis C virus infection

The history of the hepatitis C virus (HCV) is a strong example of a bench-to-bedside approach illustrating how basic scientific knowledge is applied to patient care.

Following the discovery of hepatitis B virus (HBV) in 1965[1], and hepatitis A virus (HAV) in 1973[2], a clinical entity known as post-transfusion non-A non-B (NANB) hepatitis became more evident[3]. This was regardless of the banning of commercial blood donations systems, and the implementation of HAV and HBV blood testing in 1975[3,4]. Furthermore in 1989, after years of intensive research[5], a scientific breakthrough discovered another virus consisting of a single positive strand of RNA, the HCV, classified in the *Flaviridae* family[6]. HCV was determined to be the causing agent of the post-transfusion NANB hepatitis[4,5].

In 1992, following the cloning of HCV, an evolving series of sensitive and specific anti-HCV antibody immunoassays were developed which allowed for the mandatory testing of blood collections in the United States[3]. Subsequently, third-generation immunoassays and RNA-HCV nucleic acid amplification tests improved the diagnostics of HCV for blood transfusion safety[7]. During the 90's, the HCV genotypes and subtypes, as well as their heterogeneous geographic distribution became apparent through basic research and molecular epidemiology[8]. Additionally in the United States, blood transfusions, injection drug use (IDU) and several medical procedures were identified as high-risk factors[3].

During the 90's but before 2011, the first antivirals to be marketed were standard interferon, followed by pegylated-interferon which was later combined with ribavirin[9]. Unfortunately, these antivirals achieved a low-level of sustained viral response (SVR) among patients with HCV genotype 1 compared to other genotypes[10]. However after 2011, the first and second generation direct-acting antivirals based on targeting the specific molecules of the HCV life cycle have proven more efficient regardless of genotype reaching an SVR in more than 90% or 95% of the patients who receive treatment[10].

In recent years, DNA sequencing and bioinformatics of HCV strains worldwide have allowed the evolutionary analysis and molecular tracing of the HCV epidemic across countries, mainly focusing on genotype 1a and 1b[11]. These studies have documented the prevalence of HCV in Japan since 1920, in Europe since 1940 and the United States since 1960[11,12]. Further studies have confirmed that the introduction of HCV in the United States may have occurred even earlier (in the 1950's) and is related to the use of contaminated blood obtained from commercial blood donors and the return of World War Ⅱ soldiers[13]. These reasons, along with the fact that hepatitis C is a stealthy virus causing injury to the liver in a silent manner, people who were born from 1945-1965 are currently in the target population of the "baby boomers" screening campaign for HCV in the United States[14].

Transmission of HCV infection in Latin America

Mexico's geographic location serves as a common pathway of intracontinental migration between the United States and other countries of LA. Thus, HCV could have spread from the United States to this region. However in LA, awareness of an NANB hepatitis did not intensify until the late 1970s and early 1980s[15]. During this period, the United States veterans returning from Vietnam, as well as the migration of Latin Americans to their homeland as retirees or by multiple processes of deportation and migration between countries became a major social-demographic phenomenon.

The first HCV epidemic outbreak was associated with the emergence of HIV in the mid-1980s, at a time where the presence of paid blood donors was a common situation[16]. This parenteral route of transmission may have been the primary source of dispersion in Central and South America since IDU, during those decades, was very rare in LA[17]. Furthermore, even though in the United States mandatory screening of blood-borne viruses had been implemented in 1992, it was not fully established in Mexico and LA until 1996[18,19]. This information indicates that even before 2000, blood donations contaminated with HCV were still a relevant risk factor[20]. Moreover, an additional route of HCV infection in the LA may have been the indiscriminate use of caesarean births and other medical procedures that took place during the same period[21]. Brazil and Mexico[22] are the two countries with the highest rates of cesarean sections within the Americas[23]. This could explain why Mexico has a female-to-male ratio of 2:1 of HCV infection[21].

Although partial reports were stating the frequency of HCV in countries of LA during the 1990s, it was not until after 2000 that HCV-related liver cirrhosis began to rise. In Mexico, this disease entity represents over 12 deaths per 100000 inhabitants and the estimated number of people infected with HCV may range from 1.5 to 2 million[24-26], and at least ten million in LA[26-28]. In this region the route of transmission of HCV may have been similar as it was in Mexico, taking into account that as of 2000 the use of IDU, as well as tattooing and piercing, had increased from that year

forward[17].

HCV TREATMENT IN LATIN AMERICA

HCV infection can only be prevented by evading or eradicating the virus. To achieve eradication, treatment with highly effective drugs to all who are infected is required. Currently with the introduction of the new, but very costly, direct-acting antivirals (DAAs) an enormous feeling of success is felt. This elation may be comprehensible for the developed countries, but it may not be true for many developing countries worldwide[29-32], including those in LA[33-37].

However, there are two sides to this story. On one hand, the feasibility of eradicating HCV is closer than ever due to new treatments[38]; yet, on the other hand, neither the authorities nor the physicians, recognize that many infected patients are the result of an iatrogenic spread of HCV. More than 95% of patients infected with viral hepatitis belong to a lower social class in their respective countries, and they can not afford to pay the current market prices of these new antiviral drugs[39].

Additionally, a significant challenge for the eradication of HCV is that there is more than one genotype of the human virus. There are seven genotypes and multiple subtypes[40], which have infected populations with heterogeneous genetic makeups worldwide. This point indicates that research data on HCV needs to be population-based, especially in LA. Further studies are required to identify the genotypes that circulate[41,42], the main risk factors[17,20,21] and the immunogenetic background of the population[43-45].

To date, less than 2% of the people infected with HCV have been treated with the standard pegylated-interferon/ribavirin therapy. The new DAAs, such as boceprevir, telaprevir, simeprevir, and sofosbuvir have been slowly licensed in a limited number of countries in LA. Moreover, HCV treatments are based on the United States and European guidelines which provide evidence of the SVR obtained in clinical trials carried out in populations other than LA.

Thus, under these circumstances who will have access to treatment in Latin America? A substantial body of literature has documented the multiple barriers to health care in HCV-infected patients[46-49]. Although crucial, they are not within the scope of this editorial. Alternatively, we address several issues regarding the health authorities, the medical community, and the pharmaceutical industry. These are key players involved in the prevention, diagnosis, and treatment of this disease.

KEY PLAYERS

Health authorities

The health officials have not considered viral hepatitis to be a severe health problem, which in turn has manifested ignorance or reluctance towards a state program of detection or treatment of this disease. Consequently, there are a limited number of up-to-date population-based epidemiological studies of viral hepatitis in most LA countries[35,36] sponsored by the government. The few that do exist are accomplished by the attention and personal interest of researchers, rather than the concern of the health authorities. This lack of concern from the health authorities translates into key factors and information being overlooked. Some of these factors include; lack of precision of who and how many people are infected, primary risk factors involved[50], transition in genotypes[17] and co-morbidities (obesity, diabetes, alcoholism, co-infection with HVB and HIV)[51]. Furthermore, these epidemiological studies need to be documented in the population of LA because most clinicians assume that the same SVR achieved in Caucasians will replicate in people from this region.

Medical community

In LA, including Mexico, most medical societies involved in the study of the liver have a limited number of members with a solid scientific career, based on their poor scientific productivity in indexed and high impact factor journals in their dedicated fields[52,53]. These society members are hosted by the pharmaceutical companies to attend international forums. In turn, they are the only speakers that echo their experience at the liver meetings to their fellows but do not contribute with scientific data or share their clinical experience. This circumstance has led to the situation that some members only intend to be their society's President (or Chairperson) without the actual contribution of new knowledge in their field of expertise. Moreover, it is precisely these "leaders of the field" who take part, on multiple occasions, as representatives of the health authority, sometimes playing a dual role as clinician and politician.

Therefore, when the support of the medical community is required to establish health policies that have an impact on vulnerable social groups, the members are left without a voice, and vote, on these issues. Consequently, the decisions are made in an unipersonal manner. Furthermore, if the clinician-politician now has a position within the health institutions or the government, he/she may now have a conflict of interest with his/her private practice.

Unfortunately, the lack of an official standpoint in many educational or health institutions to promote a high standard of scientific and academic quality is a serious weakness. This challenge, paired together with a high level of corruption and preferential treatment that prevails among some government authorities, makes it tough to provide treatment for this virus. This situation has created elitist groups of treating physicians being sponsored by the pharmaceutical industry. These doctors may be knowledgeable, however, in some cases, this information does not

necessarily apply to their respective countries or communities.

Furthermore, many of these physicians focus on his/her private practice and have negligible interest in understanding the reality of the disease in their community or country of origin. A reminder of the Hippocratic Oath is "We as doctors should seek the best benefit for the patients, including those who do not have access to treatment".

Likewise, in the majority of LA countries, there are no specialists in hepatology. Patients living with liver diseases are treated mainly by a gastroenterologist or internist[54]. However, with the introduction of the new effective antivirals, and the possibility of the pharmaceutical industry involvement, medical specialists from different fields now claim the liver-diseased patient. Thus, a specialty or subspecialty in hepatology supported by academic institutions should be established as soon as possible.

Pharmaceutical industry

The pharmaceutical industry has declared a responsibility to eradicate HCV by creating agreements based on the economy of each country for the marketing of the antiviral drugs. In general, the standard procedure for the pricing of these drugs is to achieve a full refund of the R and D investment in the developed countries, intermediate reimbursement in the middle-income countries, and the possibility to offer a generic product at a lower and accessible cost for the low-income countries (defined as a win-win situation).

Unfortunately, there are still some well-known challenges to overcome in many developing countries, such as corruption and slow bureaucracy to introduce these drugs[55]. Additionally, the lack of precise epidemiological information about HCV infection is an ongoing difficulty.

Another obstacle is performing the diagnostic tests. Each patient should be evaluated before treatment because not all patients that are positive for anti-HCV antibodies have detectable viral loads or have a similar grade of liver damage. Thus, a pretreatment algorithm including initial and follow-up viral loads, genotyping and identification of possible resistance mutations, and staging of liver damage (fibrosis/cirrhosis) should be considered. A conservative estimate of the costs of these tests may exceed up to $2000 to $4000 United States dollars before paying at least $10000 to $80000 United States dollars for a three-month period of treatment[56]. If the patient is a potential non-responder or present with advanced liver damage, this cost may rise even higher.

Considering these conditions several options have been proposed, such as the establishment of non-profit societies that aid in the funding of these high costs for the poor. This creates a risky situation, given the lack of transparency and poor attitudes of some key players previously mentioned, there is a chance that these "non-profit" organizations may end up as personal or family businesses, or in the hand of small groups who are in power.

Nevertheless, with the justification of "supporting updated scientific medical education", the pharmaceutical companies focus mainly on the treating physicians (clinicians) by selecting medical "leaders", doctors who have influence among the medical groups or societies, or are representatives of health institutions. Whereas, the few scholars or scientists who are knowledgeable in the field of viral hepatitis are not considered under the argument that they are not clinicians or do not treat patients.

This situation leads to the lost opportunity to be supported by the pharmaceutical industry. Thus, in the absence of proposals and sanitary laws, the pharmaceutical industry is interested in selling their drug; but the academic or research sector in the developing countries do not fall within their scope.

RECOMMENDATIONS

HCV infection imposes a large challenge in the world, and it certainly will be eradicated faster in some regions than others. In low- and medium-income countries of LA the health problem of HCV may not depend entirely on money, other nations with fewer resources are proactively establishing public-private partnerships to lower the cost of the DDAs (*e.g.*, Egypt). Hopefully, these strategies will close the gap between the number of patients who are infected (diagnosed) versus those who are treated.

The advancement of scientific knowledge and its impact on health are correlated with the progressive changes in the attitude and behavior of key players and others responsible. The increase in knowledge should benefit all people irrespective of their socio-economic status. To achieve such impact, and to reach all those who need an efficient antiviral therapy, this change in attitude needs to become a reality.

Where and how to start to face this critical situation in Latin America? One recommendation is that both politicians and authorities must consider viral hepatitis as a health problem in their respective region, and establish support strategies to investigate the burden of HCV infection. It is no longer safe to assume that any health issue can be resolved without recognizing the magnitude of the situation.

In the medical communities, a good start would be to establish a partnership between the academic sector, researchers, and physicians, instead of independently acting on their own. This collaborative shift could strengthen the figure of the MD/PhD in each medical society. This may be achieved if alongside, the academic institutions in LA make every effort towards higher standards of education and professionalism. This would greatly strengthen the quality of the medical associations with academic leaders that contribute with knowledge publishable in indexed

international journals. In countries such as Spain and Brasil, the use of the h-index (Hirsch-index) has been considered to grant membership to their respective National Academy of Medicine.

Another important change would be to educate the younger generations that the purpose of belonging to a medical society is not only to be their president or chairperson. As mentioned above, the ultimate goals are to provide prestige and effectiveness by contributing to new knowledge about their country or city in matters of health. The higher income groups require treatments, however, so does the general population. If we reconsider and always remember the Hippocratic Oath, this will help to reflect and advance the field quickly to achieve these goals.

To conclude, the disclosure of the clinicians (speakers), in regards to their sponsorship by the pharmaceutical industry, should be regulated by law and enforced by an ethical practice. Likewise, academics, scientists, medical practitioners and the pharmaceutical companies should equally engage and commit to solving the problem of HCV infection in this region of the world. Moreover, grants given by the pharmaceutical industry in LA to support research in liver disease would be extremely beneficial. These are recommendations that need to be heavily considered by all key players.

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REVIEW

Epidemiology of hepatitis E virus in Iran

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Abstract

Iran is known as an endemic country for hepatitis E virus (HEV) infection, while there are variations in the epidemiology of HEV infection throughout the country. The available epidemiological studies in different regions of Iran show HEV seroprevalence of 1.1%-14.2% among general population, 4.5% -14.3% among blood donors, 6.1%-22.8% among injecting drug users, 6.3%-28.3% among hemodialysis patients, 1.6%-11.3% among patients infected with other hepatitis viruses, 27.5% among patients with chronic liver disease, 30.8% among kidney transplant recipient patients, and 10%-16.4% among human immunodeficiency virus-infected patients. These variations reflect differences in the status of public health and hygiene, risk factors, and routes of transmission in different regions and groups. Therefore, it is necessary to review the epidemiology of HEV infection to determine the most prevalent risk factors and routes of transmission, and to evaluate the effectiveness of preventive strategies employed in the public health services of the country. Moreover, the other epidemiological aspects of HEV, including the genotypic pattern, extra hepatic manifestations, and incidence of chronic infection need to be investigated among Iranian population to expand the current knowledge on the epidemiology of HEV and to clarify the real burden of HEV infection. Therefore, this review was performed to provide a general overview regarding the epidemiology of HEV in Iran.

Key words: Hepatitis E virus; General population; Blood donors; Injecting drug users; Hemodialysis; Immunocompromised patients; Chronic liver disease; Prevalence; Epidemiology; Iran

Core tip: Iran is considered as an endemic country for hepatitis E virus (HEV) infection, while there are variations in the epidemiology and prevalence of hepatitis E throughout the country. These variations reflect differences in the life styles, status of public health, risk factors, and routes of transmission in different groups and geographical regions of Iran.

Therefore, this study was conducted to review the epidemiological aspects of HEV infection in Iran.

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INTRODUCTION

Hepatitis E virus (HEV) is the causative agent of hepatitis E infection[1]. This infection is usually asymptomatic or acute self-limiting[1] but might lead to fulminant hepatitis or even long-term chronic infection and cirrhosis with a high rate of mortality due to severe liver failure in high-risk groups, such as pregnant women, organ transplant recipient patients, immunocompromised patients, and those with pre-existing liver problems[1-6]. HEV is predominantly transmitted *via* the fecal-oral route; however, transmission of HEV through blood transfusion, organ transplantation, hemodialysis, placenta and sexual intercourse is also possible[1,7,8]. Among these, sexual transmission of HEV is less common and has mostly been reported in homosexual males[7,9].

HEV is a small spherical virus with a positive-sense RNA genome and an icosahedral non-enveloped capsid[5,10-12]. The viral genome contains three partially overlapping open reading frames[5,13,14]. HEV has been classified into the family *Hepeviridae*, the genus *Orthohepevirus*, and the species *Orthohepevirus A*[1,15]. There are four genotypes of HEV capable of causing human infection with different epidemiological features[3,16]. These genotypes have further been subdivided into 24 subgenotypes[5,17,18]. Genotypes 1 and 2 are only recognized in human beings, while genotypes 3 and 4 are found in domestic and wild animals as well[7,19-21]. Despite this genetic heterogeneity, only one serotype has been recognized so far[1,5,22].

HEV genotypes differ in their severity, pathogenecity, mortality rates, mode of transmission, age distribution, and geographical distribution[1,7-9,23]. HEV genotype 1 is frequently found in North Africa and Asia[9]. HEV genotype 2 is more common in West Africa and Mexico[7,9]. HEV genotype 3 is considered to have a worldwide distribution and is more prevalent in several European and American countries, as well as Japan, China, Australia, and New Zealand[7,9,10]. HEV genotype 4 has been reported in Asian countries and more recently in Central Europe[5,9,23]. Genotypes 3 and 4 appear to be less virulent than genotypes 1 and 2[1,24]. HEV genotype 1 is associated with the most cases of fulminant hepatitis and high mortality during pregnancy[8,10]. While HEV genotype 3 is the cause of almost all cases of chronic HEV infections worldwide[5,7,8]. Infection with HEV genotype 4 seems to be asymptomatic and mostly remains undiagnosed[6,9].

Hepatitis E is usually an asymptomatic or acute self-limited infection and only requires supportive care. However, when fulminant or chronic hepatitis arises, therapeutic intervention is an obligation[9,10]. Reduction of immunosuppressive therapy is considered as the first-line therapy in immunocompromised patients with chronic HEV infection. Nevertheless, at the same time, it can increase the risk of graft rejection[3,5,9,25-27]. In such conditions, organ transplant recipient patients benefit most from antiviral therapy, including pegylated interferon (peg-IFN) monotherapy or combination therapy with ribavirin and peg-IFN[3,10,25].

These antiviral agents are associated with severe side effects. IFN-therapy may result in acute graft rejection[3,5,8,25]. Ribavirin administration induces severe hemolytic anemia; and when the dose of ribavirin is reduced, the viral clearance is not achieved[8]. Therefore, the combination of ribavirin with a reduction in the doses of immunosuppressive drugs has been found to be the most promising treatment option[5,10,25]. In addition, these antiviral drugs should be administered with caution during pregnancy due to their teratogenicity[10,27,28]. Early delivery of fetus or termination of pregnancy should be considered as another option to save mothers' lives[28,29]. Administration of the antiretroviral drugs in human immunodeficiency virus (HIV)-positive patients can result in an increase in the proportion of T helper cells and subsequently clearance of HEV infection[25].

Considering the side effects and limitations of the currently available treatment regimens as well as the absence of a specific antiviral treatment for HEV infection, preventive measures and vaccination against HEV infection are the most desirable approaches for controlling HEV infection[25,30]. HEV vaccines using the truncated forms of capsid protein have been evaluated in human clinical trials, and one of them, HEV 239 vaccine, has been approved in china in 2011. Although this vaccine has shown promising results, it is not commercially available worldwide[5,20,27,31]. Therefore, preventive measures are still known to be the best option. Providing clean drinking water supplies, improving the hygienic infrastructure and sanitary status, washing hands and vegetables properly, boiling drinking water, and avoiding consumption of undercooked foods and unpeeled fruits are some of these preventive measures[1,5,11,23,31,32]. In addition, proper chlorination of water supplies, sanitary preparation of food, and public awareness regarding the possible routes of HEV transmission are essential to reduce the risk of exposure to HEV in the community[1,11,23].

HEV has infected one-third of the world's population[13,17]. In addition, 20 million new cases and 3.3

million acute cases of HEV infection occur globally each year[3,33], and HEV-related hepatic failure is responsible for approximately 56600 deaths per year[33,34]. The mortality rate of HEV infection is 1%-2% in the general population[17], but it may rise to 10%-25% in pregnant women[7,20] and over 75% in individuals with pre-existing liver problems[20].

HEV is a considerable global health concern. Although HEV infection was traditionally believed to be limited to developing countries, currently it is known that this infection has a worldwide distribution with different epidemiological patterns[9]. In developing countries where the infection is endemic, acute outbreaks or large epidemics of hepatitis E occur due to contamination of water supplies mainly at the time of heavy rainfall or following floods. These outbreaks are largely due to genotypes 1 and 2 and more frequently affect young adult males[1,3,5,8,9,18]. Whereas in developed countries, HEV infection is non-endemic and mostly occurs as sporadic locally-acquired disease due to consumption of contaminated food supplies. The infection is due to genotypes 3 and 4 and predominantly found among middle-aged elderly males throughout the year[1,3,5,9,18].

Apart from variations in the global epidemiological patterns of HEV, there is a wide range of variation in the prevalence and epidemiology of HEV infection within a country[5,16,35,36]. These variations reflect differences in the lifestyle, status of public health, risk factors, and routes of transmission in different regions and groups[35-37]. It is therefore necessary to review the epidemiology of HEV infection to determine the most prevalent risk factors and routes of transmission and to evaluate the effectiveness of prevention strategies employed in the public health services of a country. These epidemiological studies are not only essential for improving the current strategies to minimize the risk of acquiring HEV infection in the society but also clarify the real burden of HEV infection.

This study has the objective to review the epidemiology of hepatitis E in Iran, a vast country located in the Middle East, with an extension of about 1700000 km^2 and an estimated population of 70 million inhabitants in different provinces with various ethnicities[38].

HEV IN GENERAL POPULATION

With an overall prevalence rate of more than 5% in the general population, Iran is considered as an endemic country for HEV infection[36,39]. However, the seroprevalence of hepatitis E in the general population varies considerably in different parts of the country, ranging from 0.0% to 0.9% for anti-HEV IgM and 1.1% to 14.2% for anti-HEV IgG and the total HEV antibodies in different studies[35,39-46] (Table 1[35,39-46]). Differences in the lifestyles, risk factors, levels of exposure, the geographic regions, study population, study periods, sample sizes, time of sampling, and the diagnostic accuracy of kits used to determine anti-HEV antibodies in various studies can explain these variations[35-37]. However, the role of public health services, hygienic conditions, socioeconomic status, and environmental factors should not be dismissed[36,37,42].

The predominant mode of transmission in Iran is fecal-oral route, especially feces-contaminated drinking water; however, the other routes of transmission might also have a minor role in the spread of HEV but with undetermined importance[35,43,47]. Food-borne transmission of zoonotic origin is unlikely, since wild animal hunting and swine farming are prohibited in Iran[35]. Person-to-person transmission is most likely rare in Iran, since no association between the household size and seroprevalence rate of HEV has been reported[42]. Overall, the importance of the other probable routes of transmission in spread of HEV infection in the society still requires to be determined.

Despite the epidemiological pattern in developing countries, where HEV most often affects young adults[1,5,9], the seroprevalence rate in Iran increases with age due to cumulative exposure to HEV over time with the highest prevalence rate among middle-aged and elderly individuals aged over 50 years[35,36,40,41,45]. Another possible reason is improvement in public hygiene, sanitation, sewage disposal and drinking water supply systems, which has resulted in decreased prevalence of HEV infection in young population of the country over time[36,47].

Except two reports[44,45], in the majority of studies, the seroprevalence of HEV was higher in females compared to males. However, none of these differences were significant[35,36,39-42]. In rural areas, inhabitants were more likely to be positive for HEV serological markers compared to individuals living in urban areas[39,43]. Appropriate access to the public health services, sewage disposal systems, and safe water supplies in cities can explain these differences in the seroprevalence rates regarding the place of residence[35,37,43]. Taken together, in studies from Iran, socioeconomic status, level of sanitation, population density, age, level of education, and place of residency were found to be risk factors for acquiring HEV infection in the society[35,42,43,48].

In one study from Iran, the prevalence of anti-HEV IgG antibody in the general population was as high as 46.1% in South-West of the country[36]. The reason of this high endemicity is most likely Karun River as the drinking water source of the inhabitants, where the city sewage is also discharged in. It is also worthy to note that the participants in the mentioned study were all adults, mostly middle-aged and elderly adults[36]. Iran is located in the Middle East between HEV high endemic countries on the eastern and western borders. This considerable geographic location has affected epidemiological pattern of HEV infection in Iran[35,47].

A few occasional waterborne outbreaks of hepatitis E have also occurred in Iran[47]. The first documented outbreak was reported in Kermanshah city, West of

Table 1 Seroprevalence of hepatitis E virus in different population groups in Iran

Study population	City or province	Location	Year of study	No. of participants	Age, mean ± SD (age group), yr	No. of positive cases	HEV seroprevalence	HEV diagnosis	Manufacturer of Serology kits	Ref.
General population	Nahavand	West	2003	1824	34.7 ± 19.5 (6 to > 70)	170	9.3%	Anti-HEV IgG	DIA.PRO, Italy	Taremi *et al*[42]
General population	Sari, Mazandaran	North	2003	1080	2 to 25	25	2.3%	Anti-HEV IgG	DIA.PRO, Italy	Saffar *et al*[43]
General population	Tehran and Golestan	North-Center North-East	2006	1423	37.9 ± 13.4 (18 to 65)	105	7.4%	Anti-HEV total antibodies	DIA.PRO, Italy	Sepanlou *et al*[39]
General population	Shiraz	South	2011-2012	1030	< 1 to 95	138	13.4%	Anti-HEV total antibodies	DIA.PRO, Italy	Asaei *et al*[40]
						9	0.9%	Anti-HEV IgM		
General population	Mashhad	North-East	2009	1582	29.06 ± 18.513 (1 to 90)	225	14.2%	Anti-HEV total antibodies	DIA.PRO, Italy	Ahmadi Ghezeldasht *et al*[35]
General population	Isfahan	Center	2005	816	6 to > 50		3.8%	Anti-HEV total antibodies	DIA.PRO, Italy	Ataei *et al*[41]
General population	Tehran	North-Center	2006-2007	551	41.28 ± 16.96 (1 to 83)	51	9.3%	Anti-HEV IgG	DIA.PRO, Italy	Mohebbi *et al*[44]
General population	Ahvaz	South-West	2014	510	45.89 ± 14.63 (18 to 81)	235	46.1%	Anti-HEV IgG	DIA.PRO, Italy	Farshadpour *et al*[36]
						7	1.4%	Anti-HEV IgM		
General population	Khorramabad	West	2009	400	36 (> 20)	31	7.8%	Anti-HEV total antibodies	(ND)	Raoofi *et al*[45]
Soldier	Tehran	North-Center	2006	800	19 ± 1.2 (17 to 23)	9	1.1%	Anti-HEV IgG	DIA.PRO, Italy	Ghorbani *et al*[46]
							0.0%	Anti-HEV IgM		
Blood donor	Khuzestan	South-West	2005	400	33.3 (18 to 60)	46	11.5%	Anti-HEV IgG	HEV-EIA, Biokit, Spain	Assarehzadegan *et al*[55]
Blood donor	Tehran	North-Center	2003-2004	90	31.8 ± 11	7	7.8%	Anti-HEV total antibodies	DIA.PRO, Italy	Aminiafshar *et al*[54]
Blood donor	Tabriz	North-West	2004	399	31.4 ± 9.8	31	7.8%	Anti-HEV IgG	DIA.PRO, Italy	Taremi *et al*[49]
Blood donor	Markazi	West-Center	2012	530	36.3 ± 11.7 (18 to 71)	76	14.3%	Anti-HEV IgG	DIA.PRO, Italy	Ehteram *et al*[53]
Blood donor	Kerman	South-East	2007-2008	400	20 to 60	31	7.7%	Anti-HEV IgG	DIA.PRO, Italy	Arabzadeh *et al*[52]
Blood donor	Tehran	North-Center	2014	559	38 (18 to > 47)	45	8.1%	Anti-HEV IgG	DIA.PRO, Italy	Hesamizadeh *et al*[50]
Blood donor	Tehran	North-Center	ND	200	20 to 61	9	4.5%	Anti-HEV antibodies	DRG, Diagnostics, Germany	Keyvani *et al*[51]
Drug users (addicts)	Hamadan	West	2011-2012	131 (IDUs)	35.57 ± 8.13 (22 to 70)	8	6.1%	Anti-HEV IgG	DIA.PRO, Italy	Keramat *et al*[58]
				131 (non-IDUs)	31.57 ± 8.19 (20 to 45)	2	1.5%			
Drug users	Ahvaz	South-West	2005-2006	228	34.1 ± 6.1 (18 to 54)	35	15.4%	Anti-HEV IgG	DIA.PRO, Italy	Alavi *et al*[57]
				114 (IDUs)		26	22.8%			
				66 (Inhalant)		6	9.1%			
				48 (Oral opiate)		3	6.2%			
Hemodialysis	Hamadan	West	2010	153	< 20 to > 60	30	19.2%	Anti-HEV IgG	DIA.PRO, Italy	Eini *et al*[63]
Hemodialysis	Tabriz	North-West	2004	324	53.5 ± 15.1	24	7.4%	Anti-HEV IgG	DIA.PRO, Italy	Taremi *et al*[64]
Hemodialysis	Jahrom	South	2007	43	59.3 ± 14.4	3	7.0%	Anti-HEV IgG	DIA.PRO, Italy	Pourahmad *et al*[65]
Hemodialysis	Zanjan	West	2011	93	57.0 ± 18.5 (16 to 88)	25	26.9%	Anti-HEV total antibodies	DIA.PRO, Italy	Mobaien *et al*[66]
Hemodialysis	Jahrom and Shiraz	South	2010	80	55.69 ± 14.70 (26 to 80)	5	6.3%	Anti-HEV IgG	DIA.PRO, Italy	Zekavat *et al*[67]
Hemodialysis	Ahvaz	South-West	ND	47	55.27 ± 8.1	5	10.6%	Anti-HEV IgG	DIA.PRO, Italy	Beladi Mousavi *et al*[68]
Hemodialysis	Isfahan	Center	2012	274	59.9 ± 16.4 (21 to 80)	78	28.3%	Anti-HEV IgG	DIA.PRO, Italy	Alavian *et al*[69]
HCV-infected patients	Tehran	North-Center	ND	100	20 to 61	7	7%	Anti-HEV antibodies	DRG, Diagnostics, Germany	Keyvani *et al*[51]
HBV-infected patients	Tehran	North-Center	ND	150	20 to 61	17	11.3%	Anti-HEV antibodies	DRG, Diagnostics, Germany	Keyvani *et al*[51]
Thalassemia patients with chronic hepatitis C	Iran	Iran	2009-2010	64	25.08 ± 6.46 (12 to 76)	1	1.6%	Anti-HEV IgG	DIA.PRO, Italy	Karimi Elizee *et al*[56]

Hemophilia patients with chronic hepatitis C	Iran	Iran	2009-2010	155	30.63 ± 11.51 (12 to 76)	5	3.2%	Anti-HEV IgG	DIA.PRO, Italy	Karimi Elizee *et al*[56]
GB Virus C positive hemodialysis patients	Gorgan	North-East	2012	22	54.32 ± 12.56	0	0.0%	total anti-HEV	DIA.PRO, Italy	Kelishadi *et al*[77]
patients with chronic liver disease	Azerbaijan	North-West	2005-2006	200	48.26 ± 18.19 (10 to 87)	55	27.5%	Anti-HEV IgG	DIA.PRO, Italy	Somi *et al*[78]
HIV-infected patients	Tehran	North-Center	2012	100	38	10	10%	Anti-HEV IgG	DIA.PRO, Italy	Ramezani *et al*[79]
						0	0.0%	Anti-HEV IgM		
						0	0.0%	HEV RNA		
HIV-infected patients	Shiraz	South	2013	158	39.1 ± 8	26	16.4%	Anti-HEV total antibodies	DIA.PRO, Italy	Joulaei *et al*[80]
Kidney transplant recipient patients	Urmia	North-West	1991-2010	91	35.4 ± 14.5 (6 to 65)	28	30.8%	Anti-HEV IgG	DIA.PRO, Italy	Rostamzadeh Khameneh *et al*[81]

ND: Not defined; HEV: Hepatitis E virus.

Iran, in 1991. At the same time, a suspected outbreak was reported in Isfahan province, during which over 100 inhabitants were infected in Fereidon-Shahr. Another outbreak occurred in Lordegan, Southwest of Iran, in 1999 and affected 154 people[45,47]. The history of these outbreaks clearly implies that HEV infection is not new to Iran, and probability of future outbreaks in the country should be considered and preventive strategies for controlling transmission of HEV infection should be provided.

The prevalence rate of HEV infection in the general population is likely underestimated due to the lack of adequate population-based studies, asymptomatic nature of HEV infection, and the fact that hepatitis E is not a reportable infection in the public health system of Iran. In addition, the incidence and case fatality rate of hepatitis E in the general population of Iran are unclear and need further investigation.

HEV IN BLOOD DONORS

Even though HEV infection is an old enterically transmitted disease, it is also considered an emerging transfusion-transmitted infection[4,7]. Since only recently, HEV has been recognized as a threat to blood safety[4,6]. The possibility of HEV transmission through blood transfusion dates back to 2002 and 2004, when two molecular studies in Japan introduced HEV as a transfusion transmissible virus[6,7,18]. Since then, several studies from Japan, the United Kingdom, France, and Saudi Arabia have confirmed HEV transmission through blood transfusion[6,7,18,23].

These studies have reported high prevalence of anti-HEV antibodies, and viral RNA among blood donors[6,23]. Donor-recipient linked studies have also confirmed transmission of HEV to blood transfusion recipients[6,7]. In addition, higher incidence of hepatitis E in multi-transfused individuals compared to controls suggests this transmission[7,18,23]. Moreover, the high rate of asymptomatic or undiagnosed infection among blood donors increases the risk of HEV transmission[7,18,23]. Fortunately, these unnoticeable infections are preventable through screening of blood donations for HEV infection. Screening methods are based on the detection of anti-HEV antibodies and HEV RNA in serum or plasma samples of blood donors[1,4,7,8,16,18].

The presence of elevated liver enzymes and HEV RNA in blood is short lived, and becomes normalized or undetectable approximately 6 weeks and 3 weeks after the onset of clinical illness, respectively[5,7,16]. In some instances, viremia may persist for a longer period, especially in children after acute hepatitis E[4,7]. In addition, HEV RNA has been detected up to 3 years in immunocompromized patients, especially those with renal transplantation[4]. IgM increases during the acute phase of infection and becomes undetectable after 3-8 mo[16]. While IgG appears after the increase of IgM level and persists for years with unknown duration[5,8,16]. Therefore, anti-HEV IgG positive samples in the absence of IgM and HEV RNA are defined as past HEV infection. While a positive anti-HEV IgM test can be indicative of current

infection if HEV RNA is detected[1,16]. Some patients in viremic phase of infection do not show anti-HEV IgM responses[49]. Majority of risks are due to the presence of HEV RNA in blood of apparently healthy donors with normal levels of liver enzymes and negative anti-HEV IgM, which is indicative of asymptomatic viremia[7,18]. In these instances, blood transfusion is capable of trasmitting HEV infection despite negative serological markers. Therefore, HEV is a potential threat to blood safety[7,18].

Since 2005, Japan has implemented HEV RNA testing of all donors along with screening for elevated liver enzymes levels[7]. While some other countries perform selective HEV screening for high-risk recipients[8]. The necessity to screen all blood donations or at least a part of them for HEV infection needs to be considered in Iran.

Studies on the seroprevalence of HEV in blood donors are limited in Iran and mainly have been conducted in main cities, while the seroprevalence varies from 4.5% to 14.3% in these studies[49-55] (Table 1[49-55]). Despite this high prevalence, screening of blood donors for HEV is not performed in the blood banks of Iran until more evidence becomes available regarding the potential threat of HEV to blood safety[49,53]. In addition, the risk of incidence and transmission of hepatitis E by blood transfusion in Iran is unknown. Since the studies in Iran have only reported the rate of seropositivity, which is incapable of estimating the rate of viremic blood donors[49,53]. Therefore, additional studies to investigate the possibility of HEV transmission through blood transfusion seem to be necessary. Even if the risk of transmission through blood transfusion is low, we should not neglect the importance of this infection. Since HEV causes serious consequences in high-risk recipients, who often require blood transfusion. Therefore, access to HEV-free blood and blood products is the highest priority for this group of patients[4,7,18,50].

The seroprevalence of HEV among hemophilia and thalassemia patients in Iran is lower than expected and is in the range of that found in the general population of Iran[56]. The reason of this low prevalence may be that the blood donor population in Iran has mostly been consisted of young individuals, while HEV mostly affects middle-aged or old population in Iran[49,50,54]. This suggests that blood transfusion may not be a risk factor for transmission of HEV infection among hemophilia and thalassemia patients in Iran[56]. Still, more studies are required to confirm this issue.

HEV IN INJECTING DRUG USERS

With having approximately 180000 injecting drug users (IDUs) among Iranian adults aged 15-64 years, Iran is considered one of the countries with the highest numbers of injection drug users in the world[38]. While only two studies have assessed the possible effect of injecting drug use on the seroprevalence of HEV among IDUs in Iran[57,58]. Alavi *et al*[57] reported high seroprevalence of HEV in IDUs (22.8%) compared to inhalant drug users (9.1%) and oral opiate drug users (6.2%) and suggested an association between injection drug abuse and HEV seropositivity in Ahvaz in 2005-2006. While some other studies from France, the United States (US), and Denmark have rejected this association[59-62]. Keramat *et al*[58] reported high prevalence of HEV in IDUs (6.1%) compared to non-IDUs control group (1.5%) and found no relationship between duration of injection and HEV seroprevalence in Hamadan in 2011-2012.

These studies indicated high seroprevalence of HEV infection among IDUs in Iran[57,58], while this seroprevalence was not influenced by the type of substance abused but was associated with the route of administration[57]. According to the results of these studies, IDUs in Iran are at risk of acquiring HEV infection most likely due to exposure to infected blood through sharing syringe[58]. As a result, injection drug use was proposed as a possible route of HEV transmission. However, still more investigations are required to confirm this issue.

HEV IN HEMODIALYSIS PATIENTS

The seroprevalence of hepatitis E among patients on maintenance hemodialysis (HD) varies considerably from 4% to 28.3% in different cities of Iran[63-69] (Table 1[63-69]). The reason of this vast geographic variation in different HD centers is unknown, but it may be due to the different levels of safety strategies in HD units, as well as the public health and prevalence of HEV infection in the community[30,64,69]. In some studies, HEV seroprevalence in HD patients is lower than or in the range of HEV seroprevalence in the general population of Iran[64,65,67], indicating a low risk of exposure to HEV in these areas or maybe a negligible HEV transmission in HD centers. While in some other studies, it is noticeably higher than HEV seroprevalence in the general population, which may be indicative of parenteral transmission of HEV infection[30,63,66,69]. Similar high prevalence of anti-HEV antibodies in HD patients has been reported in Egypt (22.9%)[70], Japan (30%)[71], and Turkey (20.6%)[72]. In contrast, reports from Italy (6.0%)[73], Brazil (6.2%)[74], and Spain (6.3%)[75] have indicated a low seroprevalence of HEV among HD patients.

The seroprevalence of HEV infection among Iranian HD patients was associated with almost no risk factor in most of these studies[63,64,67,68]. While duration of HD was significantly associated with HEV seropositivity in two studies[65,69]. In addition, in one study, 41.7% of HEV seropositive HD patients had a history of blood transfusion[69]. These studies support the nosocomial transmission of HEV infection[30,47,65]. While the others indicate a rare acquisition of HEV infection through hemodialysis[64,67]. Overall, the epidemiology of HEV infection among HD patients in Iran seems to be

a controversial issue due to these variations in the results of so far conducted studies. Therefore, more extensive or comprehensive studies in different geographical regions of Iran are required to resolve these conflicts between the results and to determine the exact epidemiological pattern of HEV infection among HD patients.

Considering the clearance of a significant level of anti-HEV antibodies during the process of dialysis as well as weak antibody responses due to chronic renal disease, a considerable proportion of HEV seropositive HD patients may be reported as seronegative[30,67,69,76-81]. Except one study[65], the serum levels of liver enzymes were normal or low in HEV seropositive patients on maintenance hemodialysis due to the fast reduction of these enzymes to the normal levels[30,67]. Therefore, some HEV-infected HD patients may remain undiagnosed. These HD patients with inapparent HEV infection might be the main source of HEV transmission as a nosocomial infection in HD units[30]. Overall, neither anti-HEV antibodies nor level of liver enzymes can be valid diagnostic markers in case of HEV infection among HD patients[30]. Therefore, serious safety measures and proper screening of HD patients for hepatitis E in HD centers should be considered to prevent transmission of HEV during the process of hemodialysis.

HEV IN PATIENTS INFECTED WITH OTHER HEPATITIS VIRUSES

Viral hepatitis infections are believed to be associated with an increased risk of hepatitis E occurrence, and co-infection or superinfection with HEV will enhance the risk of liver failure[11,17,82,83]. In recent years, several studies have reported high prevalence of hepatitis E among patients with chronic viral hepatitis and supported the possibility of parenteral transmission of HEV[30,82,84], while other studies have demonstrated a low occurrence of these co-infections or superinfections and found no association between HEV and other viral hepatitis[85,86]. This variation in the prevalence of HEV among patients with other viral hepatitis reflects differences in the routes of transmission and distribution of these hepatotropic viruses in different parts of the world. Although HEV is predominantly transmitted *via* the fecal-oral route, the possibility of parenteral transmission has also been reported in endemic countries[82,87].

Currently, only a few reports are available regarding the prevalence of HEV infection among patients with viral hepatitis in Iran. Keyvani *et al*[51] reported high prevalence of anti-HEV antibody in HBV (11.3%) and HCV (7%)-infected patients compared to healthy blood donors (4.5%) in Tehran. In another study by Karimi Elizee *et al*[56], the seroprevalence of HEV among thalassemia and hemophilia patients with chronic hepatitis C was reported to be 1.6% and 3.2%, respectively, which is similar to HEV seroprevalence in Iranian general population. Kelishadi *et al*[77] reported the absence of anti-HEV IgG antibody in GB virus C positive hemodialysis patients in Gorgan. These studies were unable to determine the effect of hepatitis E on the clinical outcomes of the other viral hepatitis. Overall, data concerning dual infection with hepatitis E and the other viral hepatitis in Iran are scarce, and the routes of HEV transmission in this group of patients are unclear. Therefore, further studies are required to determine the association between HEV and other viral hepatitis in Iran.

HEV IN IMMUNOCOMPROMISED AND IMMUNOSUPPRESSED PATIENTS

HEV infection in immunocompromised and immunosuppressed patients may lead to chronic hepatitis E, with an increased risk of developing liver fibrosis and cirrhosis, and subsequently lower survival of the infected patients[9,10,27]. Chronic HEV infection is characterized by the persistent presence of detectable HEV-RNA in serum and stool for more than 6 mo (more than 3 mo in organ transplant recipient patients) along with persistently elevated liver enzymes[3,5,8,9,33]. So far, chronic hepatitis E has been observed in HIV-infected patients, organ transplant recipient patients, and those with hematological malignancies, who receive anticancer chemotherapy[5,10,25-27,88]. However, the possibility of HEV chronicization in other patients with immunosuppressive conditions is currently under investigation, and this chronic infection may identify in more categories of patients in near future[3].

More recently, some cases of chronic HEV infection have also been observed in elderly immunocompetent individuals[8]. While no report of chronic infection has been documented in pregnant women and infants[16,28]. Almost all cases of chronic hepatitis E have been observed following infection with HEV genotype 3[3,5,7,25]. The first case of chronic hepatitis E caused by HEV genotype 4 has recently been identified in a Chinese patient[3,9].

The seroprevalence of hepatitis E among organ transplant recipient patients varies from 2.3% to 43.9% in different studies[5]. While the prevalence of HEV infection based on the detection of viral RNA ranges from 0.9% to 3.5%[5]. This prevalence among transplant recipient patients with elevated liver enzymes is 4.3%-6.5%[5]. The chronicity rate of hepatitis E is approximately 60% in organ transplant recipient patients without therapeutic interventions[25,26,30].

Indeed, progression to chronicity in immunocompromised patients could be mediated by inability to clear the virus after acute infection, which is related to the degree of immunosupression and immunological status of transplant recipient patients at the time of HEV infection as well as the time period between the transplantation and incidence of HEV infection[3,5,10,29].

Therefore, suboptimal HEV-specific cellular immune responses, low lymphocyte and platelet counts, the occurrence of HEV infection immediately after transplantation, and the use of more effective immunosuppressive drugs such as tacrolimus are risk factors for the incidence of chronic hepatitis E in immunocompromised patients following exposure to HEV[1,3,5,26,88]. Even the presence of anti-HEV IgG antibodies prior to re-exposure to HEV cannot exclude the chance of reinfection in transplant recipient patients, and such reinfections may lead to chronic infection[3]. The main route of HEV transmission in immunocompromised patients seems to be fecal-oral, especially *via* consumption of contaminated food[3,5,16]. However, acquisition of HEV infection following blood transfusion and liver transplantation is also possible but seems to be uncommon[3,5,26]. Most patients with chronic hepatitis E are asymptomatic, and the rest show nonspecific symptoms, including fatigue, fever, abdominal pain, asthenia, and very rarely jaundice[5,25]. Chronic hepatitis E can rapidly progress to liver fibrosis, cirrhosis, and subsequently fatal liver failure in immunocompromised patients[3,5,25]. In addition, numerous hepatitis E-associated extrahepatic manifestations, including neurological, hematological, musculoskeletal, renal manifestations, as well as acute pancreatitis, autoimmune thyroiditis, myocarditis, mixed cryoglobulinemia, thrombocytopenia, arthralgia, Henoch-Schonlein purpura, myasthenia gravis, haemolysis, membranous glomerulonephritis associated with immunological disorders and many others have been reported in patients with acute or chronic HEV infection[7,9,25,33,89,90].

Such extrahepatic complications sometimes outshine clinical manifestations of hepatic injury, and the causative agents, hepatitis E, might not be suspected. Therefore, the probability of hepatitis E in extrahepatic manifestations should be considered[33].

Chronic hepatitis E results in graft loss and subsequently retransplantation in organ transplant recipient patients. However, recurrent hepatitis E and subsequently progressive chronic infection after retransplantation may also occur if the viral clearance is not achieved before retransplantation[5].

In this situation, early diagnosis of hepatitis E in this group of patients is the highest priority. The diagnosis should be based on the detection of HEV RNA in serum, cerebrospinal fluid (CSF) in case of neurological complications or stool samples, not levels of liver enzymes and results of serological tests[3,26,33,91]. Since various factors can elevate liver enzymes, including drugs, toxin, graft rejection, infections, and biliary tract dysfunction[30]. Furthermore, the presence of chronic HEV infection in organ transplant recipient patients is sometimes accompanied by normal liver enzymes[30]. In addition, the delay or absence of seroconversion and loss of anti-HEV antibodies are frequently observed in this group of patients due to immunosuppressive conditions, which result in suppression of antibody development over time[5,91]. Therefore, immunocompromised or immunosuppressed patients with chronic HEV infection may have normal liver enzymes and negative serological tests[26,30,81].

In these conditions of uncertainty, the awareness of physicians regarding chronic HEV infection is crucial. Since most cases of chronic HEV infection may be missed due to the lack of HEV consideration among physicians or inappropriate choose of diagnostic assays[3,25].

Reports on HEV prevalence in immunocompromised patients in Iran are scarce. Rostamzadeh Khameneh *et al*[81] assessed the seroprevalence of HEV among 91 Iranian kidney transplant recipient patients. Overall, the seroprevalence of HEV was 30.8%. Joulaei *et al*[80] reported a HEV seroprevalence of 16.4% among 158 HIV-infected individuals in Shiraz in 2013. In another study by Ramezani *et al*[79], the seroprevalence of HEV infection was found to be 10% among 100 HIV-positive individuals in Tehran in 2012. These limited studies were unable to determine the incidence and prevalence of chronic HEV infection among immunocompromised patients in Iran. Since only the seroprevalence of anti-HEV IgG antibodies has been assessed, while HEV-RNA has not been measured in these studies[81,88]. Therefore, more studies are required to gain insight into the burden of chronic HEV infection in Iran.

CONCLUSION

However, Iran is classified as an endemic region for HEV infection, but we do not know much about this infection in Iran. The available epidemiological data have demonstrated the seroprevalence of HEV infection in different groups and regions of Iran, while the presence of HEV-RNA has not been evaluated in the studies published so far. In addition, the distribution pattern of HEV genotypes is unknown in Iran.

From historical aspect, hepatitis E is not new in Iran but is underestimated due to the lack of awareness amongst physicians and inappropriate diagnosis of the infection. The importance of HEV infection as a main public health problem cannot be neglected any longer. The identification of HEV-associated extrahepatic manifestations and chronic hepatitis E in immunocompromised patients has attracted attention to the study of HEV in recent years. While these new aspects of so thought acute self-limited hepatitis remain unknown in Iran. Overall, still a long way is ahead to determine the epidemiological patterns of HEV in Iran. To approach this goal, further epidemiological investigations at the national level are needed to more clearly delineate the incidence and prevalence of HEV infection in Iran. In addition, nationwide efforts should be pursued to control and prevent HEV infection in Iran.

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ORIGINAL ARTICLE

Basic Study

Structural and molecular features of intestinal strictures in rats with Crohn's-like disease

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Abstract

AIM: To develop a new rat model we wanted to gain a better understanding of stricture formation in Crohn's disease (CD).

METHODS: Chronic colitis was induced locally by the administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS). The relapsing inflammation characteristic to CD was mimicked by repeated TNBS treatments. Animals were randomly divided into control, once, twice and three times TNBS-treated groups. Control animals received an enema of saline. Tissue samples were taken from the strictured colonic segments and also adjacent proximally and distally to its 60, 90 or 120 d after the last TNBS or saline administrations. The frequency and macroscopic extent of the strictures were measured on digital photographs. The structural

features of strictured gut wall were studied by light- and electron microscopy. Inflammation related alterations in TGF-beta 2 and 3, matrix metalloproteinases 9 (MMP9) and TIMP1 mRNA and protein expression were determined by quantitative real-time PCR and western blot analysis. The quantitative distribution of caspase 9 was determined by post-embedding immunohistochemistry.

RESULTS: Intestinal strictures first appeared 60 d after TNBS treatments and the frequency of them increased up to day 120. From day 90 an intact lamina epithelialis, reversible thickening of lamina muscularis mucosae and irreversible thickening of the muscularis externa were demonstrated in the strictured colonic segments. Nevertheless the morphological signs of apoptosis were frequently seen and excess extracellular matrix deposition was recorded between smooth muscle cells (SMCs). Enhanced caspase 9 expression on day 90 in the SMCs and on day 120 also in myenteric neurons indicated the induction of apoptosis. The mRNA expression profile of TGF-betas after repeated TNBS doses was characteristic to CD, TGF-beta 2, but not TGF-beta 3 was up-regulated. Overexpression of MMP9 and down-regulation of TIMP1 were demonstrated. The progressive increase in the amount of MMP9 protein in the strictures was also obvious between days 90 and 120 but TIMP1 protein was practically undetectable at this time.

CONCLUSION: These findings indicate that aligned structural and molecular changes in the gut wall rather than neuronal cell death play the primary role in stricture formation.

Key words: Crohn's disease; Rat model; TGF-beta; Intestinal strictures; MMP9; TIMP1

Core tip: Intestinal strictures in Crohn's disease (CD) cause hardly treatable complications in patients. The aim of this study was to find the correlation between the intestinal stricture formation, the damaged innervation of smooth muscle cells (SMCs) and the changed expression of TGF-beta 2, 3 and MMP9/TIMP1 in rats with CD by using different light- and electron microscopic and molecular biological methods. Our findings indicate that disintegration of SMCs due to the up-regulation of TGF-beta 2 and off-balance in MMP9/TIMP1 expression rather than neuronal cell death play the primary role in the formation of intestinal strictures in CD.

Talapka P, Berkó A, Nagy LI, Chandrakumar L, Bagyánszki M, Puskás LG, Fekete E, Bódi N. Structural and molecular features of intestinal strictures in rats with Crohn's-like disease. *World J Gastroenterol* 2016; 22(22): 5154-5164 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5154.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5154

INTRODUCTION

Despite the fact that the formation of obstructive strictures is the leading cause of surgical intervention in patients with Crohn's disease (CD), little is known about their etiopathogenesis, and no direct therapies are available for the effective prevention or reversal of this condition[1]. Lasting deep remission has emerged as a major therapeutic goal in CD[2,3]. This implies not only alleviation of the symptoms, but also the achievement of complete mucosal healing, with the accompanying decrease in the risk of irreversible pathological alterations of the gut wall[4]. However, total mucosal regeneration to prevent stricture formation is unattainable[5,6].

The spread of fibrosis deep into the gut wall leads to disorganization of the lamina muscularis mucosae (LMM) and thickening of all layers of the gut wall due to the accumulation of extracellular matrix (ECM) elements[5,6]. Previous studies have demonstrated that the cytokines transforming growth factor-beta (TGF-β) isoforms and the tissue-degrading matrix metalloproteinases (MMPs) are the key contributors to these processes[7,8]. Both TGF-beta and its receptors are overexpressed in the intestine of CD patients. However, the expression of the TGF-beta isoforms varies with the nature of the tissue. Fibrotic tissue exhibits a reduced expression of TGF-beta 3 and an enhanced expression of TGF-beta 2[9-11]. MMPs are secreted as inactive zymogens which must undergo proteolytic cleavage to become active, and their activity is regulated by specific tissue inhibitors of metalloproteinases (TIMPs)[12-14]. The MMPs do not simply degrade ECM as their name might suggest, but are also responsible for the homeostatic regulation of the ECM. Previous studies have shown that the gene transcription of MMP9 is inducible and that the promoter region is highly responsive to most growth factors and cytokines. They directly cleave and activate growth factors into active ligands, and therefore regulate their bioavailability and/or activity[15-17]. In consequence of these complex interactions of the regulatory processes, the development of the intestinal strictures characteristic of CD cannot be explained simply by the lower or higher expression of one or other of these factors. The key driver of stricture formation rather appears to be an off-balance between the TGF-betas, MMPs and TIMPs which develops in the chronic phase of inflammation. MMP9 is the most abundant MMP expressed in colonic tissue from CD patients, and may therefore be regarded as a biomarker in the evaluation of the clinical activity of inflammatory bowel diseases (IBDs)[18].

The intestinal symptoms common among CD patients are often related to enteric neuropathy. The evidence suggests that both the quantitative properties and function of the myenteric neurons are altered substantially by intestinal inflammation[19-22] and in fact complete loss of the myenteric neurons has been

observed in the strictured regions[23]. However, the extent to which the deficient innervation of the smooth muscle cells (SMCs) and/or the imbalance in the regulation in the molecular events behind the tissue remodelling are responsible for the stricture formation remains unclear.

We recently reported on a rat model of chronic colitis where the mortality was negligible despite the severity of the intestinal symptoms. We demonstrated that experimentally provoked recurring periods of acute inflammation exerted a preconditioning effect against the mucosal damage and reduced the rapid, significant and widespread loss of myenteric neurons observed after the induction of the colitis[24]. In the present work, we used this model to investigate the long-term consequences of acute inflammation on the structural and molecular alterations in the strictured gut wall. The aim of the study was to investigate the possible coincidence between the expressions of TGF-betas, MMP9 and TIMP1 behind the structural remodelling of the strictured gut wall. The structural findings at the light- and electron microscopic levels and the molecular findings at the mRNA and protein levels will be discussed.

MATERIALS AND METHODS

Animal model

All procedures involving experimental animals were approved from the Local Ethics Committee for Animal Research Studies at the University of Szeged. Adult male Sprague-Dawley rats weighing 200-220 g were used throughout the experiments. The animal protocol was designed to minimize pain or discomfort to the animals. The rats were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for two weeks prior to experimentation. Colitis was induced locally under pentobarbital anaesthesia (45 mg/kg *ip*) by the administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma-Aldrich, St. Louis, MO, United States; 10 mg) dissolved in 0.25 mL of 25% ethanol, as described earlier[24]. Repetitive relapsing inflammation (RRI) was mimicked through repeated administration of the same TNBS doses. The rats were treated once (n = 8), twice (n = 7) or three times (n = 8) with TNBS, 2 weeks passed between the treatments. Control rats (n = 18) received an enema of 0.25 mL of 9 g/L saline at the same time as the TNBS was administered. The rats were weighed and monitored daily for activity, bloody diarrhoea and mortality and were sacrificed 60, 90 or 120 d after the last TNBS or saline administrations.

Tissue handling

The animals were killed by cervical dislocation under pentobarbital anaesthesia. After this the last 8 cm region of the descending colon from the anus was dissected. Digital photographs were taken to evaluate the frequency and macroscopic extent of the strictures. Three colonic tissue samples were taken from each animal: the stricture itself and samples adjacent proximally and distally to it. Colonic samples of age-matched controls were also collected. Small pieces (2-3 mm) of the colonic segments for light- and electron microscopic morphometry and post-embedding immunohistochemistry were fixed in 20 g/L formaldehyde and 20 g/L glutaraldehyde solution and embedded in Epon (Electron Microscopy Sciences, Hatfield, PA, United States). Gut segments for molecular studies were cut along the mesentery and pinched flat. After longitudinal cutting, the mucosa and submucosa were removed. Half of the colon samples were immediately frozen in liquid N_2 and later processed for western blot analysis. The other half were incubated overnight at 4 ℃ in RNA Later (Qiagen, Venlo, The Netherlands) and stored at -80 ℃ until processing for quantitative real-time PCR (qRT PCR).

Light- and transmission electronmicroscopic morphometry

The Epon blocks were used to prepare semithin (0.7 μm) sections, which were stained with 10 g/L toluidine blue solution for the light-microscopic study. In the selected area of interest in the semithin cross-sections, all the layers of the gut wall were well oriented. The thicknesses of the LMM and the external circular (CM) and longitudinal (LM) smooth muscle layers were measured at random points with Image J 1.44 (National Institute of Health, Bethesda, MD, United States). The same Epon blocks were used to prepare ultrathin (70 nm) sections and the samples were mounted on nickel grids. Three grids per block were stained with uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck) and were examined and photographed with a Philips CM 10 electronmicroscope equipped with a MEGAVIEW II camera. The width of 15 tight junctions (TJs), *i.e.,* the distance between adjacent enterocytes, was measured at a magnification of × 46000 in the control samples and in the strictures by using the AnalySIS 3.2 program (Soft Imaging System GmbH, Münster, Germany). The distance between SMCs was determined to evaluate the expansion of the ECM within the muscularis externa (ME). Ten montage photographs per intestinal segment were made at a magnification of × 10500 and the distance of SMCs was evaluated in limited-size (2000 nm × 2000 nm) grids for all images, at the intersection of the grid lines, perpendicularly to the cells and calculated by using the AnalySIS 3.2 program. The mean distance was calculated by using the AnalySIS 3.2 program.

Post-embedding immunohistochemistry

The Epon-embedded tissue blocks used previously for the morphometry also served for the post-embedding immunohistochemistry of caspase 9, as described

earlier[25]. Briefly, ultrathin sections from each block were sequentially incubated with anti-caspase 9 (Sigma-Aldrich, St. Louis, MO, United States; final dilution 1:50) primary antibodies overnight, followed by protein A-gold-conjugated anti-rabbit (18 nm gold particles, Jackson ImmunoResearch, West Grove, PA, United States; final dilution 1:20) secondary antibodies for 3 h, with extensive washing between. Sections were counterstained with uranyl acetate and lead citrate, and then examined and photographed with a Philips CM10 electronmicroscope equipped with a MEGAVIEW II camera. The numbers of gold particles were counted on digital photographs at a magnification of × 25000 in 10 SMCs and at a magnification of × 34000 in 5 myenteric ganglia (MGs) per colonic segment in each experimental groups with the AnalySIS 3.2 program.

Statistical analysis

Statistical analysis of the histological results was performed by using one-way ANOVA and the Newman-Keuls test with GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, United States), and a probability $P < 0.05$ was set as the level of significance. The results were expressed as mean ± SE. The statistical methods of the study were reviewed by Mária Bagyánszki from University of Szeged.

Quantitative real-time polymerase chain reaction

Tissue samples were homogenized in AccuZol (Bioneer, Daejeon, South Korea) directly before qRT PCR. Total RNA was prepared from tissue homogenates as suggested by the manufacturer (Bioneer, Daejeon, Korea). The reverse transcription was achieved by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States) as described earlier[24]. qRT PCR was performed in an Exicycler 96 (Bioneer, Daejeon, Korea) in a total volume of 20 μL containing 10 μL of FastStart SYBR Green PCR Master Mix, 1 μL of specific primer (0.5 pmol/μL) and 50 ng of cDNA template. The PCR program began with a 15-min initial step at 95 ℃, followed by 45 cycles of 15 s at 95 ℃ for denaturation, 45 s at 60 ℃ for annealing and 25 s at 72 ℃ for extension. The sequences of primers were derived from NCBI RefSeq Database entry NM_031131.1 for TGF-beta 2 (forward: 5′ agtgggcagcttttgctc 3′ and reverse: 5′ gtagaaagtgggcgggatg 3′), NM_013174.2 for TGF-beta 3 (forward: 5′ gaagagggccctggacac 3′ and reverse: 5′ gcgcacacagcagttctc 3′), NM_031055.1 for MMP9 (forward: 5′cctctgcatgaagacgacataa 3′ and reverse: 5′ ggtcaggtttagagccacga 3′) and NM_053819.1 for TIMP1 (forward: 5′ cagcaaaaggccttcgtaa 3′ and reverse: 5′ tggctgaacagggaaacact 3′). Hypoxanthine guanine phosphoribosyltransferase (HPRT) (NBCI RefSeq Database entry: NM_012583.2; forward: 5′ gaccggttctgtcatgtcg 3′ and reverse 5′ acctggttcatcatcactaatcac 3′) was used as a housekeeping gene to normalize the expression data. The results were expressed as mean ± SD.

Western blotting analysis and gelatine zymography

Tissue samples were homogenized in TRIS-mannitol buffer and the total cellular protein was then denaturated (mixing and boiling with v/v 20 mmol/L Tris 7-9, 3 mmol/L EDTA, 20 g/L sodium dodecyl sulphate (SDS), 100 g/L mercaptoethanol and 200 g/L glycerol) from each sample as described earlier[26]. Aliquots of 10 μg of total cellular protein were electrophoresed by 100 g/L SDS-polyacrilamide gel, and transferred to nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom). Two hours after blocking (with PBS pH 7.4, 2.5 g/L Tween 20 (v/v) and 50 g/L non-fat dried milk), the membranes were probed with anti-MMP9 mouse monoclonal antibody (Abcam PLC, Cambridge, United Kingdom; final dilution 1:1000) or TIMP1 (H150) rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States; final dilution 1:1000) for 2 h, and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States; final dilution 1:2000) for 1 h at room temperature with extensive PBS-Tween 20 washing between. Immunoreaction was visualized with an Immobilon Western HRP Substrate enhanced chemiluminescence system (Millipore Corporation, Billerica, MA, United States) and scanned with a LI-COR C-DiGit™ Blot Scanner (Li-Cor Corporate, Lincoln, NE, United States).

The activity of MMP9 was determined by gelatine zymography, performed by diluting colonic homogenates in zymogram sample buffer (Bio-Rad, Hercules, CA, United States) and electrophoresing the samples in precast 100 g/L SDS-PAGE containing gelatine (20 mg/mL; Sigma-Aldrich, St. Louis, MO, United States) at 120 V until resolution was achieved. The gels were removed from their casings, gently rinsed in ddH_2O, placed onto a shaker in 1X renaturation buffer (Bio-Rad, Hercules, CA, United States) for 40 min, and then placed in 1X development buffer (Bio-Rad Hercules, CA, United States). With change of the buffer once at 20 min, the gels were next incubated at 37 ℃ for 20 h and stained with Coomassie Blue (Bio-Rad, Hercules, CA, United States) for 40 min before being destained in water for 1 h and scanned with a LI-COR C-DiGit™ Blot Scanner.

RESULTS

General observations

Despite the severity of the acute intestinal inflammation of the TNBS-treated rats, the mortality was negligible: only 2 rats died throughout the 120-d experimental period. By 1 d following the TNBS treatment, all the animals had developed symptoms such as weakness, weight loss and bloody diarrhoea. However, by 7 or 8

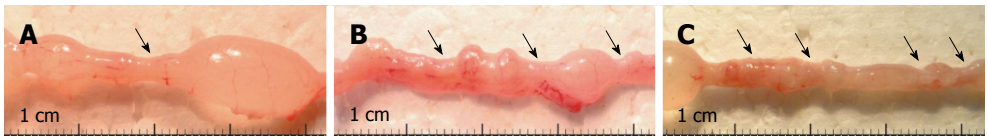

Figure 1 Representative micrographs from the distal colon of rats with chronic colitis 90 d after the first (A), second (B) or third (C) treatment with 2,4,6-trinitrobenzenesulfonic acid. The frequency and size of the strictures (arrows) increased in the time and with the number of 2,4,6-trinitrobenzenesulfonic acid (TNBS) administrations.

d after TNBS administrations, all the visible symptoms accompanied by acute inflammation had resolved. By day 60 following TNBS treatments, all the rats that had previously been exposed to acute colitis had regained their initial body weight and strictures had appeared in each TNBS-treated group. We, therefore, investigated the structural and molecular characteristics of the strictured gut wall from this timepoint on. Whereas the numbers and sizes of the strictures increased in time and with the number of TNBS treatments, they always developed within the previously inflamed colonic areas (Figure 1) and, once they had appeared, their structure and molecular characteristics did not differ. To avoid repetitions therefore, representative results will be presented here, obtained after the processing of tissue samples collected exclusively after the third TNBS administration.

Light microscopy

Representative images of toluidine blue-stained semithin sections of colon where the thickness of the ME was measured are shown in Figure 2. Such colonic sections were collected for measurements on days 90 and 120 following TNBS administrations and also from age-matched controls. The strictured colonic regions displayed normal mucosal architecture and clearly defined, yet thickened muscle layers (not shown). Morphometric analyses revealed the approximately 2-fold thickening of the LMM and layers of the ME in the strictured region relative to the control samples on 90 d. While further significant thickening of the ME was measured beyond day 90 after TNBS administrations, the thickness of the LMM at later than 90 d was similar to that in the controls (Figure 2).

Transmission electronmicroscopy

Transmission electronmicroscopic examination of the colonic epithelium in the strictured region on days 90 and 120 after TNBS administrations showed that the apical surface of the enterocytes with intact brush-border and closed TJs was similar to that in the controls (Figure 3). The width of the TJs between adjacent enterocytes was evaluated morphometrically and was always found to be less than 3 nm (data not shown). However, autophagosome-like double-membrane vesicles of different sizes were frequently seen within the enterocytes (Figure 3).

Because of the excess accumulation of ECM elements in the strictured colonic regions, the SMCs had moved away from each other significantly by day 90 after TNBS administrations, and by 120 d there was more than 2-fold increase in the distance between adjacent SMCs as compared with the controls (Figure 4). Because of the ECM deposition, the SMCs also moved away from the MGs (Figure 4). By day 120 post-TNBS treatments, swollen and empty confluent vacuoles and autophagosomes were frequently seen in the SMCs and also in their close environment, together with different cell organelles in the strictured colonic areas (Figure 4). The vast majority of the axons appeared normal, but necrotic axons were seen rarely in the MGs (Figure 4). Quantitative post-embedding immunohistochemistry in the strictured areas revealed a progressive increase in the number of gold particles indicating caspase 9 antigen in the SMCs and MGs relative to the control samples (Figure 5). The caspase 9-labelling gold particles in the MGs were mainly associated with the mitochondria (Figure 5), the ultrastructure of which was well preserved even 120 d after TNBS treatments.

Quantitative changes in TGF-beta, MMP9 and TIMP1 mRNA and protein expression

On day 90, the TGF-beta 2 mRNA was up-regulated, while the TGF-beta 3 mRNA was down-regulated in the strictured gut wall and also in the colonic segments adjacent proximally and distally to the strictures as compared with the controls. The TGF-beta 2 mRNA expression progressively increased, while the TGF-beta 3 mRNA expression further decreased by day 120 in all three segments (Figure 6A).

A marked overexpression of MMP9 mRNA was detected in all the colonic segments examined on days 90 and 120 after TNBS treatments (Figure 6B). At the same timepoints, the TIMP1 mRNA expression was up-regulated in the colonic segments adjacent proximally and distally to the strictures, but was down-regulated in the strictures themselves (Figure 6B). MMP9 and TIMP1 expression was also evaluated at the protein levels. Although a high amount of MMP9 protein was demonstrated in the tissue samples from the control rats, the progressive increase in the amount of MMP9 protein in the strictures was obvious between days 90 and 120 (Figure 6C). Gelatine zymography demonstrated that an active form of MMP9 protein rather than pro-MMP was expressed (Figure 6C). While the amount of TIMP1 protein also decreased acutely between days 90 and 120 in the control samples, it was

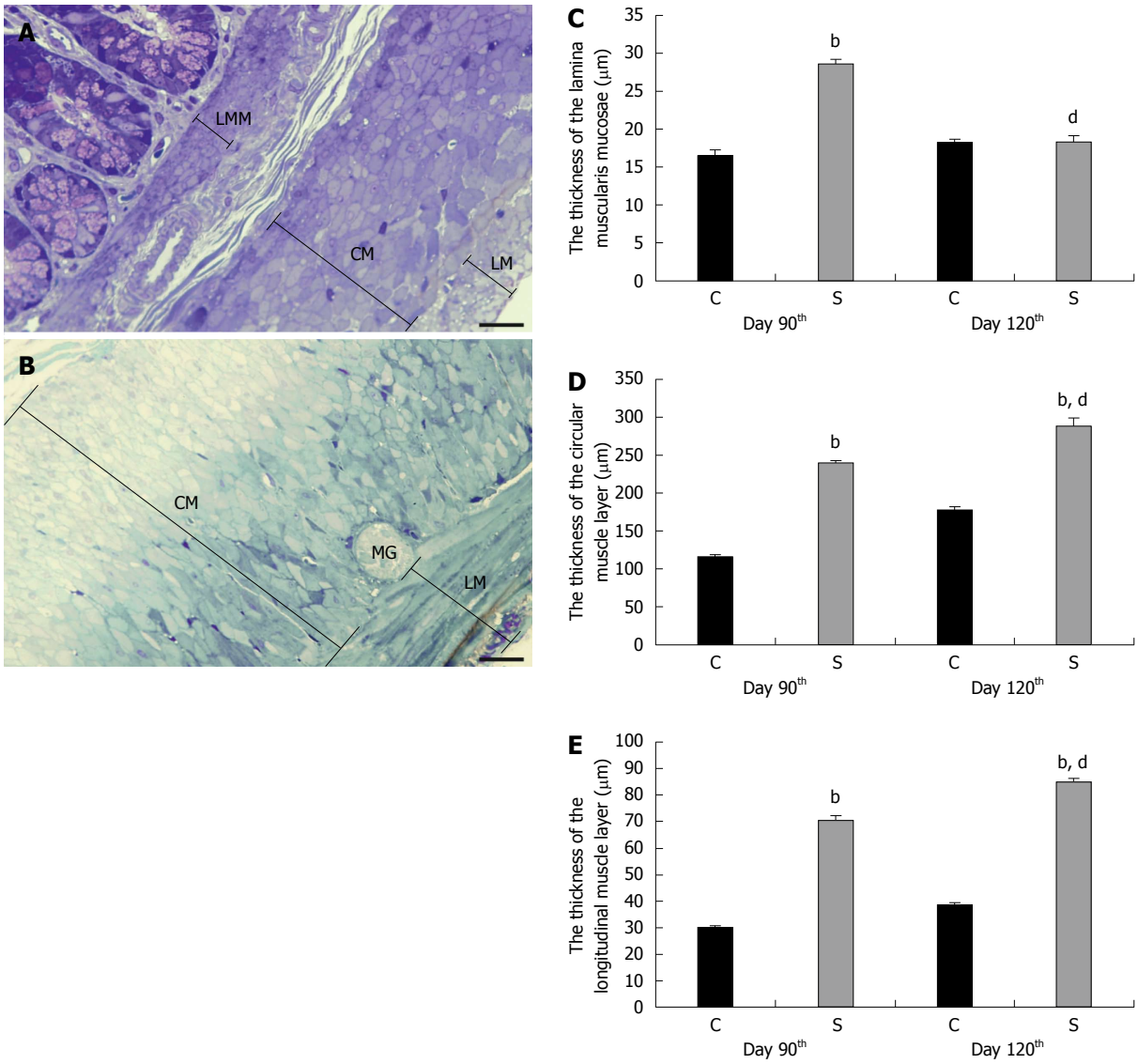

Figure 2 Thickness of the smooth muscle layers in the colon of control animals and in rats treated three times with 2,4,6-trinitrobenzenesulfonic acid. A: Representative light micrographs of a toluidine blue-stained semithin section from the colon of a control rat; B: TNBS-treated rat on day 120 of the experimental period. Bar: 25 μm. Significant thickening of the LMM (C), CM (D) and LM (E) was demonstrated in the strictured gut wall of the TNBS-treated rats (S) relative to the controls (C) on day 90. Whereas a further significant thickening was measured in the CM and LM on day 120, the thickness of the LMM was similar to that in the controls at this timepoint. Data are expressed as mean ± SE. $^{b}P < 0.001$ TNBS-treated groups *vs* age-matched controls; $^{d}P < 0.001$ 2,4,6-trinitrobenzenesulfonic acid (TNBS)-treated group on day 90 *vs* TNBS-treated group on day 120. LMM: Lamina muscularis mucosae; CM: Circular muscle layer; LM: Longitudinal muscle layer; MG: Myenteric ganglion.

practically undetectable in the strictures (Figure 6C).

DISCUSSION

We recently reported on a rat model in which alleviated inflammatory damage in association with the persistent up-regulation of HO-1 were salient features in the acute phase of intestinal inflammation induced by repeated TNBS administrations[24]. The same model was used in the present work to investigate the structural and molecular events leading to the formation of a strictured gut wall. Concerning the long-term consequences of the acute inflammation in this model, all the visible symptoms had resolved by day 60 after TNBS administration, the body weight of the treated rats was similar to that of the age-matched controls, and intestinal strictures developed in all of the rats that had previously displayed intestinal inflammation. These findings accord well with the clinical observations that mucosal healing and clinical remission alone cannot be treatment endpoints in CD, because this does not prevent later stricturing[27,28]. The increases in size and frequency of the strictures observed here after 60 d provide experimental evidence in favour of the view that strictures, once present, gradually progress and, once fibrosis develops, it cannot be reversed[29].

Aligned thickening of all the muscle layers in the strictured gut wall until up to day 90 after TNBS administrations was characteristic. Whereas the thic-

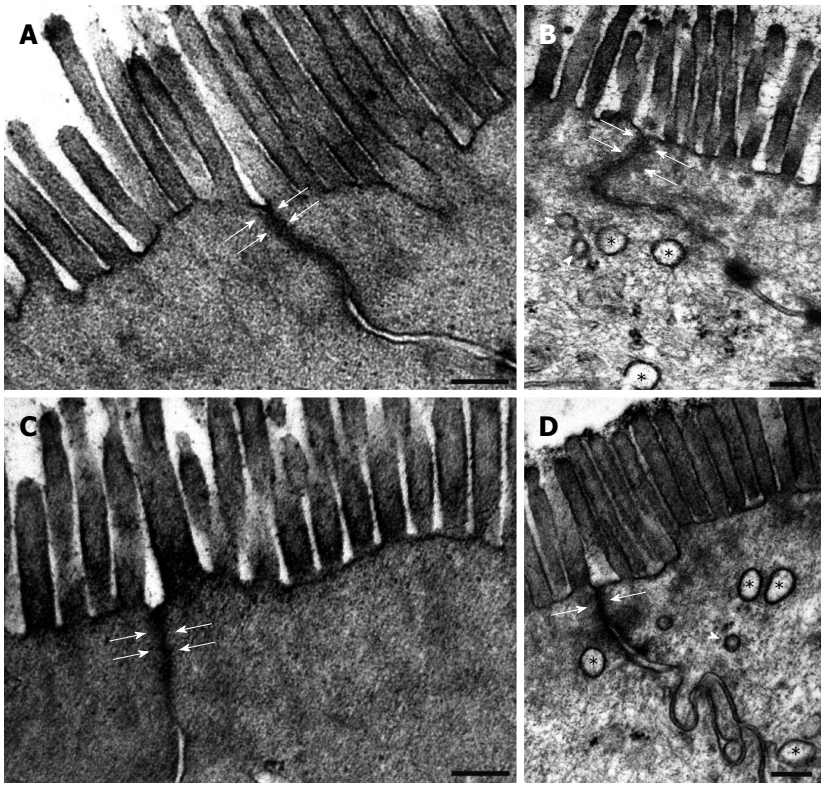

Figure 3 Representative electron micrographs of two neighbouring enterocytes from the colon of control animals (A, C) and in rats treated three times with 2,4,6-trinitrobenzenesulfonic acid. On days 90 (B) and 120 (D) following the third 2,4,6-trinitrobenzenesulfonic acid (TNBS) administration, both the microvillar surface and the width of the apical intercellular tight junctions (arrows) of the enterocytes in the strictured regions were similar to those in the age-matched controls (A on day 90 and C on day 120). However, autophagosomes (asterisks) and lysosomes (arrowheads) were commonly observed within the epithelial cells of the strictured gut wall. Bars: 200 nm.

kening of the ME progressed further and became a decisive element of the strictured gut wall, the thickness of the LMM did not change after day 90, and did not differ from that in the controls by the end of the experimental period. Since the LMM is most involved in maintaining the mucosal integrity[30], we suppose that the earlier cessation of excess ECM deposition in the LMM is a consequence of the differential regulation of inflammation-related events here through cytokines derived from the epithelium[31,32].

At 90 and 120 d following TNBS administrations, transmission electronmicroscopy showed that the structures of the epithelium necessary to maintain the barrier functions were intact. However, the frequent presence of double-membrane autophagosomes indicated high levels of intracellular stressors in the previously affected epithelium. It has been well documented that induction of autophagy is a determining factor for the maintenance of cellular homeostasis in chronic colitis[33,34]. The importance of autophagy in the pathogenesis of chronic intestinal inflammation has also been demonstrated by genome-wide association studies which indentified a link between the genes involved in autophagy regulation and IBDs[35,36].

While the rapid and widespread loss of myenteric neurons was a characteristic feature of the onset of acute inflammation[24], the precise timing of the cellular events in the chronic phase leading to the intestinal stricturing here showed that the SMCs in the ME were affected first in these processes. Since the excess deposition of ECM in the ME was sustained throughout the experimental period, the SMCs progressively moved away from each other and also from the MGs, leading eventually to deficient innervation and severe cellular damage. After day 60 following TNBS treatments, the appearance of autophagosomes, the leakage of cellular contents and the increasing number of gold particles labelling caspase 9 expression indicated that all three types of cell death mechanisms had already progressed in the SMCs by day 90 when necrotic axons were only rarely seen in the MGs. As the pathological environment became more extensive with time, by day 120 after TNBS administration locally severe neuronal injury also occurred in the strictured tissue as a significant sign of chronic inflammation, similarly as described in other models[23,37]. As regards the timing of the events, we presume suppose that the neuronal injury is a consequence and not the cause of the stricturing processes.

Evidence from both animal models[38,39] and human studies[18,40,41] has suggested that the up-regulation of TGF-beta 2 and of MMP9 may be considered to be biomarkers in the post-inflammatory tissue

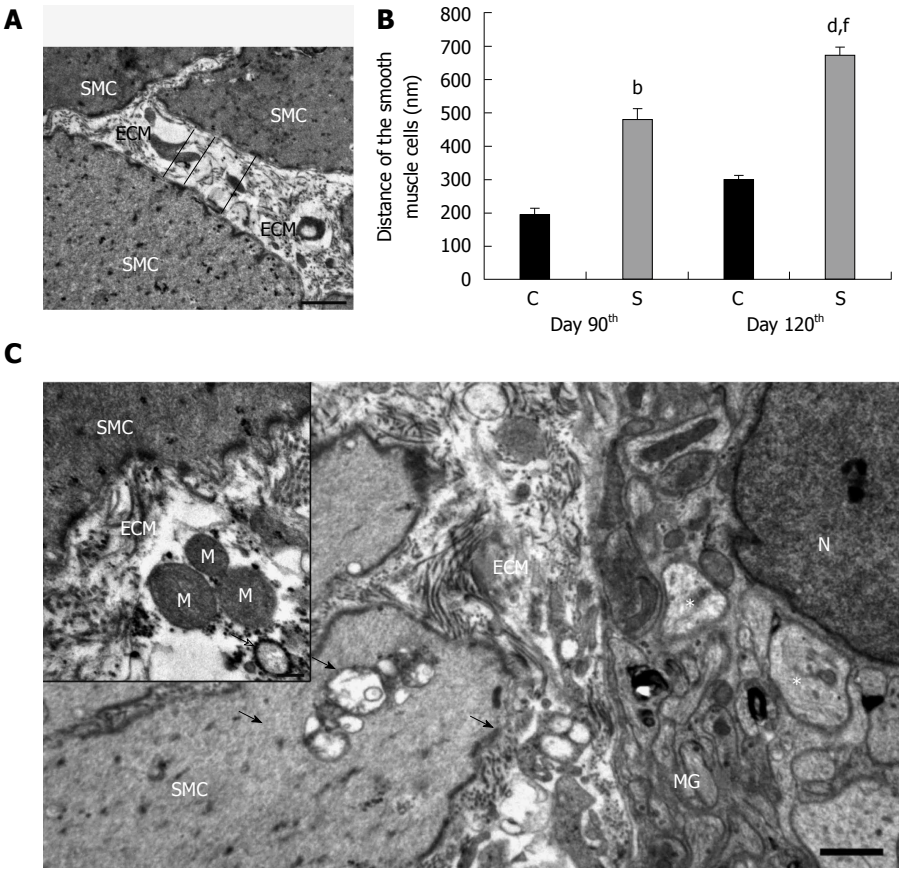

Figure 4 Ultrastructural alterations within the colon of control animals and in rats treated three times with 2,4,6-trinitrobenzenesulfonic acid. Excess deposition of extracellular matrix (ECM) was observed within the smooth muscle layers in the ultrathin sections derived from the strictured region (A); Electronmicroscopic morphometry revealed that the distance between adjacent smooth muscle cells (SMCs) was significant larger in the strictured gut wall of the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-treated rats (S) as compared with the gut wall of the control rats (C) on day 90 (B); A further significant increase in the mean separation distance of the SMCs was recorded on day 120 post-TNBS treatment (B). Data are expressed as mean ± SE. $^{b}P < 0.01$, $^{d}P < 0.001$ TNBS-treated groups *vs* age-matched controls; $^{f}P < 0.001$ TNBS-treated group on the day 90 *vs* TNBS-treated group on day 120. Representative electron micrograph of the strictured colonic area 120 d after the third TNBS administration (C). Because of ECM accumulation, the SMCs also moved away from the myenteric ganglia (MGs). Swollen and empty confluent vacuoles of different sizes (arrows) were frequently seen in the SMCs and also in their close environment. Rupture of the plasma membrane and subsequent leakage of the cell organelles into the microenvironment, *e.g.*, the mitochondria (M) and autophagosomes (hollow arrow), were frequently seen in the intercellular spaces (insert). However, the vast majority of the axons appeared normal; necrotic axons were rarely seen in the MGs (asterisks). N: Nucleus. Bars: 1 μm and 200 nm (insert).

remodelling leading to stricturing in CD. The mRNA expression profile of the TGF-beta isoforms, the up-regulation of TGF-beta 2 and the down-regulation of TGF beta 3 in the colonic segments examined in our model accorded well with the distinctive expressional profile of the secreted TGF-beta isoforms in human CD primary intestinal myofibroblasts[41]. The spreading of this characteristic expression pattern both proximally and distally to the strictures indicated the bidirectional diffusion of the disease along the colon in our model. We also detected progressive up-regulation of MMP9 mRNA in all three colonic segments, suggesting again the proximally and distally directed diffusion of the pathological environment. However, the MMP9 up-regulation in the strictured gut wall was coupled with the down-regulation of TIMP1, and an increased amount of active MMP9, but no TIMP1 protein was detected here, indicating a stricture-specific off-balance in the production of proteases and their inhibitors. This expression pattern is very reminiscent of that which develops in the fistulae in approximately one-third of patients with CD[42]. The apparent differences in expression profiles between our study and the literature data in tissue samples prepared from the control guts could be explained by the different methodological approaches. The novelty of our studies was that we prepared tissue homogenates for molecular studies not from the mucosa overlying the strictures, but exclusively from the ME, where the background events of the chronic inflammation leading to stricture formation actually occurred.

In conclusion, The structural and molecular events leading to stricturing as a long-term consequence of acute intestinal inflammation that were demonstrated earlier in animal models and in human studies also characterized the stricture formation induced in our rat model by repeated TNBS administrations. Since the exact timing of the stricturing processes was possible in this model, we reached the conclusion that, in contrast with the general view, the ME, and not the

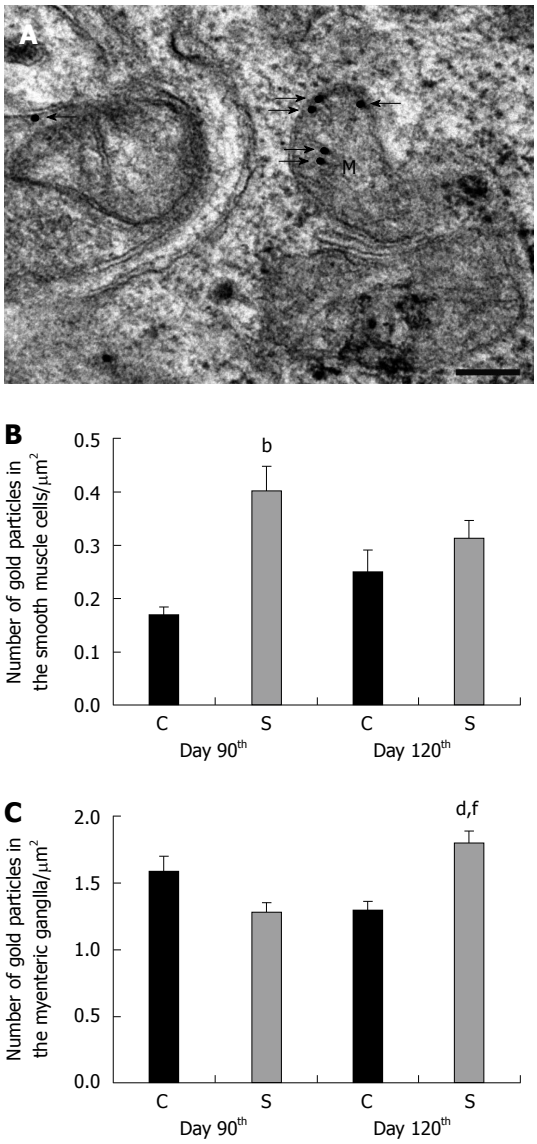

Figure 5 Post-embedding immunogold labelling for caspase 9 in the smooth muscle cells and myenteric ganglia in the colon of control animals and in rats treated three times with 2,4,6-trinitrobenzenesulfonic acid. Representative electron micrograph of a myenteric ganglion (MG) from the strictured gut wall (S) 120 d after the third 2,4,6-trinitrobenzenesulfonic acid (TNBS) administration (A). The 18 nm gold particles labelling caspase 9 immunoreactivity (arrows) were mainly associated with mitochondria (M). Bar: 200 nm. The number of gold particles in the S was increased significantly in the smooth muscle cells on day 90 (B) and also in the MGs on day 120 (C) as compared with the gut wall in the control rats (C). Data are expressed as mean ± SE. $^{b}P < 0.01$, $^{d}P < 0.001$ TNBS-treated groups *vs* age-matched controls; $^{f}P < 0.01$ TNBS-treated group on the day 90 *vs* TNBS-treated group on day 120.

epithelial barrier or the MGs, was the primary target of the events leading to stricture formation. Moreover, this TNBS-induced rat model has provided the first experimental demonstration of the molecular diffusion of the disease both proximally and distally along the gut wall. The off-balance in MMP9/TIMP1 expression profile found strictly within the border of the strictures may well allow use of this model to investigate the molecular mechanisms leading to fistulated CD.

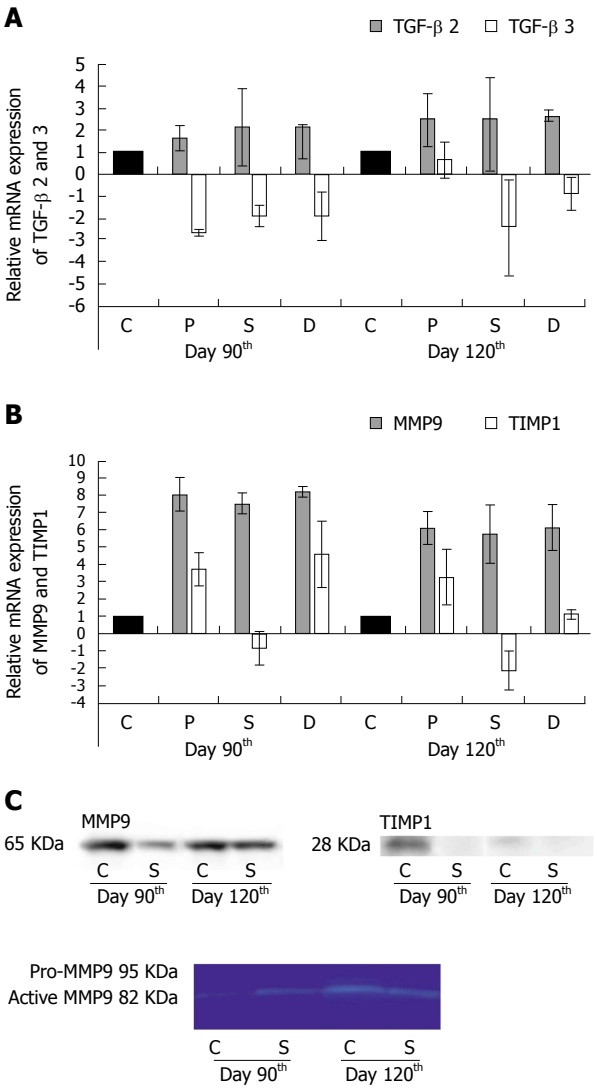

Figure 6 Relative mRNA and protein expression of transforming growth factor-beta 2 and 3, matrix metalloproteinase 9 and tissue inhibitor of metalloproteinases 1 in the colon of control animals and in rats treated three times with 2,4,6-trinitrobenzenesulfonic acid. TGF-beta 2 was up-regulated in the strictured region (S) and also in the adjacent proximal (P) and distal (D) segments of the colon as compared with the controls (C) on day 90 and day 120 (A). TGF-beta 3 gene repression was detected both 90 and 120 d after the third TNBS treatment in each colonic segment (A). The marked overexpression of MMP9 mRNA was confirmed in each colonic segment in the chronic phase of the inflammation (B). TIMP1 mRNA expression was detected in the P and D colon segments at both timepoints examined, but in the S the gene was down-regulated (B). Data are expressed as mean ± SD. 90 d after the third TNBS treatment, a decreased MMP9 protein level was detected in the S relative to the C (C, upper). Nevertheless, on day 120 the MMP9 protein expression was similar to that in the C. The activity of MMP9 was determined by gelatine zymography (C, lower). An active form of the MMP9 protein rather than pro-MMP9 was expressed in the C and S segments at both timepoints examined. Well-detectable amounts of TIMP1 protein were revealed only in the control samples from day 90 (C, right side).

COMMENTS

Background

Intestinal strictures are characteristic complications of Crohn's disease (CD) affecting more than one third of all patients. Its can lead to partial or total

intestinal obstruction with potentially life-threatening consequences. Although the treatment of the chronic complications of CD is a serious medical problem, the pathogenesis, factors, and cell types involved in stricture formation are largely unknown.

Research frontiers

Despite of the huge amount of animal models and human studies, the structural and molecular events leading to stricturing as a long-term consequence of acute intestinal inflammation are still not clear until today. Besides, the ultrastructure of the intestinal strictures is still unknown.

Innovations and breakthroughs

This TNBS-induced rat model has provided the first experimental demonstration of that, in contrast with the general view, the muscularis externa, and not the epithelial barrier or the myenteric ganglia, was the primary target of the events leading to stricture formation.

Applications

The authors hypothesize form the results derived our rat model with chronic colitis and very low mortality that the experimentally provoked recurrent relapsing inflammations characteristic to CD can provoke the recrudescence of the strictures post-surgically despite of the complete mucosal healing and restoring myenteric neuronal injury.

Terminology

The authors described earlier that experimentally provoked repetitive relapsing inflammations develop preconditioning effect by speeding up mucosal healing and restoring myenteric neuronal injury.

Peer-review

This is a well-written manuscript with carefully designed and described experiments. The observations are interesting and certainly add to our knowledge in the inflammation-induced fibrosis in the large intestine.

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ORIGINAL ARTICLE

Basic Study

Apoptosis induced by a low-carbohydrate and high-protein diet in rat livers

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Abstract

AIM: To determine whether high-protein, high-fat, and low-carbohydrate diets can cause lesions in rat livers.

METHODS: We randomly divided 20 female Wistar rats into a control diet group and an experimental diet group. Animals in the control group received an AIN-93M diet, and animals in the experimental group received an Atkins-based diet (59.46% protein, 31.77% fat, and 8.77% carbohydrate). After 8 wk, the rats were anesthetized and exsanguinated for transaminases analysis, and their livers were removed for flow cytometry, immunohistochemistry, and light microscopy studies. We expressed the data as mean ± standard deviation (SD) assuming unpaired and parametric data; we analyzed differences using the Student's *t*-test. Statistical significance was set at $P < 0.05$.

RESULTS: We found that plasma alanine aminotransferase and aspartate aminotransferase levels were significantly higher in the experimental group than in the control group. According to flow cytometry, the percentages of nonviable cells were 11.67% ± 1.12% for early apoptosis, 12.07% ± 1.11% for late apoptosis, and 7.11% ± 0.44% for non-apoptotic death in the experimental diet group and 3.73% ± 0.50% for early apoptosis, 5.67% ± 0.72% for late apoptosis, and 3.82% ± 0.28% for non-apoptotic death in the control diet group. The mean percentage of early apoptosis was higher in the experimental diet group than in the control diet group. Immunohistochemistry for autophagy was negative in both groups. Sinusoidal dilation around the central vein and small hepatocytes was only observed in the experimental diet group, and fibrosis was not identified by hematoxylin-eosin or Trichrome Masson staining in either group.

CONCLUSION: Eight weeks of an experimental diet resulted in cellular and histopathological lesions in rat livers. Apoptosis was our principal finding; elevated plasma transaminases demonstrate hepatic lesions.

Key words: Apoptosis; Liver injury; High-protein diet; High-fat diet; Low-carbohydrate diet

Core tip: Obesity is a serious and growing health problem. A high-protein, high-fat, and low-carbohydrate diet known as the Atkins diet has been adopted since the 1970s. Many people adhere to this diet in an attempt to lose weight, and it has recently been introduced for children with difficult-to-control seizures and elderly suffering from Alzheimer's and Parkinson's diseases. The benefits and effects of the Atkins diet remain unclear, especially in hepatic metabolism. Since the primary metabolic reactions involving macronutrients occur in the liver, it is essential to understand the potential hepatic lesions that can result from dietary modifications.

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INTRODUCTION

The prevalence of obesity is increasing worldwide among adults, youth, and children; this serious health problem requires widespread public mobilization in the search for solutions. Extremely high numbers of people adhere to special diets in an attempt to lose weight[1]. A high-protein, high-fat, and low-carbohydrate diet was announced by the American cardiologist Robert C. Atkins in the mid-1970s as the best and healthiest way to become slim[2]. This so-called "Atkins diet" or "protein diet" has continued to be viewed as "a matter of love or hate"[3]. This diet was recently introduced for children with difficult-to-control seizures and elderly with Alzheimer's and Parkinson's diseases[4]. Despite numerous medical publications related to the Atkins diet, the results from a majority of such studies are inconclusive and have failed to demonstrate benefits and effects, especially in hepatic metabolism[5].

Several studies have reported associations between a high-protein diet and alterations in the liver[6], intestinal mucosa[7], kidneys[8,9], pancreas[10], adipose tissue[11], and bones[9,12]. Since the primary metabolic reactions involving macronutrients occur in the liver, it is essential to understand the potential hepatic lesions that may result from dietary modifications. It has been shown that high-fat or low-carbohydrate diets can cause hepatic steatosis related to excessive demand for fatty acids from diet and from adipose tissues as a consequence of gluconeogenesis[5]. In a recently published study, a high-protein diet (independent of the amount and type of fat or carbohydrate) was found not to lead to steatosis and may actually reverse it[13].

Hepatic cells are important targets for lesions in the presence of excessive dietary components since they are absorbed through the intestinal mucosa and quickly reach the liver through the portal vein[6]. Among macronutrients, carbohydrates and fat are largely responsible for the observed alterations since they promote changes in gene transcription and glycolytic and lipogenic enzymes [sterol responsive binding protein 1/2 (SREBP) and the mammalian target of rapamycin - mTOR), insulin, and adipokines][14].

Hepatocytes respond to injuries *via* various mechanisms, of which the most important are autophagy, apoptosis, and non-apoptotic death[15]. Autophagy may be considered to be an adaptive process associated with different types of liver injury, such as nutrient deprivation, insufficient growth factor, hypoxia, and the accumulation of fat in hepatocytes. Autophagy can be reversed if conditions improve. The cell digests its own components for use as an energy substrate; and when these components are insufficient to maintain cell homeostasis, either apoptosis or non-apoptotic death occurs[16,17]. Apoptosis, a cellular suicide program, is a natural process that is necessary to remove damaged, senescent, or mutagenic cells that have completed their mission. However, this process may lead to the development of various liver diseases. It is responsible for the development of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) when the capacity of hepatocytes to store free fatty acids from the diet is exceeded[18]. Apoptosis is an active process that requires energy; when energy is insufficient, the process decelerates and non-apoptotic death begins. Non-apoptotic death differs from apoptosis in many ways since it sparks an

inflammatory response that can aggravate the initial lesion[15,18].

Additional histopathological studies of livers of animals fed a low-carbohydrate and high-protein diet are required since the results to date have been conflicting[19,20].

Here, we hypothesized that a high-protein (59.46%), high-fat (31.77%), and very low-carbohydrate (8.77%) diet can cause hepatocyte lesions, as identified by flow cytometry and immunohistochemistry (IHC). We aimed to correlate the cytometry findings with light microscopy alterations and plasma transaminase levels.

MATERIALS AND METHODS

Animals

The experimental study was performed from March-May 2015 in the Experimental Nutrition Laboratory of the Nutrition College, Fluminense Federal University, Niterói, RJ, Brazil. The animal protocol minimized pain and discomfort to the rats. The experiment used 20 female Wistar rats (*Rattus norvegicus*) ranging in age from 11-13 wk. The animals weighed 211-249 g and were reared at the Laboratory Animal Facility of the Oswaldo Cruz Foundation, Ministry of Health, Rio de Janeiro, Brazil. The animals were kept in group cages with four animals each, for adaptation, over the course of 5 d, receiving water and laboratory diet *ad libitum*. After this period, the rats were separated randomly into two groups of 10 animals each [the control diet group (CDG) and the experimental diet group (EDG)] and individually housed in polypropylene cages with controlled temperature (24 ± 2 ℃) and humidity (60% ± 10%) and an alternating light-dark cycle consisting of 12 h of lightness and darkness.

Diets

The CDG diet consisting of the AIN 93M diet[21] was formulated for the maintenance of adult rats by the American Institute of Nutrition in 1993; we based the elaborated EDG diet on the Atkins diet. Both groups received water and an *ad libitum* diet for 8 wk. The diets were prepared by Pragsoluções Biociências Comércio e Serviços, LTD, Jaú, São Paulo, Brazil. The control diet had the following composition: carbohydrate (76.98%), protein (13.56%), and fat (9.46%). The experimental diet was composed of carbohydrate (8.77%), protein (59.46%), and fat (31.77%). The amount of vitamins, minerals, L-cysteine, choline, and fiber were the same in the two groups, and tert-butylhydroquinone was calculated as 0.002 mg per gram of fat, all based on AIN 93M determinations (Tables 1 and 2).

Experimental procedures and sample collection

On the morning of the day of sacrifice, all of the animals underwent vaginal smears to determine their estrous cycle phase. Animals in estrus were separated and given no more access to food. After 8 h of fasting, the animals were anesthetized *via* an intraperitoneal injection of a solution containing 11.50 mg/100 g body mass of ketamine and 0.10 mg/100 g body mass of xylazine and were exsanguinated by cardiac puncture[22]. They were then sacrificed one at a time, alternating between the experimental and control group. The blood was placed in a heparinized tube and centrifuged for 20 min at 314 rad/s, and the plasma was separated and stored at -80 ℃ until analysis. The liver was removed after withdrawing the blood, and six liver fragments measuring 1 cm^3 each were washed gently with NaCl 0.9%, submerged in a recipient with the same solution and stored in the freezer at -4 ℃ for 2 h prior to performing flow cytometry.

Table 1 Composition of control diet (AIN-93M)

Ingredients	g/100 g	CH (g)	PTN (g)	LIP (g)	FI (g)
Cornstarch	46.5	39.52			
L-cysteine	0.18		0.18		
Choline bitartrate	0.25		0.25		
Mineral mix	3.50	0.77			
Vitamin mix	1	0.97			
Tert-butylhydroquinone	0.008				
Fiber	5				5
Soybean oil	4			4	
Casein (> 85% Protein)	14		11.06		
Sucrose	10	10.00			
Dextrinized cornstarch	15.5	13.95			
Kcal (%)	338.8	260.84	45.96	32	
Macronutrients (%)	100	76.98	13.56	9.46	

The control diet group was fed this diet for 8 wk, elaborated by the American Institute of Nutrition in 1993 for laboratory rodents, with adequate percentages of macronutrient carbohydrate (CH), protein (PTN) and Lipids (LIP), vitamins, minerals and fiber (FI).

Table 2 Composition of experimental diet (based on the Atkins diet)

Ingredient	(g/100 g)	CH (g)	PTN (g)	LIP (g)	FI(g)
Agar	2				2
L-cysteine	0.18		0.18		
Choline bitartrate	0.25		0.25		
Mineral mix	3.5	0.77			
Vitamin mix	1	0.97			
Tert-butylhydroquinone	0.028				
Fiber	5				5
Sucrose	6	6			
Casein(> 85% protein)	20		16		
Powdered chicken breast	60		36	12	
Soybean oil	2			2	
Kcal (%)	352.68	30.96	209.72	112	
Macronutrients (%)	100	8.77	59.46	31.77	

The experimental diet group was fed this diet for 8 wk, elaborated by the authors based on the Atkins diet. The percentage of macronutrients was 2:1 carbohydrate (CH) + protein (PTN)/lipids (LIP) with fiber (FI). The others ingredients had the same amount as recommended for the AIN-93M.

Analytical methods

We measured plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels using

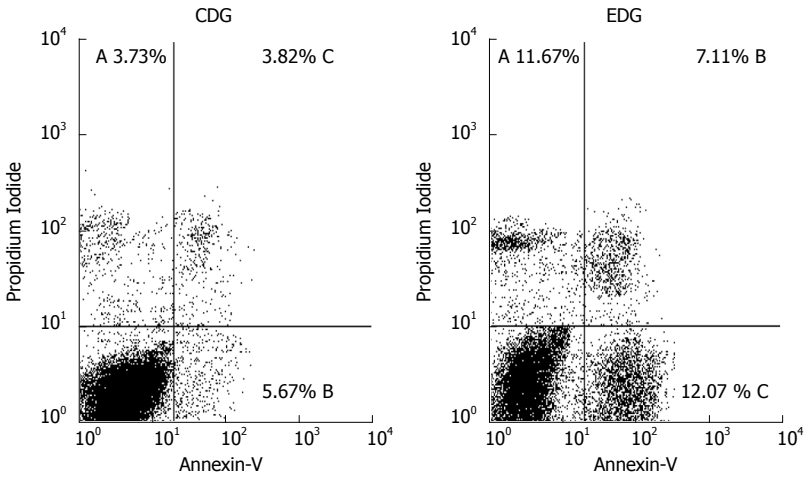

Figure 1 Flow cytometry in hepatocytes from the experimental diet group and control diet group at 8 wk. Annexin-V was used to identify late apoptosis and non-apoptotic death and propidium iodide for early apoptosis. A-% early apoptosis; B-% non-apoptotic death; C-% late apoptosis; D-% viable cells. EDG: Experimental diet group; CDG: Control diet group.

automatic analysis (Vitalab Selectra E, Vital Scientific, Spankaren, Netherlands) with commercial kits from BioSystems Reagents and Instruments (Barcelona, Spain) located in the Multidisciplinary Research Support Laboratory (LAMAP), School of Medicine, UFF, Niterói, RJ, Brazil. The flow cytometry used the following fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I components: 10X Annexin V Binding Buffer; FITC Annexin V; propidium iodide solution from BD Pharmingen (San Diego, CA, United States). The flow cytometer was the FACF-Calibur BD model. The liver fragments were fixed in Bouin's solution, processed in graded alcohols and xylene, embedded in paraffin blocks, stained for optical microscopy [hematoxylin-eosin (HE) and Trichrome Masson (TM) stains], and prepared for IHC. Alexa fluor 647 rat anti-mouse blimp-1 from BD Pharmingen was used for identifying autophagy by IHC. We performed flow cytometry and IHC in the Biomedical Science Institute of the Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil. We relied on a Zeiss (Oberkochen, Germany) Axioscop 20 microscope and Canon (Tokyo, Japan) G10 camera JPG with 14.7 megapixels at the Pathology Department of the School of Medicine, UFF, Niterói, RJ, Brazil for the optical microscopy.

Statistical analysis

The results of this study are presented using descriptive statistics, such as arithmetic mean and standard deviation. The two-tailed unpaired Student' *t* test was used to compare means between the two groups. It was considered that 95% confidence interval contains the true difference between the means ($P < 0.05$). An *F* test was performed to prove that the data came from two groups that have identical standard deviations (and thus identical variances). The computer program used was Graphpad-Prism version 6.0e (La Jolla, CA, United States) for Mac OS X, 2015, and WinMDI 2.9 was used for flow cytometry. The statistical analyses were reviewed by a biomedical statistician. The IHC and optical microscopy were based on observational evaluation by an expert.

RESULTS

Plasma transaminases

Plasma ALT and AST levels were significantly higher in the EDG than in the CDG. The mean ALT in the EDG was 61.70 ± 4.16 U/L compared with 28.10 ± 4.06 U/L in the CDG ($P < 0.0001$). AST in the EDG was 238.30 ± 15.85 U/L and 179.20 ± 13.86 U/L in the CDG ($P = 0.0117$).

Flow cytometry

Flow cytometry revealed a significantly higher percentage of nonviable cells in the EDG compared with the CDG (30.85% ± 2.20% and 13.22% ± 1.43%, respectively; $P < 0.0001$).

In the EDG, the percentages of nonviable cells were 11.67% ± 1.12% for early apoptosis, 12.07% ± 1.11% for late apoptosis, and 7.11% ± 0.44% for non-apoptotic death. In the CDG, the comparable values were 3.73% ± 0.50% for early apoptosis, 5.67% ± 0.72% for late apoptosis, and 3.82% ± 0.28% for non-apoptotic death (Figure 1).

When comparing the nonviable cells in the two groups, only the mean percentage of early apoptosis was statistically significant (Table 3).

Considering only non-apoptotic death and total apoptosis (early + late), the EDG demonstrated 23.99% ± 2.12% non-apoptotic death and 76.01% ± 2.12% total apoptosis ($P < 0.0001$); the CDG exhibited 29.20% ± 1.29% non-apoptotic death and 70.80% ± 1.29% total apoptosis ($P < 0.0001$).

Immunohistochemistry

IHC was negative for autophagy in both groups.

Table 3 Nonviable cells in experimental diet group and control diet group

	Early apoptosis	Late apoptosis	Non-apoptoic death
EDG[1]	37.34% ± 1.30%	38.67% ± 1.73%	24.00% ± 2.12%
CDG[1]	28.43% ± 1.19%	42.37% ± 1.10%	29.20% ± 1.29%
P-value	< 0.0001	0.0882	0.0512

[1]Each value expressed as mean ± SD (n = 10) significance $P < 0.05$. EDG: Experimental diet group; CDG: Control diet group.

Optical microscopy

Upon examining the livers of the rats in the EDG, the pathologist identified marked sinusoidal dilation around the central vein, with smaller perisinusoidal hepatocytes compared with rats in the CDG, which showed no alteration. The central vein was normal in both groups. Five animals in the CDG had isolated periportal cytoplasmic microvesicles compared with three animals in the EDG. Small and heterogeneously distributed structures were found in the liver of all animals in both groups; these structures likely corresponded to deposition of glycogen. In the EDG, these structures decreased and even absent in some zones. The pathologist observed acute and chronic inflammatory periportal cells in both groups, which are considered to be normal in rats. Fibrosis was not identified by HE or TM in either group (Figure 2).

DISCUSSION

As expected, we found that a high-protein, high-fat, and low-carbohydrate diet caused cellular and histopathological lesions in the livers of experimental rodents. ALT and AST were increased in the EDG compared with the CDG. Since these tests are considered to be precise liver function tests, our results confirmed the presence of liver damage involving hepatocyte destruction with plasmatic membrane disruption and late-phase apoptosis and non-apoptotic death in the EDG[23,24].

In a study by Jean *et al*[25], a group of animals that received a diet consisting of 50% protein exhibited high ALT and normal AST compared with controls that received a modified AIN-93M diet. The results were interpreted as hepatic lesions since ATL is a specific liver enzyme located in the hepatocyte cytoplasm; AST can also be expressed by muscles and kidneys[23,25]. Oarada *et al*[6] demonstrated that when rats were fed increasing amounts of protein (35%, 40%, 45%, and 50%), ALT and AST increased to the same degree. These authors accordingly concluded that protein-independent of other macronutrients and energy consumption-was a risk factor for liver injury. In a recent study, Kostogrys *et al*[26] found no changes in plasma transaminase levels with a diet of 50.0% protein, 37.7% fat, and 12.3% carbohydrate, although the liver was enlarged compared to animals receiving the AIN93-M diet. Comparing our results with those noted in the literature may be difficult since the percentages of macronutrients fed to the rats vary from one study to another.

Flow cytometry confirmed the hepatic damage, as demonstrated by increased plasma transaminase levels; 30.85% of the hepatocytes were nonviable in the EDG compared with 13.22% in control animals. Nonviable cells in the CDG included 3.73% early apoptosis, 5.67% late apoptosis, and 3.82% non-apoptotic death. These findings in the control group can be considered to be physiological since apoptosis and non-apoptotic death represent a continuous process that is responsible for maintaining the balance between proliferation and cellular death. Non-apoptotic death is part of the same process since it is the ultimate fate of cells that undergo apoptosis[24].

Apoptosis was markedly increased in the EDG, with 11.67% early apoptosis, 12.07% late apoptosis, and 7.11% non-apoptotic cells exhibiting a non-physiological state. Any dysregulation of apoptosis is deleterious and results in tissue damage[15]. Similar results were found by Chiang *et al*[27] who demonstrated that mice receiving an 8-wk diet with 60% protein exhibited changes in bodyweight, liver histology, and expression of apoptosis and fibrosis.

Another important finding of the present study was that the percentage of early apoptosis, degeneration of mRNA, was significantly higher in the EDG (37.34%) compared with that in the CDG (28.43%) ($P < 0.0001$), indicating that apoptosis was progressing in the liver[18]. This finding might be evidence that the rats did not adapt to the experimental diet over the 8 wk of the study.

IHC was negative for autophagy in both groups. Autophagy was likely not found in this study because the percentage of nonviable cells increased (*i.e.*, the cytoprotective mechanism was probably suppressed), and apoptosis continued to be active since early apoptosis was higher in the experimental group than in the control group. The two pathways are controlled by common mechanisms: when autophagy is inhibited, apoptosis is induced[16-18]. For comparison, we note that Garbow *et al*[5] fed animals a similar diet and found autophagy among others alterations.

A histological examination of the rats' livers in the EDG revealed sinusoidal dilation around the central vein with smaller perisinusoidal cells compared to the livers of control animals. Similarly Bollo *et al*[28] found small hepatocytes around the central vein in alpine chamois during winter and considered this change an adaptation to under-nutrition. Bollo *et al*[28] asserted that the central vein region was a metabolic zone from which nutrients were distributed to the rest of the organ. The hepatocytes atrophied, and the size of the core was reduced. This probable adaptation to inadequate nutrition could be considered a strategy to minimize energy expenditures.

Cytoplasmic microvesicles were observed in five rats fed the control diet and three rats fed the

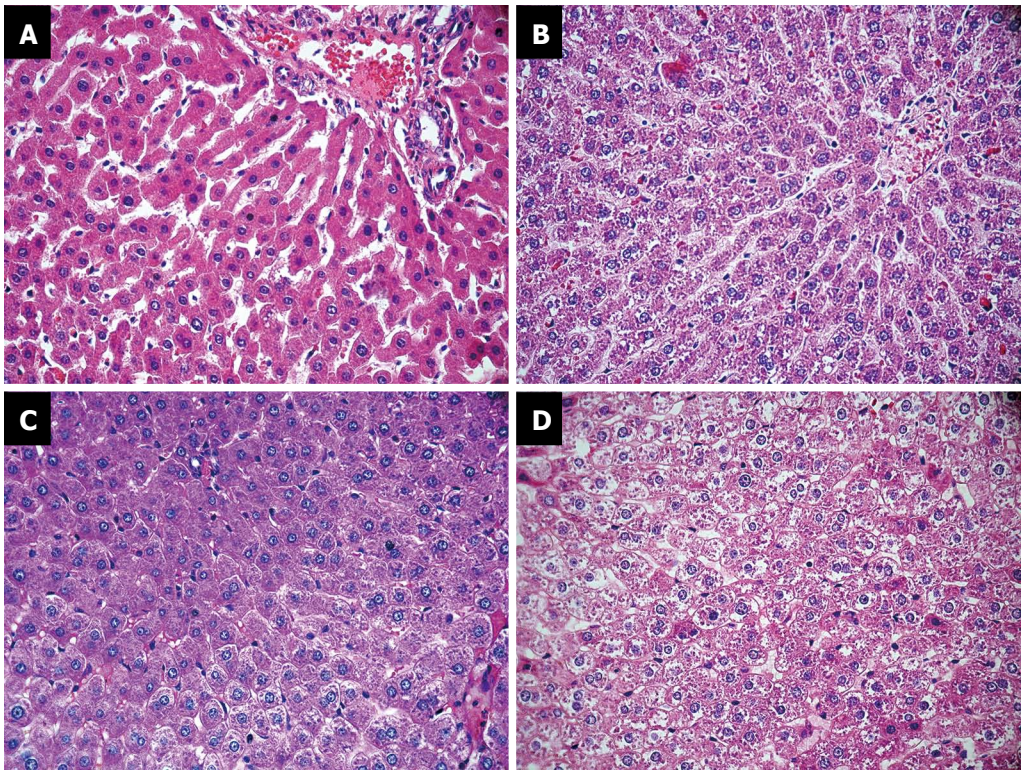

Figure 2 Staining of liver fragments at 8 wk: experimental diet group and control diet group. A: Normal central vein, functional sinusoidal dilation, and small hepatocytes in the EDG (HE, original magnification 400 ×); B: Normal central vein, sinusoidal cells, and hepatocytes in the CDG (HE, original magnification 400 ×); C: Zones without glycogen in the EDG (HE, original magnification 400 ×); D: Homogeneous distribution of glycogen in the CDG (HE, original magnification 400 ×). EDG: Experimental diet group; CDG: Control diet group; HE: Hematoxylin and eosin.

experimental diet. The stain method that we used was unable to differentiate fat from water. Other authors have recovered similar results. Lacroix *et al*[19], in an experimental study with rats fed a high-protein diet (50%) versus a normal-protein diet (14%) over the course of 6 mo, found no serious histological lesions in either group. Rats in the normal-protein diet group exhibited more microvesicular hepatic steatosis than rats in the high-protein group. Caraballo *et al*[13] showed that rats fed a high-protein and high-fat diet did not develop steatosis compared with rats fed a high-fat and low-protein diet; they concluded that the high-protein diet had an anti-steatotic effect on rat livers regardless of the amounts of others macronutrients that the rats ingested. On the other hand, Garbow *et al*[5] found hepatocellular damage, inflammatory response, severe hepatic steatosis, apoptosis, and autophagy in mice that were fed a ketogenic diet (low-carbohydrate, low-protein, high-fat) for 12 wk. Only Caraballo *et al*[13] used a stain specific to fat. For energy, high-protein diet associated gluconeogenesis improves glucose disposal from amino acids and glycogen and reduces fat deposition; neo-lipogenesis does not occur[20]. York *et al*[29] showed that a low-carbohydrate diet might be an option to treat patients with NAFLD and NASH since such a diet improves liver histology and reduces fat deposits, insulin resistance, and metabolic syndrome. Although the prevalence of NAFLD in rich countries is between 20% and 30% and this condition is considered to be part of the Metabolic Syndrome, which is responsible for high morbidity and mortality, many aspects of its pathology and treatment remain only partially understood. Apoptosis is considered an important point of NAFLD lesion and a common mechanism of hepatic injury[15,30].

We found that the amount of glycogen was likely lower in the experimental group and that both groups exhibited a heterogeneous glycogen distribution. Since the animals of each group were sacrificed in an alternating fashion, changed in glycogen content cannot be due to the duration of fasting. It was not possible to be certain that it was glycogen since no specific stain (periodic acid-Schiff reagent PAS) was conducted. Caraballo *et al*[13] reported similar results in animals fed a high-protein and high-fat diet. However, with a low-fat and low-protein diet, glycogen was not decreased and was instead concentrated in the periportal area. With a high-fat and low-protein diet, the glycogen was concentrated in the pericentral area[28]. In contrast, when Azzout-Marnich *et al*[20] compared two diets with 14% and 50% protein, respectively, glycogen levels were not different between the groups, but hey considered glyconeogenesis to be the primary pathway of the high-protein diet metabolism. Caraballo *et al* and Azzout-Marniche *et al*[20] also did not use a specific stain for glycogen.

We found no evidence for inflammation in the liver, as expected, since apoptosis (76.01%) was the primary mechanism of hepatocyte damage in the experimental group, not non-apoptotic death (23.99%). Furthermore, literature results have shown that apoptosis does not lead to a local inflammatory response[18].

Although neither group exhibited evidence of fibrosis, apoptosis may be considered not only an important mechanism of liver injury but also a contributor to liver fibrosis[27,31,32]. It is probable that if the rats had received the experimental diet for a longer period of time, fibrosis may have eventually appeared.

Additional studies are necessary to determine the exact mechanism by which changes in the percentage of macronutrients induce apoptosis. The lack of suitable nutrients may be considered to be a possible hypothesis. Gluconeogenesis, the primary metabolic pathway associated with a low-carbohydrate diet, leads to an increased production of keto acids and fatty acids, but they may not be the better energy substrate for hepatic cells[33]. When gluconeogenesis occurs, mitochondria exert important functions in energy metabolism (*i.e.*, the oxidation of fatty acids and oxidative phosphorylation for the production of adenosine triphosphate). Dysregulation of this pathway results in energy deficiencies and/or the production of reactive oxygen species that may be responsible for cell damage[34-36].

In conclusion, this study furthers our understanding of the hepatic cellular and histological changes caused by a high-protein, high-fat, and low-carbohydrate diet. The findings revealed that rats fed this diet had elevated levels of plasma transaminases and a higher percentage of nonviable cells in flow cytometry, which is evidence of hepatic lesions. Apoptosis was the principal pathway of hepatic injury. The primary findings from the optical microscopy-small hepatocytes and a decreased amount of glycogen-correlated well with changes in flow cytometry and can be attributed to modification of essential macronutrients to the liver. No inflammation or fibrosis was found in the livers of either the experimental or control animals. A better understanding of the mechanism of hepatic lesions associated with a high-protein, high-fat, and low-carbohydrate diet requires investigating the metabolic effects of diet in the liver.

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COMMENTS

Background

The authors did an experimental study comparing two groups of rats, one fed an experimental diet based on Atkins' (59.46% protein, 31.77% fat, and 8.77% carbohydrate) and the other an AIN-93M diet, so that they could study the effect of the experimental diet on hepatic metabolism. The modification of dietary components might result in hepatic cell damage since they are important targets for lesions when excessive macronutrients are absorbed and go through the intestinal mucosa to reach quickly the liver through the portal vein.

Research frontiers

The prevalence of obesity is increasing and may be considered a serious health problem. Worldwide, many people adopt various diets to lose weight without a particular orientation. The Atkins diet is a low-carbohydrate and high protein diet formulated by Dr. Robert Atkins in 1972. More recently, it was introduced for children with difficult-to-control seizures and elderly with Alzheimer's and Parkinson's diseases. Despite these indications, the effect of the diet on hepatic metabolism remains unclear.

Innovations and breakthroughs

This study demonstrated a strong association between low-carbohydrate, high-protein, and high fat diet and hepatic apoptosis, as identified by flow cytometry. These changes were correlated with alterations found by optical microscopy and may be due to metabolic changes.

Applications

A better understanding of the mechanism underlying hepatic lesions associated with this diet in rats and its effects on human metabolism is essential. The article emphasizes that the use of this diet should be used with caution for children, adults, and elders.

Terminology

Alanine aminotransferase) and aspartate aminotransferase are two enzymes found mainly in the liver that are considered hepatic markers of liver damage. Flow cytometry is a biophysical laser technology that was employed in this cell study; it detects cells in early apoptosis, late apoptosis, and nonapoptotic death.

Peer-review

Rats fed a low-carbohydrate, high-protein, and high-fat diet for 8 wk have hepatic cellular damage, as demonstrated by flow cytometry.

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ORIGINAL ARTICLE

Basic Study

Dominating expression of negative regulatory factors downmodulates major histocompatibility complex Class-Ⅱ expression on dendritic cells in chronic hepatitis C infection

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Conflict-of-interest statement: Authors declare no conflict of interest.

Data sharing statement: Technical appendix, statistical code and dataset are available from corresponding author at arora.sunil@pgimer.edu.in. Participants consent form was not taken for data sharing but the presented data are anonymized and risk of identification is low.

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Abstract

AIM: To elucidate the molecular mechanisms leading to development of functionally impaired dendritic cells (DCs) in chronic hepatitis C (CHC) patients infected with genotype 3 virus.

METHODS: This prospective study was conducted on the cohorts of CHC individuals identified as responders or non-responders to antiviral therapy. Myeloid DCs were isolated from the peripheral blood of each subject using CD1c (BDCA1)$^+$ DC isolation Kit. Monocytes from healthy donor were cultured with DC growth factors such as IL-4 and GM-CSF either in the presence or absence of hepatitis C virus (HCV) viral proteins followed by LPS stimulation. Phenotyping was done by flowcytometry and gene expression profiling was evaluated by real-time PCR.

RESULTS: Non-responders [sustained virological response (SVR)-ve] to conventional antiviral therapy had significantly higher expression of genes associated with interferon responsive element such as *IDO1* and *PD-L1* (6-fold) and negative regulators of JAK-

STAT pathway such as *SOCS* (6-fold) as compared to responders (SVR+ve) to antiviral therapy. The down-regulated genes in non-responders included factors involved in antigen processing and presentation mainly belonging to major histocompatibility complex (MHC) Class-Ⅱ family as *HLA-DP*, *HLA-DQ* (2-fold) and superoxide dismutase (2-fold). Cells grown in the presence of HCV viral proteins had genes down-regulated for factors involved in innate response, interferon signaling, DC maturation and co-stimulatory signaling to T-cells, while the genes for cytokine signaling and Toll-like receptors (4-fold) were up-regulated as compared to cells grown in absence of viral proteins.

CONCLUSION: Underexpressed MHC class-Ⅱ genes and upregulated negative regulators in non-responders indicate diminished capacity to present antigen and may constitute mechanism of functionally defective state of DCs.

Key words: Dendritic cells; Hepatitis C; Non-responders; Negative regulators; Major histocompatibility complex Class-Ⅱ genes

Core tip: The study was aimed to understand the mechanisms of dendritic cells dysfunction during chronic hepatitis C (CHC) infection. The findings highlight the association between different immune response genes and viral persistence in non-responders to antiviral therapy. Up regulation of negative regulators and down-regulation of molecules involved with antigen presentation seems to associate with non-responsiveness to antiviral therapy. Some novel pathways can be targeted to achieve better management of CHC patients.

Tomer S, Chawla YK, Duseja A, Arora SK. Dominating expression of negative regulatory factors downmodulates major histocompatibility complex Class-Ⅱ expression on dendritic cells in chronic hepatitis C infection. *World J Gastroenterol* 2016; 22(22): 5173-5182 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5173.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5173

INTRODUCTION

Hepatitis C virus (HCV), a positive sense single stranded RNA virus, infecting around 180 million people worldwide, is becoming a significant global health problem[1,2]. Transmitted through infected blood and body fluids, it is responsible for chronic hepatitis, which ultimately leads to life threatening liver diseases like fibrosis, cirrhosis, steatosis and finally causing hepatocellular carcinoma (HCC), thus a need for liver transplant[3]. Genotype 3 of HCV being more prevalent in South Asia, accounts for more than 50% of all the genotypes[4]. Although more patients infected with genotype 3 respond successfully to therapy as compared to genotype 1, yet approximately 25%-30% patients fail to achieve sustained virological response (SVR) and are considered as non-responders (NR) to antiviral therapy containing IFN-α and ribavirin, offered till recently[5,6].

Dendritic cells (DC) are professional Antigen-presenting cells (APC) having the unique property to induce a primary immune response[7]. Antigen uptake, processing and presentation to naïve T-cells for activation of the immune system are the main functions carried out by DC. There have been studies reported from our laboratory which showed that DC are numerically, functionally and phenotypically dysfunctional in patients infected with CHC[8,9]. The study also showed that functionally defective monocyte-derived dendritic cells (moDC) from CHC patients who did not achieve SVR, failed to reconstitute the capacity to mature, indicating that the dysfunctional status of DC in CHC patients was directly associated with the persistence of the virus.

The expression of many genes is responsible for regulation of DC maturation. They involve genes associated with antigen processing and presentation[10], interferon α, β response[11,12], cytokine signaling[13], adhesion and migration[14], phagocytosis[15], interferon responsive elements[16], anti-inflammatory process[17], negative regulators of JAK-STAT pathway[18] and genes involved in TLR mediated signaling[19].

Effective cellular immune response directed against HCV is mediated through T-cell and DC crosstalks[20]. A subdued adaptive immune response in chronic HCV patients might be due to the suboptimal antigen presentation and signaling via impaired DCs in these individuals. So, in the proposed study, we wanted to find out whether this non-responsiveness to standard anti-viral therapy in a proportion of CHC patients is associated with the virus-modulated expression of certain genes, which may culminate into dysfunctional status of DC (maturation as well as functional defects). So, we planned to investigate the expression levels of a set of selected genes in the myeloid dendritic cells (MDC) of CHC patients with the hypothesis that, an analysis of the association of immune response genes and non-responsiveness to therapy may reveal molecular mechanisms of DC dysfunction in the non-responders.

MATERIALS AND METHODS

Ethical statement

The study was approved by the Institute Ethics Committee of the PGIMER, Chandigarh (Reg. No. NKG/947). An informed written consent was obtained from all the subjects before taking blood samples.

Subjects and sampling

A total of 20 CHC patients were recruited for the study. Patients were divided into two groups on the basis of response to therapy in terms of SVR *i.e.,* HCV RNA negative at 24 wk after cessation of antiviral therapy. Patients achieving SVR (SVR+ve) were considered as "Responders" whereas those who failed to achieve SVR (SVR-ve) were termed as "non-responders". Responders (n = 10, MDC-R) and Non-responders (n = 10, MDC-NR) were recruited on the basis of inclusion and exclusion criteria. Inclusion criteria included patients positive for anti-HCV antibodies and serum HCV RNA, HCV RNA genotype 3 only, no prior history of any treatment for HCV, negative for auto-antibodies (ANA, SMA, LKM, AMA and PCA) and non-viral factors (alcoholism, inherited metabolic disorders). Exclusion criteria included patients with HBV, HCV genotype 1, 2 or 4, HIV and other co-infections, patients with regular use of hepato-toxic drugs and alcohol intake and any evidence of auto-immune or metabolic disease. Venous blood was taken in heparin vacutainer vials (BD) from each recruited patient in the hepatology clinic of PGIMER, Chandigarh. Age and sex matched healthy volunteers were recruited as control subjects (HC; n = 10). Inclusion criteria for HC included those subjects who had normal liver function tests with no history of jaundice or viral hepatitis infection in the past.

PBMC isolation and enrichment of myeloid dendritic cells using magnetic beads

Plasma was stored at -80 ℃ before isolation. From heparinised blood, peripheral blood mononuclear cells (*PBMCs*) were isolated by ficoll-hypaque density gradient centrifugation using Hisep (Himedia, Mumbai, India). MDC enrichment was performed by using CD1c (BDCA-1)$^+$ Dendritic Cell Isolation Kit (MiltineyiBiotec, Germany) following manufacturer's instructions. Briefly, the procedure included two steps: In the 1st step, CD1c (BDCA-1) expressing B cells labeled with CD19 magnetic microbeads got depleted by separation over a MACS column placed in a magnetic field of a MACS Separator. In the second step, CD1c (BDCA-1)$^+$ MDC labeled with CD1c-Biotin and Anti-biotin microbeads in B cell depleted flow-through fraction were retained within the column and eluted after removing the column from magnetic field. These cells (MDC) were used for further experiments.

Flow cytometric analysis for purity check

PBMCs and MDCs (10 μL each) were stained with fluorochrome-labeled antibodies (2 μL): Allophy-cocyanin (APC)-conjugated anti-HLA-DR, Fluorescein isothiocyanate (FITC)-conjugated Lineage Cocktail 1 (Lin1: CD3, CD14, CD16, CD19, CD20, CD56) and Phycoerythrin-Cy5 (PE-Cy5) - conjugated anti-CD11c from BD Biosciences (San Jose, CA, United States) for 15 min in the dark. Cells washed with staining buffer for 5 min at 1400 rpm were re-suspended in buffer for acquisition on Flowcytometer (FACS Calibur, BD, United States). Percent purity was calculated.

Generation of monocyte-derived DCs from HCs PBMCs

Monocyte-derived dendritic cells (moDCs) were derived according to the method described by Romani *et al*[21] and modified in our laboratory[8]. Cells were cultured in the presence (moDC-Ag) or absence (moDC-N) of HCV viral proteins. Briefly the PBMCs were isolated from venous blood as described above. Cells were suspended in RPMI 1640 medium (Sigma-Aldrich) and monocytes were made to adhere for 2 h at 37 ℃ (Plate adherence method). After incubation, non-adherent cells were removed. Adherent cells were cultured in the DC culture medium (DCCM) consisting of RPMI 1640 supplemented with: 2 mmol/L L-glutamine, 5 mmol/L HEPES buffer, 100 IU/mL penicillin and 100 μg/mL streptomycin, 10% fetal bovine serum (GIBCO), 20 ng/mL recombinant human GM-CSF (Peprotech Asia) and 20 ng/mL recombinant human IL-4 (Peprotech Asia) at 37 ℃ in a humidified incubator with CO_2 volume fraction, 50 mL/L CO_2 for six days. The cells were cultured in different sets as: either in presence or absence of viral proteins: core, NS3, NS4 and NS5 (Peprotech Asia). At the end of six days, these moDCs were stimulated with bacterial lipopolysaccharide (LPS) and further cultured for 48 h in maturation cocktail which comprised of DCCM with LPS (500 ng/mL). On the 8th day, moDCs were harvested and gene expression studies were carried out.

Gene expression analysis (RNA extraction, cDNA preparation and real time PCR)

MDCs and moDCs were centrifuged and dissolved in 1 mL TRIzol (Sigma, United States). RNA was extracted and reverse transcribed to cDNA using the RT^2 First Strand Kit (Qiagen, Germany) according the manufacturer's protocol, and cDNA was stored at -20 ℃ till further use. A custom PCR array (RT^2 Custom Profile PCR Array Human, Qiagen) was designed which included a panel of immune-stimulatory genes (ISGs) and genes involved in DC functioning (Table 1). Real-time PCR was undertaken using RT SYBR Green Master Mix (Qiagen, Germany) in a 96-well PCR plate pre-dispensed with primers in a Light Cycler 480 (Roche, Germany). Values were normalized against housekeeping genes (*GAPDH, β-actin*) in the same sample. Each experiment included positive PCR control (PPC), reverse transcription control (RTC) and human genomic DNA contamination (HGDC) control. Ct values were obtained for calculation of delta-CtCt and further analysis.

Statistical analysis

Statistical analysis for viral load (baseline and 4 wk) and other clinical features were done using GraphPad Prism software v 5.03 statistical package. Parametric

Table 1 List of selected genes for custom PCR array

CD209	CSF1R	ADAMDEC1	PDCD1	HLA-DPB1	HLA-DQA1	HLA-DQB1	HMOX1	ITGB2	CD40	*CD80*	*CD86*
CD83	*LY75*	*LAMP3*	*ARHGDIB*	*CCL5*	*CCL8*	*TLR2*	*CCL22*	*CCR7*	*CXCR3*	*CXCR4*	*CXCL6*
CXCL9	*CXCL10*	*CXCL11*	*CXCL12*	*CXCL16*	*ITGAX*	*ICAM1*	*VCL*	*TLR8*	*NFKB1*	*NFKB2*	*CD1A*
CD1B	*CD1C*	*CD52*	*S100A4*	*RELB*	*IDO1*	*CD274*	*IFNAR1*	*IFNAR2*	*IRF1*	*IRF3*	*CD44*
IRF7	*IRF9*	*STAT1*	*STAT2*	*ADAR*	*EIF2AK2*	*IFI6*	*IFI27*	*IFI35*	*OAS1*	*OAS2*	*OAS3*
PRKRA	*SOD2*	*MX1*	*MX2*	*ISG15*	*ISG20*	*IFIT1*	*FAS*	*LITAF*	*IFIT3*	*IFITM1*	*ITIH2*
GBP1	*GBP2*	*PIAS1*	*PIAS2*	*SOCS1*	*SOCS2*	*SOCS3*	*SOCS4*	SOCS5	IL28B	TRIM22	RARRES3
TAP1	*TAP2*	*RELA*	*TLR3*	*TLR4*	*TLR7*	*TLR9*	*GAPDH*	*ACTB*	*HGDC*	*RTC*	*PPC*

Table 2 Clinical characteristics of study subjects

Parameter	Responder	Non-responder
Mean viral load (IU/mL)	6.09 ± 0.29	5.97 ± 0.81
Mean age (yr)	42.0 ± 2.8	47 ± 2.9
Male/Female	6/4	8/2
Mean TB/CB (mg/dL)	0.75 ± 0.10	1.25 ± 0.20
Mean AST (U/L)	94.77 ± 23.40	102.3 ± 12.7
Mean ALT (U/L)	155.4 ± 45.2	111.2 ± 18.3
Mean AP (U/L)	94.50 ± 8.20	160.00 ± 24.44
Mean A/G (mg/dL)	1.25 ± 0.10	1.74 ± 0.10
(Fibrosis) Median LSM (kPa)	6.10	23.50 (P = 0. 01)

TB/CB: Total bilirubin/conjugated bilirubin; AST: Aspartate transaminase; ALT: Alanine transaminase; AP: Alkaline phosphatases; A/G: Albumin/globulin; LSM: Liver stiffness measurement.

and non-parametric *t*-tests were carried out and $P < 0.05$ was considered significant. For flow cytometry results, Cellquest software (BD Biosciences, United States) was used. Analysis of up-regulated and down-regulated genes was done using web based online software RT^2 Profiler PCR array data analysis version 3.5 software. To check interactions and associations between different genes, string software available online was used.

RESULTS

Clinical and demographic details of patients

A total of 20 patients were recruited for the study. Their clinical and demographic parameters like gender, age, genotype, liver enzyme (AST-ALT) levels, total bilirubin/conjugated bilirubin, Alkaline Phosphatase (ALP) levels were recorded (Table 2). At the baseline, there was no significant difference in the viral loads of responders vs non-responders, but when compared between baseline *vs* 4 wk (at RVR - rapid virological response) the viral load became undetectable in responders, while remained detectable in non-responders although was significantly decreased (Figure 1). Also, the degree of liver fibrosis (LSM - liver stiffness measurements) which provides useful information in prognostication, therapeutic planning, and assessment of the impact of treatment in chronic liver diseases, was significantly increased in non-responders ($P < 0.05$), which suggests that the persistence of virus in the liver leads to cirrhosis of the liver.

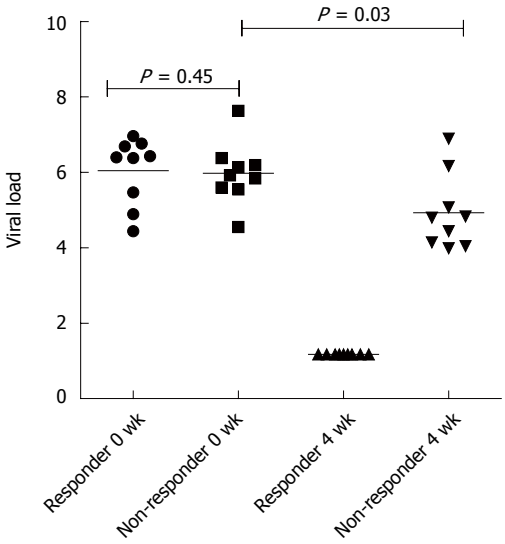

Figure 1 Difference between viral load in responders to antiviral therapy (at 0 and 4 wk) *vs* non-responders (0 and 4 wk). No significant difference in the viral loads of responders vs non-responders at baseline, but when compared between baseline *vs* 4 wk (at RVR - rapid virological response) the viral load became undetectable in responders, while remained detectable in non-responders.

Flow cytometric analysis of MDC

For phenotyping and purity of the isolated MDC, the cells negative for Lineage (CD3, CD14, CD16, CD19, CD20, CD56) and dual positive for CD11c and HLA-DR were gated. Percent enrichment of MDC was 65% after magnetic sorting as compared to 10% in PBMCs before sorting.

Gene expression profiles by PCR array

The gene expression profiles of the selected genes (as in Table 1) using custom-designed PCR array are shown in the heat map of genes indicating the differentially expressed genes (Figure 2). The genes upregulated or down-regulated are shown in Figure 3.

Upregulated genes

Non-responders (MDC-NR) *vs* Responders (MDC-R) group: Genes involved in negative signaling of JAK-STAT pathway, such as suppressor of cytokine signaling (*SOCS1*; six-fold, *SOCS2, SOCS4* and *SOCS5* all two-fold) and genes involved with down-modulation of immune response such as Indoleamine 2,3-Dioxygenase (*IDO1*) and Programmed death-

Figure 2 Heat map showing the expression of all the genes in different groups recruited. Graphical representation showed up-regulated and down-regulated genes in all the recruited groups. NR: Non-responder; HC: Healthy controls; HT: Healthy treated; RESP: Responder.

ligand 1 (*PD-L1*) were found to be significantly upregulated (two-fold or more) in non-responders as compared to responders to therapy (Figure 4).

Further, the genes for antiviral innate response such as TLR, ISGs and JAK/STAT pathway were also found to be up-regulated in non-responders as compared to responders to antiviral therapy.*TLR3* which is activated by viral RNA (HCV RNA) was four-fold up-regulated whereas *TLR4* and *TLR7* showed two-fold up-regulation in non-responders. The genes for Interferon regulatory factors (*IRF 7* and *IRF 9*) and the Interferon stimulatory genes (*ISG15* and *ISG20*) were also found to be significantly (six-fold) upregulated in non-responders. Further, the genes involved in JAK-STAT signaling, the *STAT1* and *STAT2,* showed two-fold upregulation along with the increased expression of IFN-induced proteins with tetratricopeptide repeats (*IFIT1*; six-fold, *IFIT3*; two-fold), IFN-Inducible transmembrane family (*IFITM1*; two-fold), Interferon-induced GTP-binding protein encoding gene (*MX1*, *MX2*; both two-fold), 2′,5′-oligoadenylate synthetase (*OAS1*; six-fold, *OAS2*; two-fold), IFN-inducible genes (*IFI6*; two-fold, *IFI27*; two-fold, *IFI35*; four-fold), Adenosine deaminase acting on RNA (*ADAR*; two-fold) and eukaryotic translation initiation factor 2-alpha kinase 2 (*EIF2AK2*; two-fold). Also gene associated with apoptosis such as Fas cell surface death receptor (*FAS*) showed increased expression (two-fold) in non-responders to antiviral therapy.

moDC from healthy donor differentiated in presence (moDC-Ag) or absence (moDC-N) of viral proteins: Sixteen genes were upregulated in the moDC differentiated from monocytes grown in presence of HCV viral proteins as compared to the cells grown in absence of proteins. Amongst these, included the chemokine and their receptor genes (*CXCR3*, *CXCR6*, *CXCL12*, *CCL8*; all two-fold) and Toll-like receptor genes (*TLR2*, *TLR4*, *TLR9*; all two-fold).

Downregulated genes

Non-responders (MDC-NR) *vs* Responders (MDC-R) group: The genes downregulated in non-responders as compared to responders included the genes belonging to MHC-Class Ⅱ family (*HLA-DPB1*, *HLA-DQA1*, *HLA-DQB1*) and Superoxide dismutase (*SOD*), the enzyme involved in transforming toxic superoxide anion radicals into hydrogen peroxide and oxygen for protecting DNA from oxidative stress showed two-fold reduced expression in non-responders as compared to responders (Figure 4).

DCs from healthy donor grown with (moDC-Ag) or without (moDC-N) viral proteins: A decreased expression of 21 genes in the cells grown in presence of viral proteins was observed as compared to the cells grown in absence of proteins. The genes found down-regulated (two-fold) include the ones involved in innate response and Interferon signaling (*EIF2AK2, IFI27, OAS1, OAS2, MX1, IFIT1, IFIT3, GBP1, GBP2, ISG20*); the genes involved with DC maturation (*CD83, LY75, LAMP3*) and genes involved in delivering co-stimulatory signals to T-cells (*CD40, CD80, CD86*) (Figure 4).

DISCUSSION

Non-responsiveness to antiviral therapy has been linked to defective phenotype of MDC by previous reports including our laboratory[8,9]. This indicates a direct association of immune defects with response to treatment in CHC, which could be attributed to many reasons such as (1) defect in IFN-α interactions with its receptors on MDCs; (2) defect in signal transduction machinery after this interaction; and (3) abnormal expression of certain transcription factors and immune response genes which are involved with the activation and maturation of DCs.

Although it has already been reported that there are functional and maturation defects in MDCs during CHC infection, yet the molecular mechanisms involved have not been fully elucidated[8]. The present study was designed with a view to understand these mechanisms and the role of different immune response genes that are involved in regulation of DC functions and may be associated with non-responsiveness to therapy and viral persistence during CHC. In order to achieve the objectives, gene expression profiles were studied in MDC isolated from the peripheral blood of CHC patients put on standard anti-viral treatment consisting of

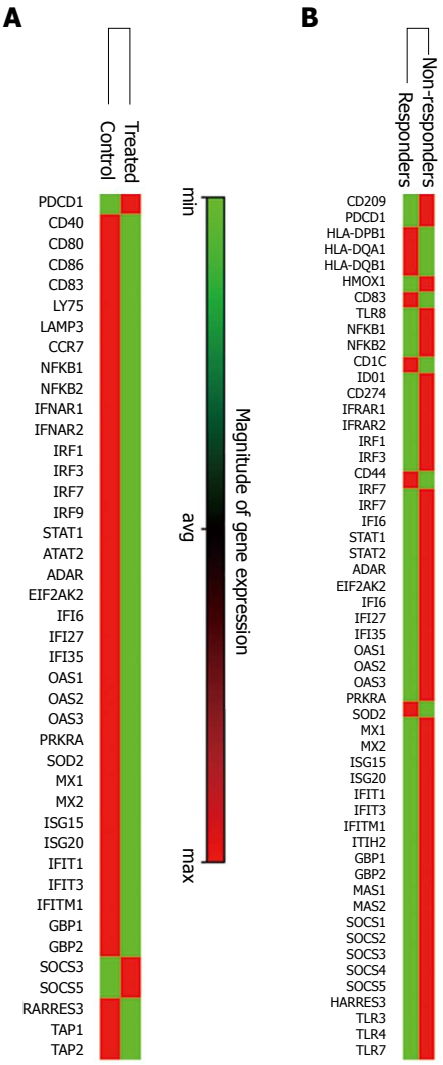

Figure 3 Clustergram showing up-regulated and down-regulated gene expression in dendritic cells of A: moDCs from healthy volunteer differentiated in presence of HCV viral proteins (Test group) and moDCs differentiated in absence of viral antigens (control group); B: Responders and non-responders. Graph showing the differentially expressed genes in different groups. Green represents the lower expression of a particular gene and red represents the higher expression of a particular gene in that particular group as compared to control.

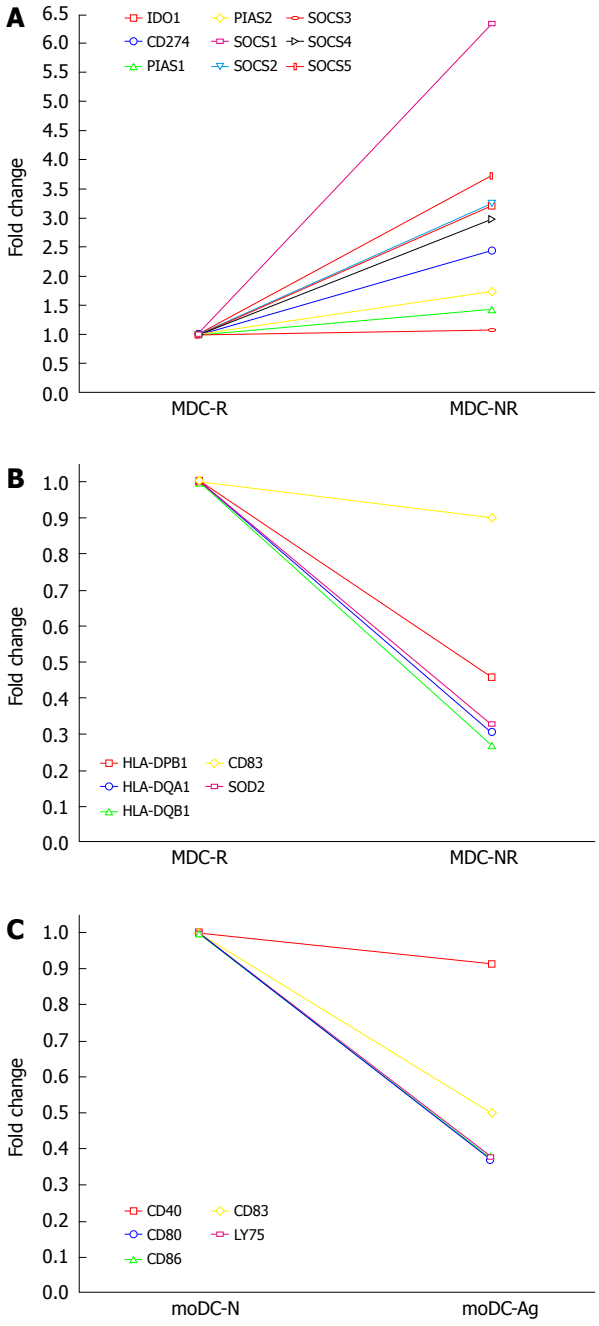

Figure 4 Multigroup plot showing A: up-regulation of down modulatory genes (negative regulators) in MDC-NR (non-responders) as compared to MDC-R (responders); B: Down-regulated genes in MDC-NR (Non-responders) as compared to the MDC-R (Responders); C: Down-regulation of genes involved with DC maturation and co-stimulatory molecules in the cells differentiated in the presence of viral antigens (moDC-Ag) as compared to cells without antigens (moDC-N). The genes which are negative regulators of JAK-STAT such as suppressors of cytokine signaling (SOCS), protein inhibitors of activated STATs are up-regulated in non-responders as compared to responders whereas the genes which belong to MHC class Ⅱ family such as HLA-DPB1, HLA-DQA1 and HLA-DQB1 and dendritic cell maturation marker such as CD83 is down-regulated in non-responders as compared to responders.

Type 1 IFN and ribavirin, some of those who achieved SVR were termed "responders" and those who did not achieve SVR were termed "non-responders". The differentially expressed genes were identified after analysis of the expression profile results. Further, these findings were confirmed in an *ex vivo* moDC model where gene expression profiles were analyzed in monocytes from a healthy donor, differentiated to DC, either in presence or absence of some HCV proteins, using same custom-designed PCR array. Interestingly results from both these experiments although not exactly overlapping, yet revealed the set of genes down-regulated in "non-responders" or in cells grown in presence of viral proteins were those, which are involved with DC maturation and function. Similarly the genes that were found to be up-regulated in these cells were mainly of the negative regulators of DC functions suggesting that the continuous presence of virus or viral proteins in individuals infected with HCV, would facilitate the development of functionally

defective phenotype of DCs in these individuals.

The monocytes cultured and differentiated in the presence of HCV viral proteins to dendritic cells in a culture system *ex vivo,* in the present study, induced the development of a defective phenotype of DCs with hampered maturation capabilities in a similar manner and also confirmed the findings from CHC patient experiments as described above. The analysis of gene expression profiles of these cells revealed downregulated expression of some important genes associated with DC maturation (*LAMP3* and *LY75*), co-stimulatory signaling (*CD80* and CD86) and many immune-stimulatory genes, the ISGs (*EIF2AK2*, *IFI27*, *OAS1*, *OAS2*, *MX1*, *ISG20*, *IFIT3*, *IFIT1*, *GBP1* and *GBP2*), which are the first line of defense in innate antiviral immunity, suggesting that persistence of HCV down-modulates the host defense mechanisms and make conditions favorable for its own survival. These results are consistent with the earlier reports, which also indicated that different HCV viral proteins disrupt the host IFN signaling and ISGs to establish chronic infection[22].

Entry of the virus in the host results in up-regulation of many TLRs like *TLR2*, *TLR3*, *TLR4*, *TLR7*, *TLR8* and *TLR9* on PBMCs and monocytes[23]. The expression of *TLR3*, *TLR4* and *TLR7* was also found to be increased on the MDC of non-responders in our study, suggesting the immune activation due to the constant presence of viral RNA. Besides, *RARRES* (Retionic acid receptor responder protein 1), which is also activated by viral RNA, was also upregulated in non-responders. The *TLR7* and *RARRES* cause *IRF7* activation and induction of Type1 IFN gene, leading to activation of JAK-STAT signaling and upregulated expression of *STAT1*, *STAT2* and *IRF9*, which further lead to enhanced expression of ISGs namely *IFIT1*, *IFIT3*, *ISG15*, *ISG20*, *ADAR*, *GBP1*, *PRKRA*, *EIF2AK2* (PKR), *IFITM1*, *MX1*, *MX2*, *OAS1*, *OAS2*, *IFI16*, *IFI27* and *IFI35*. The administration of exogenous IFN and upregulation of ISGs may be effective to a certain limit because virus replication and copy number gets significantly reduced from baseline to week 4 (RVR) in these patients, but complete removal was not achieved as viral load was still detectable, which suggests that there are other factors that are associated with the persistence of the virus.

The possible reasons may be attributed to dampening of the immune response (functionally impaired immature CD4+ cells) by Type 1 IFN, which leads to impaired T-cell immunity as evident in these patients. Moreover the genes important for optimal antigen presentation like MHC-Ⅱ (*HLA-DP*, *HLA-DQ*) and co-stimulatory molecules (*CD80*, *CD86*) as well as the homing receptors like *CCR7* were all found to be down-regulated in MDC from non-responder patients as compared to responders in our study, which again supports the hypothesis that such maturation defective DCs with hampered antigen-presenting and migration capabilities would be responsible for the generation of functionally impaired set of immature T-cells, which are incapable of clearing the virus in CHC. Also, it has been reported earlier that excessive or prolonged IFN-αβ signaling is associated with severe disease in HIV infection and favors the replication of virus in the host[23]. Although HCV has different mechanisms to down-regulate Type 1 IFN production, still ISGs are induced in infected hepatocytes in most of the chronically infected CHC patients. Earlier reports suggest that HCV patients having high pre-existing levels of ISGs are less likely to respond to IFN-α therapy in comparison to those with lower levels[24].

Besides, the genes for factors such as *SOCS1*, *SOCS2*, *SOCS4* and *SOCS5* which negatively regulate the inflammatory pathways such as JAK-STAT signaling[25]; *PDL1* responsible for exhaustion of T-cell function and its blocking on DCs shown to enhance T-cell activation[26,27]; *IDO1* which alters DC by decreasing its APC function and capable of suppressing local T-cell immune responses and promoting systemic tolerance[28], were all up-regulated in non-responders. Our findings corroborate the study reported earlier that up-regulation of PD-1 and SOCS-1 inhibitory molecules mediates functional impairment of the early immune response during HCV infection[29].

The expression of CD209 (DC-SIGN), a molecule expressed more on immature DCs and involved in innate immune responses also plays a critical role in viral pathogenesis, was also upregulated in non-responders[30,31]. Immature DCs bind more strongly to E1 and E2 (HCV Envelope proteins) through DC-SIGN with a difference in internalization pathway. These HCV viral like particles are targeted to non-lysosomal compartment in immature DCs and are protected from lysosomal degradation[32]. Thus, HCV may use DC-SIGN as an entry portal and facilitate viral infection of nearby hepatocytes and also use these DCs as reservoirs resulting in establishment of viral infection. HIV gp120 also binds to DC-SIGN and results in horizontal and vertical transfer and also helps in spreading the virus in the host[33].

HCV induces chronic increase in hepatic oxidative stress which plays an important role in pathogenesis of HCV[34]. Expression of genes of the factors involved with stress conditions such as Heme Oxygenase (*HMOX1*) was up-regulated in non-responders, suggesting higher oxidative stress in these patients. Since the *HMOX1* catabolizes heme to bilirubin, this might be responsible for significantly higher bilirubin levels observed in non-responders as compared to responders. The *HMOX1* being mainly expressed in immature DCs and its overexpression induces down-regulation of co-stimulatory molecules on DCs, might be responsible for inhibition of T-cell proliferation in CHC[35]. On the other hand, *SOD* responsible for transforming toxic superoxide anion radicals, showed down-regulated expression in non-responders resulting in increased levels of reactive oxygen species (ROS) and oxidative stress in these patients, might

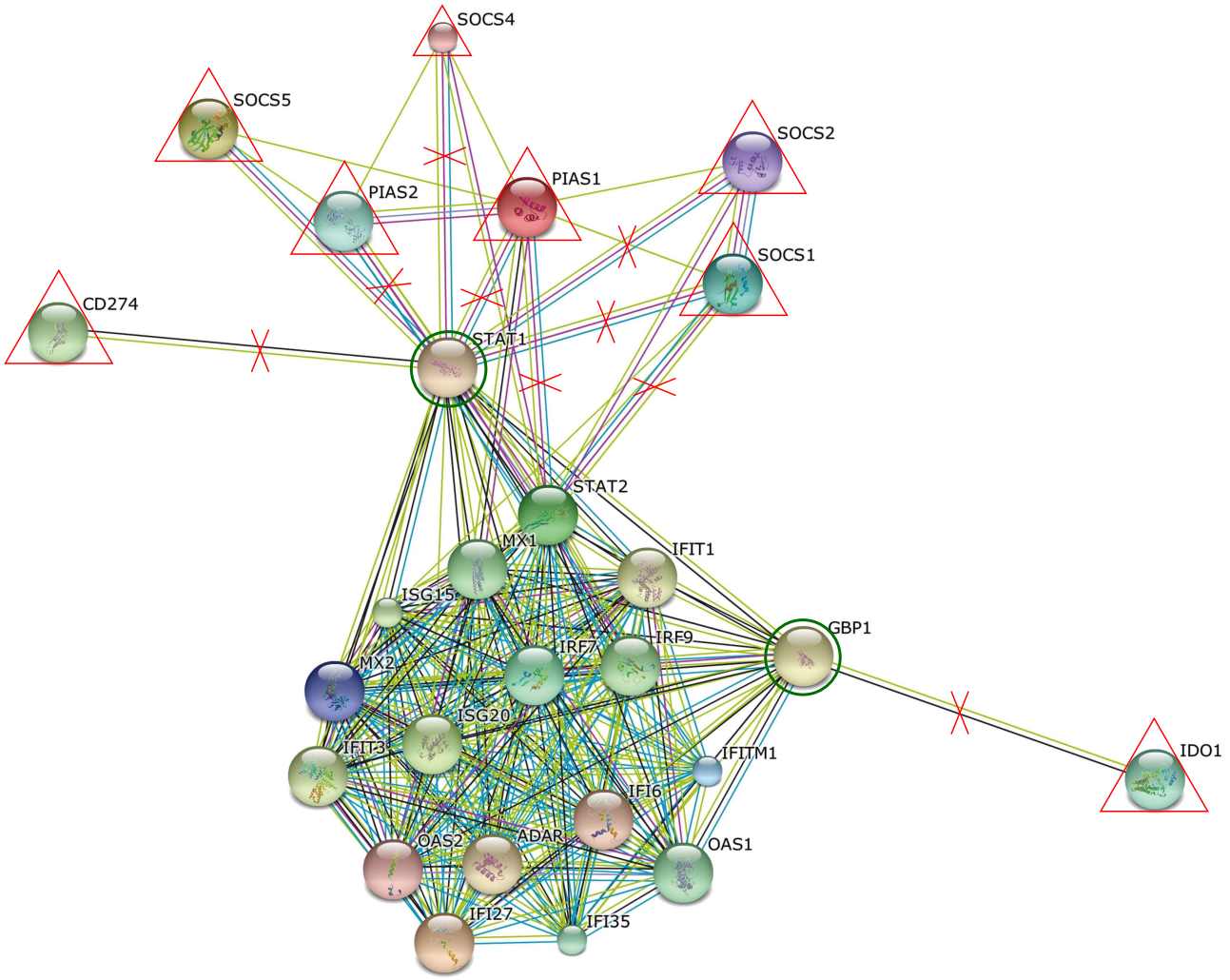

Figure 5 Interactions between different interferon stimulatory genes and negative regulatory genes of immune system. Using string software, possible interaction pathways between different Interferon Stimulatory genes and Negative regulatory genes were drawn. Negative regulators such as SOCS, PIAS when upregulated results in the inhibition of *STAT* and *GBP* genes which in turn result in the induction of transcription of many genes involved in innate immunity and interferon signaling such as *IRF7*, *IRF9* and *ISG20*. ISG: Interferon stimulatory genes.

be responsible for increased apoptosis of cells, which is also supported by up-regulated expression of *FAS* gene observed in these patients in our study. Thus, these patients are unable to destroy superoxide anion radicals, which are normally produced within the cells and are toxic to biological systems. Earlier report has also shown increased levels of *HMOX1* and decreased levels of *SOD2* in PBMC of patients with CHC[36].

In summary, our study indicates that there is up-regulation of negative regulators and down-regulation of molecules involved with maturation and antigen-presentation on DCs of non-responders. This imbalanced state, possibly modulated by the continuous replication of HCV, results in the generation of maturation-defective phenotype of DCs which are not capable of presenting the viral antigens to the naïve T-cells and lead to the generation of functionally defective immature T-cells incapable of clearing the virus (Figure 5). Whether this defective state of DCs in these patients is the cause or effect of viral persistence, is not really clear, but possibly this vicious cycle might be the cause of non-responsiveness to anti-viral therapy. Never the less, the study points to some novel pathways that may be targeted to achieve better management of this chronic disease.

COMMENTS

Background

In patients infected with hepatitis C, it had already been reported that dendritic cells are numerically, functionally and phenotypically dysfunctional. Also functionally defective monocyte-derived dendritic cells (DCs) from chronic hepatitis C (CHC) patients who did not achieve sustained virological response (SVR) failed to reconstitute the capacity to mature, indicating the dysfunctional status of DC in CHC patients, however the molecular mechanisms regulating this defect have not been elucidated.

Research frontiers

Previous experiments have indicated that CHC patients having dysfunctional dendritic cells led to therapy non-responsiveness in these patients.

Innovations and breakthroughs

The expression profile of selected genes related to hepatitis C virus (HCV) infection have been studied in various hepatocytes cell lines but, the role

of dendritic cells in non-responsiveness to antiviral therapy has not been elucidated as yet.

Applications

The molecular profile of dendritic cells on their role to standard therapy may be used in better prognosis and will help in designing newer therapeutic modalities that might help in better management of non-responders who do not respond to extended regimens to antiviral therapy.

Terminology

SVR - HCV RNA negative 24 wk after cessation of treatment. It is the best predictor of a long-term response to treatment.

Peer-review

The paper of Tomer S *et al* discusses the effects of HCV on DCs isolated from PBMC of IFNα-treated HCV patients. The comparisons in gene expression have been done between the responders and non-responders to treatment *vs* DC from healthy donors exposed or not to HCV proteins. This is an interesting attempt to elucidate the role of HCV-infection in impairment of DC function and to link this situation to non-responsiveness to IFNα treatment, which provides a promising connection to translational research.

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ORIGINAL ARTICLE

Basic Study

miR-106b promotes cancer progression in hepatitis B virus-associated hepatocellular carcinoma

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Abstract

AIM: To investigate the effect of miR-106b on tumor progression in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC).

METHODS: A total of 120 patients who underwent liver resection for HCC at National Cheng Kung University Hospital were enrolled in the present study. MicroRNA (miRNA) array was first used to screen the miRNA expression profiles in HCC patients. The clinical records were retrospectively analyzed, and correlations with the miRNA expression profiles were evaluated. The mRNA expression levels of the miR-106b-25 cluster (miR-106b, miR-93 and miR-25), and MCM7 in tumor and non-tumor samples were quantitated using quantitative real-time reverse transcription-polymerase chain reaction (q-RT-PCR) analysis, and correlations in the levels of miR-106b, miR-93 and miR-25 expression were calculated. Kaplan-Meier overall and disease-free survival rates of HBV-associated HCC patients were analyzed using the log-rank test based on miR-106b expression. The comparison of the miR-106b expression levels in patients with different clinical outcomes was analyzed using Mann-Whitney *U* tests. Furthermore, a hepatitis B virus X protein (HBx) expression plasmid was transfected into Huh7 and Hep

3B cells. The expression levels of the miR-106b-25 cluster and MCM7 in HBx-expressing Huh7 and Hep 3B cells were detected using q-RT-PCR.

RESULTS: miRNA array screening showed that miR-106b and its cluster, miR-93 and miR-25 were up-regulated in HCC patients ($P < 0.01$). The value of miR-106b expression in HBV-associated HCC patients was significantly higher than that in HCV- ($P < 0.05$) or non-B/non-C- ($P < 0.001$) associated HCC patients. The expression of the miR-106b-25 cluster was significantly higher in tumor tissue ($P < 0.001$) and associated with the host gene, MCM7, in clinical specimens from HBV-associated HCC patients. Furthermore, the expression levels of miR-106b, miR-93 and miR-25 were positively correlated in HBV-associated HCC tissues (miR-106 *vs* miR-93, $r = 0.75$; miR-93 *vs* miR-25, $r = 0.69$; miR-106b *vs* miR-25, $r = 0.33$). The overall and disease-free survival curves showed that high-miR-106b expression was correlated with the poor prognosis of HBV-associated HCC. HCC differentiation was significantly correlated with miR-106b expression ($P < 0.05$). Lower miR-106b expression levels resulted in the well differentiation of HCC. Moreover, the expression of the miR106b-25 cluster and MCM7 was up-regulated in Huh7 and Hep 3B cells after transfection with the HBx expression plasmid.

CONCLUSION: The data obtained in the present study suggests that HBx enhances miR-106b transcription to promote tumor progression in HBV-associated HCC.

Key words: miR-106b; Hepatitis B virus; Hepatocellular carcinoma; Tumor progression; Hepatitis B virus X protein

Core tip: The role of miR-106b in tumor progression of hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) and how it be regulated are still unclear. In this study, we analyzed the expression levels of miR-106b in HBV-associated HCC tissues and correlated the data with clinical records of patients. Our results indicated that miR-106b expression was up-regulated and related with tumor progression in HBV-associated HCC. In addition, hepatitis B virus X protein may contribute to enhance the transcription of miR-106b. These findings provide potential diagnostic and therapeutic targets for HBV-associated HCC.

Yen CS, Su ZR, Lee YP, Liu IT, Yen CJ. miR-106b promotes cancer progression in hepatitis B virus-associated hepatocellular carcinoma. *World J Gastroenterol* 2016; 22(22): 5183-5192 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5183.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5183

INTRODUCTION

Hepatocellular carcinoma (HCC) ranks among the 10 most common cancers in the world and is a major cause of cancer death in Southeast Asian countries[1,2]. The carcinogenesis of HCC is a multi-factor, multi-step and complex process, associated with chronic and persistent hepatitis B virus (HBV) infection[3,4]. Chronic hepatitis infection causes liver inflammation damage, subsequent fibrosis, liver cell regeneration and liver cell proliferation leading to the malignant transformation of the liver[5]. Most HCC patients die as a result of the rapid tumor progression and hepatic resection or transplantation is the only potential curative treatment for HCC patients when the HCC is diagnosed early[6]. However, the effective diagnostic and therapeutic targets remain unclear.

Several mechanisms of HBV-related tumorigenesis have been proposed[5]. HBV X (HBx) protein has recently been implicated as an oncoprotein in HBV-related tumorigenesis and HCC progression[7,8]. Previous studies have shown that HBx modulates cytoplasmic signal transduction pathways, such as Ras/Raf-1, through the transactivation of cellular signaling molecules to promote HCC proliferation[9].

MicroRNAs (miRNAs) are small non-protein coding gene (19-22 or 19-25 nucleotides) with important role in the regulation of gene expression at the post-transcriptional level[10]. Studies have demonstrated that miRNA plays a role in the regulation of fundamental cellular processes, including development and proliferation, cell fate determination and apoptosis[10,11]. Nearly 60% of human genes are controlled through miRNAs[11]. Several studies have shown that miRNA might affect on numerous types of cancer, and the dysregulation of miRNA has been associated with certain cancer types[11-14]. The dysregulated miRNA promotes or suppresses tumorigenesis through the down-regulation tumor suppressor gene or oncogene expression[15,16]. The miR-106b-25 polycistron is located within intron 13 of the minichromosome maintenance protein 7 (MCM7) genes on chromosome 7q22.1[17]. The results of previous sequence study indicated that miR-106b-25 is homologous with the known oncogene miR-17-92[18]. Previous studies have shown that the miR-106-25 cluster is overexpressed as a group of oncogenic miRNAs in many cancer types including prostate cancer, breast cancer, and gastric cancer[14,19-21]. MCM7, the host gene of the miR-106b-25 cluster, belongs to a family of the minichromosome maintenance (MCM) complex, comprising six replication proteins including MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7 (termed MCM2-7). Previous studies have implicated MCM7 in the replication licensing and synthesis of DNA[22,23]. The expression of MCM7 can be a prognostic indicator in diverse cancers, such as prostate cancer, ovarian cancer, endometrial cancer,

Table 1 Characteristics of hepatocellular carcinoma patients in the present study[1]

Characteristics	Patient numbers *n* (%)		
	1st Cohort (*n* = 12)	2nd Cohort (*n* = 108)	Total (*n* = 120)
Gender			
Male	8 (67)	86 (80)	94 (78)
Female	4 (33)	22 (20)	26 (22)
Age (yr)			
< 50	0 (0)	15 (14)	15 (13)
⩾ 50	12 (100)	93 (86)	105 (87)
Viral infection			
HBV	5 (42)	108 (100)	113 (94)
HCV	6 (50)	0 (0)	6 (5)
Non-B/Non-C	1 (8)	0 (0)	1 (1)
HCC differentiation			
Well	1 (8)	20 (19)	21 (18)
Moderate	8 (67)	76 (70)	84 (70)
Poor	3 (25)	11 (10)	14 (11)
Unknown		1 (1)	1 (1)
Pathological staging			
Stage Ⅰ	1 (8)	39 (36)	40 (33)
Stage Ⅱ	9 (75)	51 (47)	60 (50)
Stage Ⅲ	2 (17)	18 (17)	20 (17)

[1]A total of 120 hepatocellular carcinoma (HCC) patients were divided into two cohorts. A total of 12 HCC patients with distinct types of HCC were included in the 1st cohort, and the remaining 108 hepatitis B virus (HBV)-associated HCC patients were enrolled in the 2nd cohort.

etc[24-26]. Moreover, the dysregulation of MCM7 might be involved in tumor development and associated with the miR-106b-25 cluster.

In the present study, we analyzed the expression levels of miR-106b in HBV-associated HCC tissues and correlated the data with the clinical records of patients to clarify the role of miR-106b in tumor progression and regulation in HBV-associated HCC. These results indicated that miR-106b expression is up-regulated and associated with tumor progression in HBV-associated HCC. In addition, HBx might enhance miR-106b transcription. Thus, these findings highlight a potential diagnostic marker and a therapeutic target for HBV-associated HCC.

MATERIALS AND METHODS

Patients and HCC tissue

A total of 120 patients who underwent liver resection for HCC at the National Cheng Kung University Hospital from September 2012 to July 2015 were enrolled in the present study. Informed consent regarding use of specimens for this research was obtained from all patients and all protocols were reviewed and approved through the National Cheng Kung University Hospital Institutional Review Board. The patients were regularly followed up at clinical visits every 1 to 3 mo after curative surgery. The patients included 94 (78%) males and 26 (22%) females ranging in the age from 34 to 90 years (mean age 61.6 years). The median follow-up time was 35 mo (range, 1 to 118.8 mo). At the end of the follow-up, 25 patients had died of disease. HCC patients were divided into two study cohorts: 12 patients with distinct types of HCC were included in the 1st cohort to screen the miRNA expression profile using miRNA array, and the other 108 HBV-associated HCC patients were enrolled in the 2nd cohort for further analysis of the role of miR-106b in HBV-associated HCC. The characteristics of the HCC patients are listed in Table 1. The HCC tissue specimens were collected during surgery. The clinical records of the patients were retrospectively analyzed and correlated with the miRNA expression profiles. In the survival analysis, the mean of miR-106b level in adjacent non-tumor tissues was defined as the threshold (0.4 arbitrary unit from q-RT-PCR analysis). Samples with miR-106b expression levels higher than the threshold were classified into the "high-expression of miR-106b" group. Patients with levels lower than the threshold were classified into "low-expression of miR-106b" group. The overall and disease-free survival rates of patients were calculated using the Kaplan-Meier analysis.

Cells

Human hepatocellular carcinoma, Hep-3B 2.1-7 and Huh7 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) and minimum essential medium (MEM, Hyclone) containing 10% fetal bovine serum (FBS, GIBCO) and 100 IU of penicillin, 100 μg of streptomycin, and 0.25 μg of amphotericin B per milliliter, respectively. The cells were cultured in a humidified incubator with 5% CO_2 at 37 ℃.

RNA extraction and real-time RT-PCR

Total RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. A total of 500 ng RNA was used to synthesize cDNA using a quantitative reverse transcription (RT) kit (Qiagen). The expression levels of miRNAs were analyzed using the TaqMan MicroRNA Assay Kit (Applied Biosystems) according to the manufacturer's instructions. The mRNA levels of human GAPDH and MCM7 were detected using the validated specific primers/probes of TaqMan Gene Expression Assays (Thermo Fisher Scientific) and the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific). Real-time PCR assays were performed using the StepOne Real-Time PCR System (Applied Biosystems). The signals for miRNAs and inducible cellular MCM7 mRNAs were normalized to a small nuclear RNA, RUN48 and the mRNA signal of the housekeeping gene, human GAPDH.

Plasmid and transfection

The HBx protein expression plasmid was isolated and purified using the Plasmid Midi Kit (Qiagen). The Plasmid was transiently transfected into Hep-3B 2.1-7 and Huh7 cells using HyfectTM DNA transfection

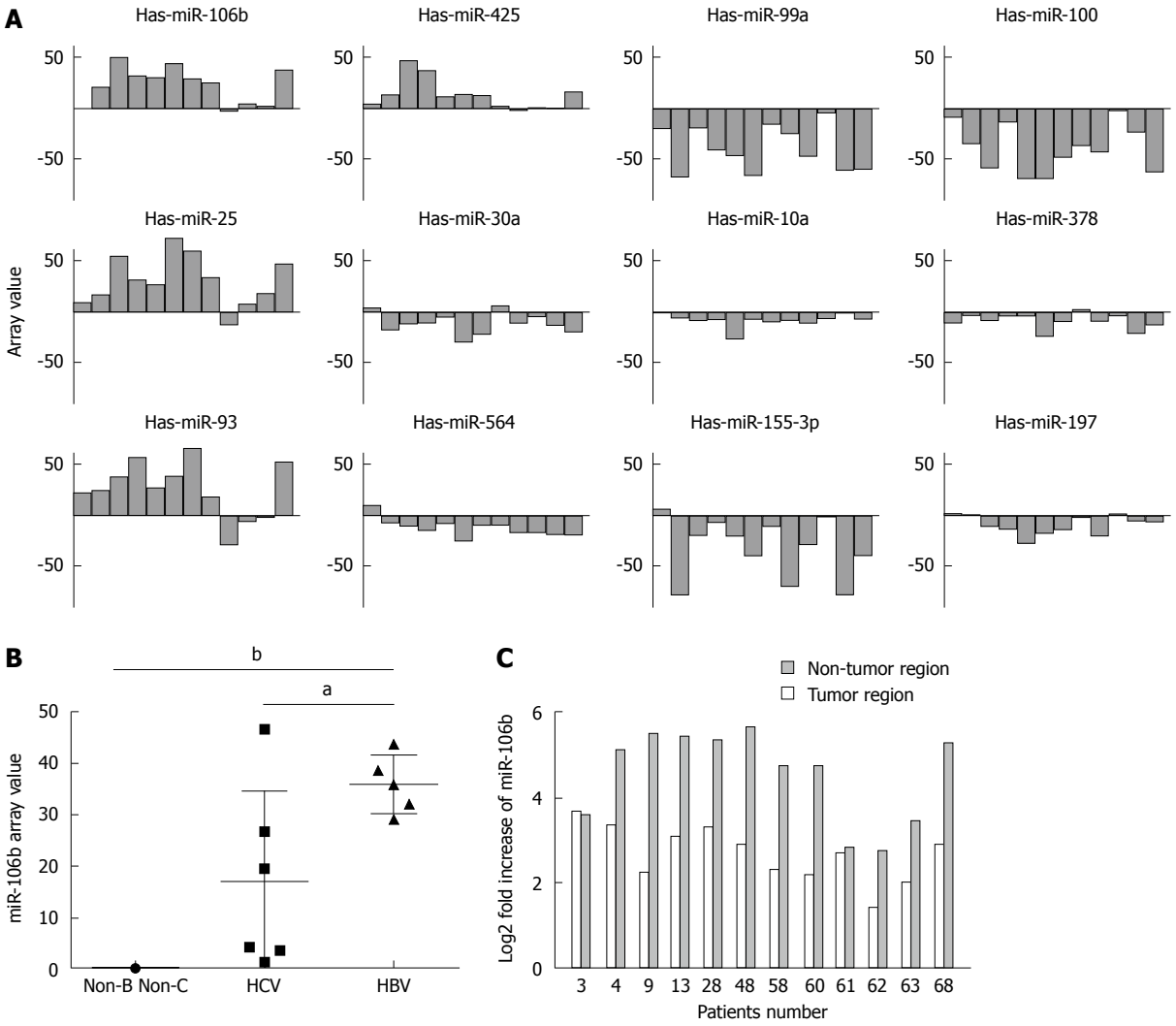

Figure 1 miRNA array analysis of the miRNA expression patterns in patients with distinct types of hepatocellular carcinoma (*n* = 12). A: The top 12 miRNAs significantly dysregulated in the tumor regions of hepatocellular carcinoma (HCC) patients based on statistical results ($P < 0.01$); B: miR-106b expression levels in tumor regions of patients with hepatitis B virus (HBV) -associated HCC, HCV-associated HCC, and non-B/non-C HCC; C: q-RT-PCR analysis of miR-106b expression values in the tumor and adjacent non-tumor regions of patients. Fold-increases were calculated after comparing the results with the miR-106b expression levels in normal liver samples. Data represent the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* HCV.

reagent (LEADGENE) according to the manufacturer's instructions. The expressions of HBx protein in the cell lines was confirmed using q-RT-PCR with HBx-specific primers/probe.

Statistical analysis

Statistical evaluation was completed using GraphPad Prism software version 5.01 (GraphPad, Inc., San Diego, CA, United States). The normal distribution of variables was assessed prior to selecting the tests to use for statistical analyses. The *W* value for the Shapiro-Wilk's method and the *D* value for the Kolomogorove method were used in the tests for normality. The values of miRNAs and MCM7 mRNA were analyzed using either the nonparametric one-way analysis of variance or unpaired *t* test, and the survival rates were analyzed using log rank analysis. The correlation between the patient outcomes and the miR-106b expression profiles were analyzed using Mann-Whitney *U* tests. The results are expressed as the mean ± SEM. A *P* value of less than 0.05 ($P < 0.05$, $P < 0.01$, $P < 0.001$) was considered significant.

RESULTS

miR-106b expression was up-regulated in HCC patients

Dysregulated miRNAs is a common characteristic of human tumors that could play an important role in oncogenesis or tumor suppression. To investigate the different miRNA expression profiles in HCC patients, we used miRNA array to analyze the miRNA expression patterns in 12 patients with distinct types of HCC including HBV-associated HCC, HCV-associated HCC, and non-B/non-C HCC in the 1st study cohort. The top 12 dysregulated miRNAs in HCC patients are listed in Figure 1A. miR-106b and the members of its associated cluster, miR-93 and miR-25 were up-regulated in HCC patients (Figure 1A). The value of

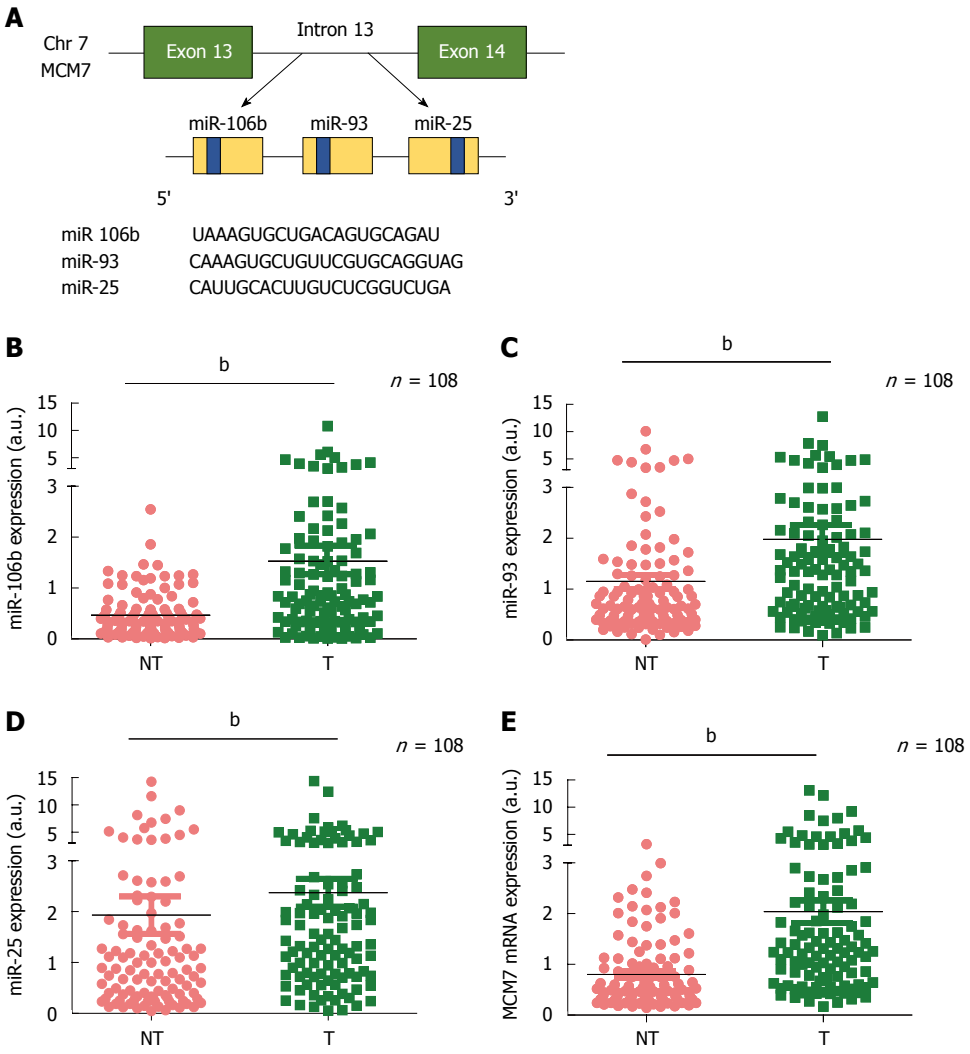

Figure 2 The mRNA expression levels of the miR-106b-25 cluster and MCM7 in tumor and non-tumor regions of hepatitis B virus-associated hepatocellular carcinoma patients (*n* = 108). A: Schematic representation of the miR-106b-25 cluster of miRNA (miR-106b, miR-93 and miR-25) within the 13th intron of the MCM7 gene. The yellow boxes represent pre-miRNAs. The blue boxes represent mature miRNAs; B-E: miR-106b (B), miR-93 (C), miR-25 (D), and MCM7 (E) expression levels in the tumor and non-tumor regions of hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) patients, determined using q-RT-PCR. $^{b}P < 0.001$ NT *vs* T. a.u.: Arbitrary unit.

miR-106b expression in HBV-associated HCC patients was significantly higher than that in HCV- ($P < 0.05$) or non-B/non-C- ($P < 0.001$) associated HCC patients (Figure 1B). Furthermore, the levels of miR-106b expression in the 12 patients were confirmed using q-RT-PCR. In most cases, the values of miR-106b in the tumor regions were higher than in non-tumor regions (Figure 1C). These results indicated that miR-106b was significantly up-regulated in the tumor regions of HCC patients, particularly HBV-associated HCC patients.

miR-106b-25 cluster was co-transcribed with its host gene, MCM7 in HBV-associated HCC

miR-106b is located in an intergenic region embedded within intron 13 of the MCM7 gene in chromosome 7q22.1. This miRNA belongs to a cluster comprising miR-93 and miR-25 (Figure 2A). To determine whether the miR-106b promoter transcribes the associated gene or this gene is co-transcribed with the host gene, MCM7, Mass array EpiTyper was performed to detect the methylation landscape of MCM7 in the 12 patients. The results demonstrated that only the promoter and 3'-UTR of MCM7 could be detected within the methylation landscape suggesting that miR-106b is also co-transcribed with its host gene, MCM7 in HCC (data not shown). To further confirm whether the expression of miR-106b is higher in the tumor tissues of HBV-associated HCC patients, we expanded the sample size to 108 patients and validated the miRNA levels through q-RT-PCR in the 2nd study cohort. The results showed that the miR-106b levels were significantly higher in tumor tissues compared with normal tissues ($P < 0.001$) and this phenomenon was observed in more than 70% of HBV-associated HCC patients (Figure 2B). In addition, the expression of miR-93 and miR-25 was significantly up-regulated in the tumor tissues of HBV-associated HCC patients ($P <$

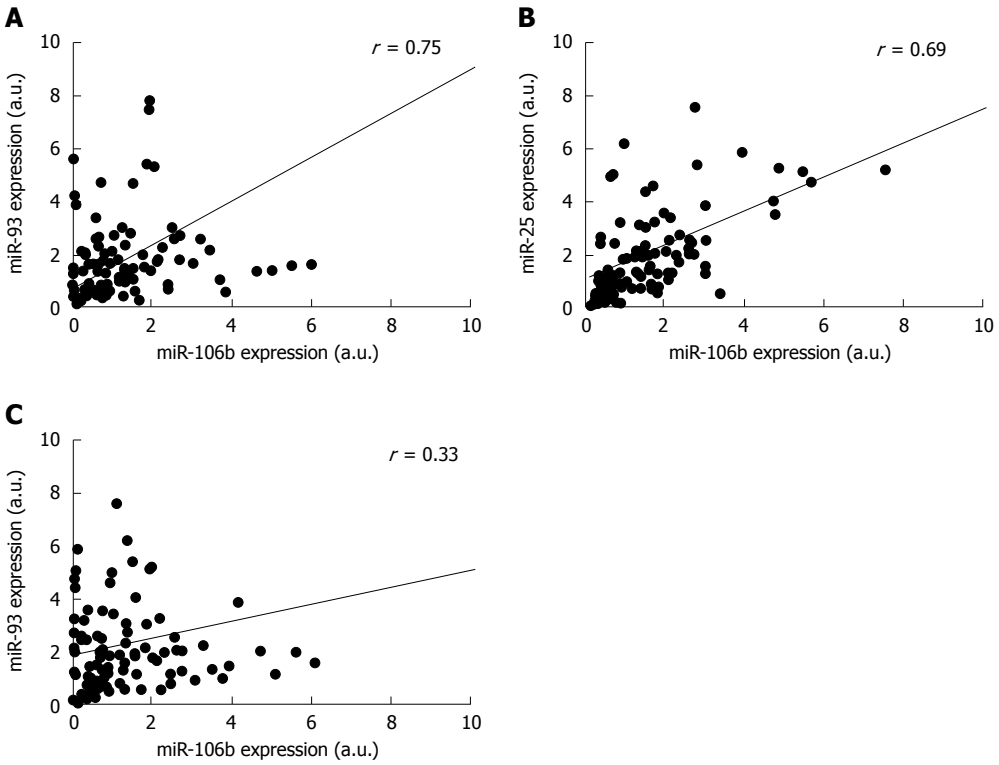

Figure 3 The correlation analysis miR-106b *vs* miR-93, miR-106b *vs* miR-25, and miR-93 *vs* miR-25 expression in tumor regions of patients with hepatitis B virus-associated hepatocellular carcinoma (*n* = 108). A: The correlation between miR-106b and miR-93 expression; B: The correlation between miR-106b and miR-25 expression; C: The correlation between miR-93 and miR-25 expression. Data represent the correlation coefficient (r). a.u.: Arbitrary unit. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus.

0.001) (Figure 2C and D). The mRNA expression level of the MCM7 gene was also significantly increased in tumor regions compared with the adjacent non-tumor regions ($P < 0.001$) (Figure 2E). Furthermore, the expression of miR-106b, miR-93 and miR-25 showed a positive correlation in HBV-associated HCC tissues (miR-106 *vs* miR-93, $r = 0.75$; miR-93 *vs* miR-25, $r = 0.69$; miR-106b *vs* miR-25, $r = 0.33$) (Figure 3A-C). These results indicated that the miR106b-25 cluster is up-regulated in the tumor regions and co-transcribed with its host gene, MCM7 in HBV-associated HCC.

Up-regulation of miR-106b expression corresponds with decreased survival time in HBV-associated HCC patients

We further evaluated the relationship between the miR-106b expression and clinical outcomes in HBV-associated HCC patients; the patients were divided into two groups, high miR-106b expression and low miR-106b expression and the overall and disease-free survival rates in these two groups were analyzed. The data showed a negative correlation between the miR-106b expression level and survival time of HBV-associated HCC patients (Figure 4A and B). Relatively poor overall and disease-free survival rates were observed for the individuals in the high miR-106b expression group (overall survival in the 5th year: 65%; disease-free survival in the 5th year: 40%) compared with the low miR-106b expression group (overall survival in the 5th year: 93%; disease-free survival in the 5th year: 57%) ($P < 0.05$). These results demonstrated that poor prognosis is correlated with HBV-associated HCC patients with higher miR-106b expression.

miR-106b expression levels are correlated with HCC differentiation

The demographic and clinical features of patients were retrospectively analyzed and correlated with the miR-106b expression profiles to determine the specific features associated with miR-106b expression. HCC differentiation but not underlying liver disease, microvascular invasion, tumor number, tumor size, recurrence after surgery, and pathological staining were significantly correlated with miR-106b expression (Table 2). The miR-106b expression level in patients with well HCC differentiation (2.24 ± 0.44 a.u.) was significantly lower than that in patients with moderate (5.32 ± 1.00 a.u.) and poor HCC differentiation (4.85 ± 1.02 a.u.) (well *vs* moderate, P=0.0359; well *vs* poor, P=0.0145). These results indicated that low levels of miR-106b expression result in well HCC differentiation.

HBx promotes miR-106b expression in HCC cells

Next, we investigated the mechanism of how miR-106b expression is regulated in HBV-associated HCC. HBx protein is necessary for HBV replication and acts as a trans-activator for the modulation of the signaling

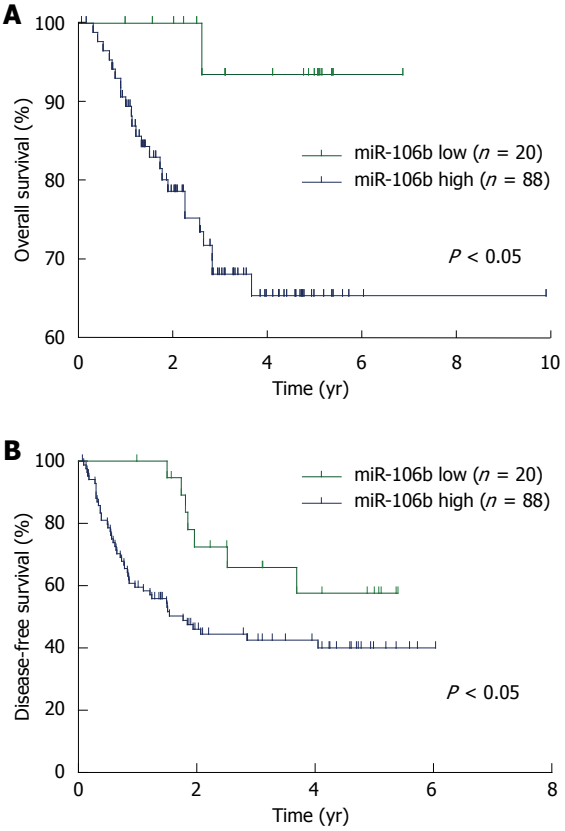

Figure 4 Kaplan-Meier overall and disease-free survival curve of patients with hepatitis B virus-associated hepatocellular carcinoma based on miR-106b expression (*n* = 108). Hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) patients were divided into two groups including miR-106b low (*n* = 20) and miR-106b high (*n* = 88) groups based on the expression levels of miR-106b. A: Overall survival curve; B: Disease-free survival curve. Log-rank test was used for statistical analysis. $P < 0.05$ was considered significant.

Table 2 Correlation analysis of clinical outcomes with miR-106b expression profiles (*n* = 108)[1]

	Patient numbers *n* (%)	*P* value	Significant[2]
Underlying liver disease		0.374	NS
Liver cirrhosis	39 (36)		
Non-cirrhosis	69 (64)		
HCC differentiation			
Well *vs* moderate	24 (22) *vs* 74 (69)	0.036	[a]
Well *vs* poor	24 (22) *vs* 9 (8)	0.015	[a]
Moderate *vs* poor	74 (69) *vs* 9 (8)	0.238	NS
Unknown	1		
Microvascular invasion		0.856	NS
Yes	30 (28)		
No	78 (72)		
Tumor number		0.798	NS
Single tumor	87 (81)		
> 1 tumor	21 (19)		
Tumor size (cm)[3]		0.812	NS
< 5	72 (67)		
⩾ 5	36 (33)		
Recurrence after surgery		0.687	NS
Yes	58 (54)		
No	50 (46)		
Pathological staging			
Stage Ⅰ *vs* stage Ⅱ	39 (36) *vs* 51 (47)	0.968	NS
Stage Ⅰ *vs* stage Ⅲ	39 (36) *vs* 18 (17)	0.625	NS
Stage Ⅱ and stage Ⅲ	51 (47) *vs* 18 (17)	0.575	NS
		Total patients, *n* = 108	

[1]The patient characteristics are summarized based on the clinical outcomes. Demographic and clinical features of patients were retrospectively analyzed and correlated with the miR-106b expression profiles; [2]The correlation between miR-106b expression values and the patient numbers of each clinical outcome parameter were analyzed using Mann-Whitney *U* tests. [a]$P < 0.05$ was considered significant; [3]Tumor size represents the maximum diameter of the tumor nodule. The diameter of the largest nodule was 16 cm. NS: No significant difference; HCC: Hepatocellular carcinoma.

pathways involved in the HBV replication and HCC development. To determine whether HBx protein contributes to the regulation of miR-106b expression in HBV-associated HCC, we used an HBx protein over-expression system. Huh7 and Hep 3B cells were transiently transfected with an HBx-expression plasmid, and subsequently the expression levels of the miR-106b-25 cluster and MCM7 were analyzed. The levels of miR-106b, miR-93, and miR-25 were significantly increased (all $P < 0.05$), peaking at 6 h post transfection in both Huh7 and Hep 3B cells (Figure 5A and B). The MCM7 mRNA levels were also gradually increased after 6 h post transfection compared with the un-transfected control group in both Huh7 ($P < 0.01$) and Hep 3B ($P < 0.05$) cells (Figure 5C and D). These results suggested that the HBx protein might contribute to the up-regulation of the miR-106b-25 cluster and MCM7 in HBV-associated HCC.

DISCUSSION

HCC ranks as the third leading cause of cancer-related deaths worldwide with increasing cases in many countries[1,27]. Increasing evidence has shown that many miRNAs are dysregulated in HCC and play a crucial role in the development of HCC by affecting cell proliferation, apoptosis, migration, *etc*[28]. Therefore, the identification of a key miRNA associated with tumor progression in HCC could provide an accurate marker for diagnosis and a new direction for a novel therapeutic approach. In the present study, we found that miR-106b plays an important role in tumor progression in HBV-associated HCC.

Recent studies have demonstrated that several miRNAs are involved in the life cycle and infectious processes of HBV and HBV-associated liver diseases, including fibrosis, cirrhosis and HCC[29,30]. Herein, we observed the up-regulation of the miR-106b-25 cluster in HCC, particularly in HBV-associated HCC. The expression of miR-106b, miR-93 and miR-25 was positively correlated in HBV-associated HCC tissues, consistent with the results of previous study showing a similar correction pattern in gastric cancer, suggesting the co-transcription of miR-106b-25 in biosynthesis[31]. Interestingly, the correlation coefficients between each other were not consistent, indicating that additional mechanisms might be involved in the regulation of miRNA expression. Notably, the up-regulation of

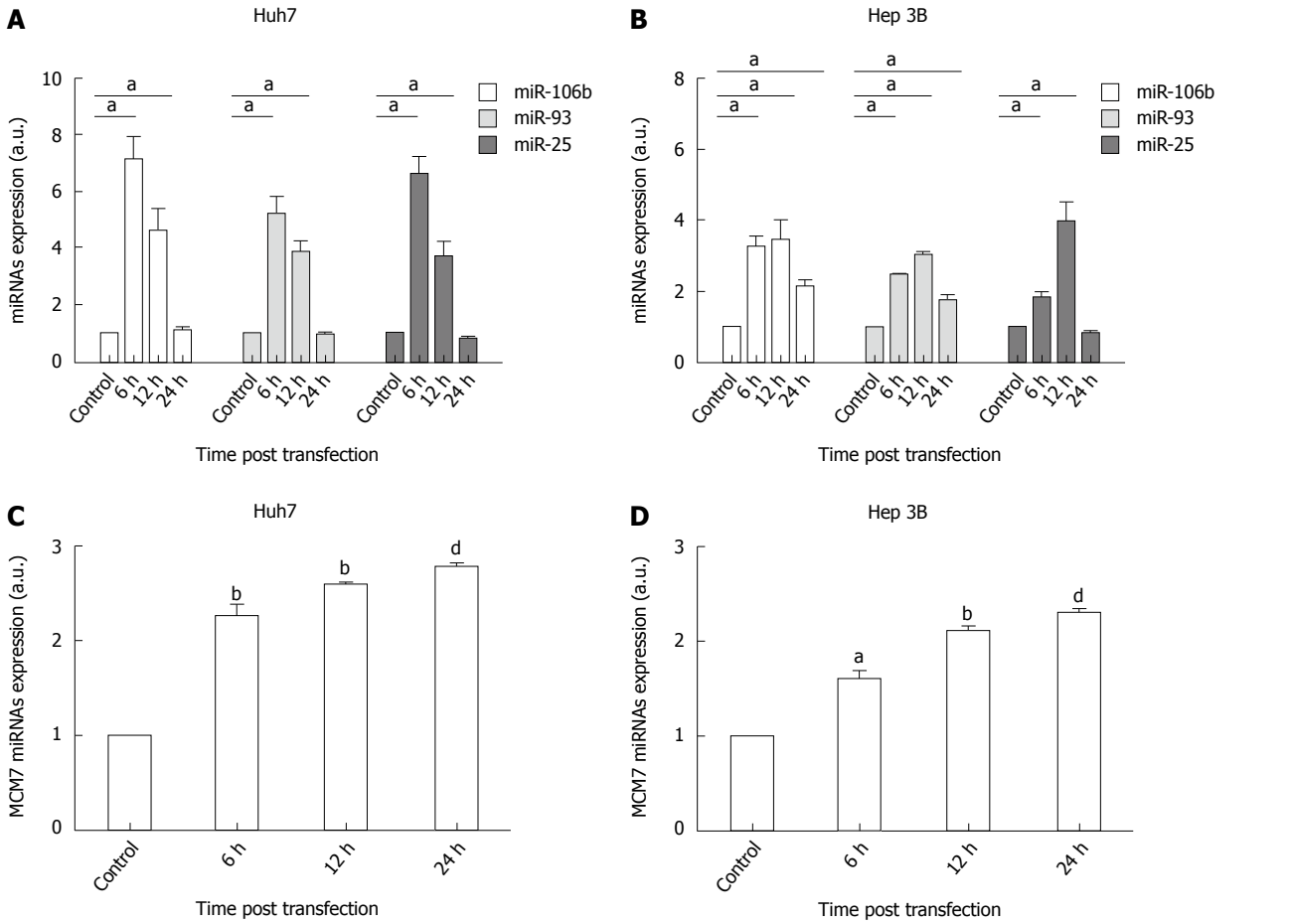

Figure 5 The mRNA expression levels of miR-106b-25 cluster and MCM7 in hepatitis B virus X protein-transfected hepatocellular carcinoma cell lines (*n* = 3). The hepatitis B virus X protein (HBx) protein expression plasmid was transiently transfected into Huh7 and Hep-3B cells. miR-106b, miR-93, miR-25, and MCM7 expression levels at 0 (control), 6, 12, and 24 h post transfection were detected using q-RT-PCR. A: miRNAs expression levels in HBx-transfected Huh7 cells; B: miRNAs expression levels in HBx-transfected Hep-3B cells; C: MCM7 expression levels in HBx-transfected Huh7 cells; D: MCM7 expression levels in HBx-transfected Hep-3B cells. Data represent the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control. a.u.: Arbitrary unit. HCC: Hepatocellular carcinoma.

miR-106b but not miR-93 and miR-25 expression corresponds to decreased survival times, increased recurrence rates and HCC differentiation in HBV-associated HCC patients (data not shown). This effect might reflect the different biological functions of individual miRNAs. Previous studies have indicated that miR-106b and miR-93 directly target the cell-cycle inhibitor, CDKN1A (p21), and miR-25 inhibits cell apoptosis through the down-regulation of a the pro-apoptotic gene, *BCL2L11* (Bim), in gastric cancer[31]. Other studies have shown that miR-106b is not only involved in cell cycle inhibition but might also play an anti-apoptosis role in cancer cells[32]. The data obtained in the present study, support the idea that miR-106b has a greater effect on promoting tumor progression compared with the other members in the same cluster.

However, HBV-associated protein regulates the expression of several miRNAs to assist with viral replication and survival and HCC development[33,34]. In a previous study, we showed that HBx protein up-regulates mTOR signaling through IKKβ to increase cell proliferation and VEGF production in HCC[35]. Furthermore, HBx protein is highly expressed in the cytoplasm of hepatocytes after HBV infection, thereby promoting tumorigenesis through the induction of mitochondrial dysfunction, involving several signaling pathways associated with tumorigenesis through the regulation of non-coding RNAs (ncRNAs) and epigenetic changes[8,34,36]. The results of present study showed that HBx over-expression promoted the transcription of miR-106 in HCC cell lines. This finding might explain why miR-106b was remarkably up-regulated in HBV-associated HCC but not in other types of HCC. However, the precise regulatory mechanism of HBx in miR-106b expression should be further investigated.

Based on these results, the miR-106b-25 cluster was co-transcribed with its host gene, MCM7 in HBV-associated HCC. Previous studies have indicated that MCM proteins are involved in critical steps of DNA synthesis[37]. MCM proteins bind to DNA replication origins during the initiation step, and subsequently the MCM proteins provide the helicase activity to unwind the template DNA ahead of the fork for DNA elongation. In primary gastric tumors and normal mucosa, the mRNA expression of MCM7 is precisely

correlated with the expression of the miR-106b-25 cluster[18]. The detailed regulatory mechanism between miR-106b-25 and MCM7 and whether MCM7 is involved in the miR-106b-mediated influence on HBV-associated HCC need to be further examined.

In conclusion, the results of present study indicate that miR-106b is up-regulated and co-transcribed with its host gene MCM7 in HBV-associated HCC. The up-regulation of miR-106b expression corresponds with a decrease in survival time, and an increase in the recurrence rate and HCC differentiation in HBV-associated HCC patients. Furthermore, HBx over-expression increased the RNA levels of the miR-106b-25 cluster and MCM7 in human hepatocellular carcinoma cell lines. These results suggest that HBx enhances the transcription of miR-106b to promote tumor progression in HBV-associated HCC. These findings provide a potential diagnostic marker and therapeutic target for HBV-associated HCC.

COMMENTS

Background

MicroRNAs (miRNAs) are involved in the progression of numerous types of cancers. Chronic hepatitis B virus (HBV) infection is one of the major risks for hepatocellular carcinoma (HCC), and through the regulation of miRNA expression, the virus promotes carcinogenesis in HCC.

Research frontiers

HBV infection not only induces liver inflammation but also produces viral oncoproteins to influence HCC progression. Previous studies have reported that many miRNAs are dysregulated in the HBV-associated HCC. However, the role of miRNA in tumor progression and regulation remains unclear. In the present study, the authors report that the hepatitis B virus X protein (HBx) protein enhances miR-106b expression to promote HCC progression.

Innovations and breakthroughs

Previous studies regarding the role of miRNA in tumors are limited in the use of correlation analyses and use small-scale cohort studies to address this issue. In addition, the function of the HBx protein in HCC progression is controversial. This work represents the first large-scale cohort study demonstrating that the miR-106b-25 cluster and its host gene, MCM7, are overexpressed in HBV-associated HCC. The results also suggest that the HBx protein enhances miR-106b transcription to promote tumor progression.

Applications

Because miR-106b is up-regulated in patients with HBV-associated HCC and correlates with poor disease outcome, these finding could provide a novel diagnostic marker and a therapeutic target for HBV-associated HCC.

Peer-review

In this manuscript the authors investigated the effect of miR-106b on tumor progression in HBV-associated HCC in a clinical model. This study provides evidence that enhanced transcription of miR-106b with its host gene MCM7 in HBV-associated HCC is associated with tumor progression and poor outcome. This study is well designed and the results are acceptable to draw the conclusions stated herein.

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ORIGINAL ARTICLE

Basic Study

Chitooligosaccharides promote radiosensitivity in colon cancer line SW480

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Abstract

AIM: To investigate the anti-proliferation and radiosensitization effect of chitooligosaccharides (COS) on human colon cancer cell line SW480.

METHODS: SW480 cells were treated with 0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL of COS for 48 h. CCK-8 assay was employed to obtain the cell survival ratio of SW480 cells, and the anti-proliferation curve was observed with the inhibition ratio of COS on SW480 cells. The RAY + COS group was treated with 1.0 mg/mL of COS for 48 h, while both the RAY and RAY+COS groups were exposed to X-ray at 0, 1, 2, 4, 6 and 8 Gy, respectively. Clonogenic assay was used to analyze cell viability in the two groups at 10 d after treatment, and a cell survival curve was used to analyze the sensitization ratio of COS. The RAY group was exposed to X-ray at 6 Gy, while the RAY+COS group was treated with 1.0 mg/mL of COS for 48 h in advance and exposed to X-ray at 6 Gy. Flow cytometry was employed to detect cell cycle and apoptosis rate in the non-treatment group, as well as in the RAY and RAY + COS groups after 24 h of treatment.

RESULTS: COS inhibited the proliferation of SW480 cells, and the inhibition rate positively correlated with the concentration of COS ($P < 0.01$). Cell viability decreased as radiation dose increased in the RAY and RAY+COS groups ($P < 0.01$). Cell viabilities in the RAY+COS group were lower than in the RAY group at all doses of X-ray exposure ($P < 0.01$), and the sensitization ratio of COS on SW480 cells was 1.39. Compared with the non-treatment group, there was a significant increase in apoptosis rate in both the RAY

and RAY + COS groups; while the apoptosis rate in the RAY+COS group was significantly higher than in the RAY group ($P < 0.01$). In comparing these three groups, the percentage of G_2/M phase in both the RAY and RAY + COS groups significantly increased, and the percentage of the S phase and G_0/G_1 phase was downregulated. Furthermore, the percentage in the G_2/M phase was higher, and the percentage in the S phase and G_0/G_1 phase was lower in the RAY + COS group *vs* RAY group ($P < 0.01$).

CONCLUSION: COS can inhibit the proliferation of SW480 cells and enhance the radiosensitization of SW480 cells, inducing apoptosis and G_2/M phase arrest.

Key words: Chitooligosaccharides; Cancer of colon; Radiotherapy; Radiosensitization; Apoptosis; Cell cycle

Core tip: In this study, the colorectal cancer cell line SW480 that is homologous with colon-rectum was used as the research tool. It was confirmed that chitooligosaccharides (COS) not only directly blocked SW480 cell proliferation, but also enhanced radiotherapy effects. Furthermore, COS induced a large amount of SW480 cell apoptosis, and induced a large number of cells to remain in the G2/M phase with radiation-sensitive killing effect. Thus, the sensitivity of SW480 cells to radiation was effectively enhanced 1.39 times. This is beneficial for the therapeutic effect.

Han FS, Yang SJ, Lin MB, Chen YQ, Yang P, Xu JM. Chitooligosaccharides promote radiosensitivity in colon cancer line SW480. *World J Gastroenterol* 2016; 22(22): 5193-5200 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5193.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5193

INTRODUCTION

According to the latest Chinese cancer epidemic profile survey, the 2010 cancer morbidity and mortality rates in China were 235.23/100000 and 148.81/100000, respectively. Among these cancers, colorectal cancer incidence and mortality have shown an obvious predominantly evident upward trend again, causing this to be the focus of medical experts[1-4]. In China, colorectal cancer has a prevalence of 16.14%, allowing a leap up to fifth place among cancers; and the incidence in males is as high as 18.75%. In 2012, over 250000 colorectal cancer cases were added nationwide; this total accounts for 18.6% in the world[5,6]. China has become a big focus at the forefront of colorectal cancer research, since efficient and low toxicity treatments are needed. Since the discovery of radiation, radiotherapy has lasted for centuries as the main indispensable weapon against cancer that is active in clinic. Statistics have shown that more than 70% and 50% of cancer patients are in need of this kind of therapy in China and the United States, respectively[7,8]. Although radiotherapy has great significance for cancer treatment, killing cancer cells could injure healthy tissues, causing malignant complications. This has been a researcher's hurdle that is difficult to bypass. However, radiosensitizers then emerged as a necessity of the times. Chitooligosaccharides (COS) are products of chitin, having good solubility and a high absorption rate, making the ratio of the carbohydrate polymer more advantageous for biological applications; and they have become the new favorite of medical researchers[9,10]. Although there have been many reports about COS anticancer effects[11-14], there are few studies on the applications of radiation sensitization. In this study, human colon cancer SW480 cells were selected and treated with COS application in parallel with radiation, to verify that COS can enhance radio sensitivity for colorectal cancer cell line; this is reported below. We expect to further explore this superior and low-damage anticancer therapies.

MATERIALS AND METHODS

Seven COS concentration levels were established and 3-hole samples were simultaneously cultured in parallel with each level SW480 cells were subcultured to the logarithmic phase (human colon cancer cells SW480; Shanghai Cell Institute of Chinese Academy of Sciences). After digestion, the cells were diluted to a concentration of 5×10^4 cells/mL according to the 0.1 mL/hole access in a 96-well plate. A suitable environment was set (CO_2 incubator, Shanghai Gemtop Scientific Instrument CO.,Ltd.) for adherent growth, and diluted COS (Chitooligosaccharides, Shanghai Huich Biotech Inc.) was replaced after 24 h with fresh medium at 0.11 mL/hole, and added into each well at COS concentrations of 0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL After 48 h of COS application, CCK-8 reagent (CCK-8 Kit, Shanghai Liruibio Technology Co., Ltd.) was added along the pore walls at 0.01 mL/hole. Then, the culture plate was tapped to mix reagent and culture medium. After four hours of sufficient reaction, OD absorbance at $\lambda = 450$ nm was detected at all levels. The experiment was repeated three times to investigate the inhibitory effect of COS on SW480 cell proliferation. Accordingly, 1.0 mg/mL of COS concentration was selected for follow-up studies.

Six X-ray dose levels were established and levels were divided into the RAY group and RAY+COS group; each group was simultaneously cultured in parallel with the 3-hole sample. According to 0-, 1- and 2-Gy dose levels at 200/well, 4- and 6-Gy dose levels at 400/well, and 8-Gy dose level at 800/well inoculation amounts, respectively, different concentrations of single-cell suspensions in 6-well culture plate were set in an incubator with a suitable environment for growth

Table 1 Inhibition of chitooligosaccharides for the proliferation of SW480 cells

COS concentration (mg/mL)	OD (mean ± SD)	Inhibition rate
0	1.019 ± 0.007	-
0.5	0.969 ± 0.005[1]	4.91%
1.0	0.908 ± 0.008[2]	10.89%
2.0	0.804 ± 0.006[3]	21.10%
3.0	0.692 ± 0.007[4]	32.09%
4.0	0.580 ± 0.006[5]	43.08%
5.0	0.433 ± 0.008[6]	57.51%
P	0	-
F	137.6	-

Compared with COS concentrations in the 0 mg/mL Group: [1]P = 0.000, q = 17.437; [2]P = 0.000, q = 31.326; [3]P = 0.000, q = 80.047; [4]P = 0.000, q = 99.096; [5]P = 0.000, q = 142.849; [6]P = 0.000, q = 165.379. COS: Chitooligosaccharides.

adherence. After six hours, appropriate amounts of COS were added into each well to reach a 1.0 mg/mL concentration in the RAY + COS group, while equal amounts of infiltrating medium were added into each hole and cultured for 48 h in the RAY group. Both groups were stamped with 1-cm thick tissue analogs in the culture plates and X-ray irradiated (Electron linear accelerator, Nanjing Chuang Rui Ying Biotechnology Co., Ltd.) at a distance of 100 cm with a dose rate of 2 Gy/min. Incubation continued for 10 d, the cells were washed, fixed and stained again; then, the number of cells were counted as 50 or more units of cell clusters. The experiment was repeated three times for statistical data, and the cell survival curve was draw up from the final slope of the D_0 value obtained by sensitizing ratio SER = $D_{0\,(RAY)}/D_{0\,(RAY+COS)}$.

The three groups were established simultaneously in parallel with the 3-hole samples. The concentration of 1 × 10^5/mL cell suspension was inoculated into 6-well culture plate with a suitable growth-adherent environment for 24 h. In the RAY + COS group, the correct amount of COS was added to reach a 1.0 mg/mL concentration. In the non-treatment group and RAY group, equal amounts of medium were added. In the RAY group and RAY + COS group, infiltration was carried out for 48 h and samples were exposed to 6-Gy X-ray irradiation. After replacing with fresh medium, cells were cultured for 48 h. After digestion, rinsing, dilution and other treatments were performed to determine the cell cycle stage and extent of apoptosis in each group.

Statistical analysis

Using SPSS19.0 statistical software for statistical analysis, OD value, survival rate, apoptosis rate and cell cycle distribution ratios were presented as mean ± SD. OD values for each COS concentration level and cell survival rates at X-ray dose levels were compared among multiple groups using ANOVA analysis and compared between the two groups using SNK-q test. For the RAY group and RAY + COS group, cell survival rates under different X-ray doses, cell cycle control experiments among the three groups, and apoptosis rates between the three groups were compared by *t*-test. $P < 0.05$ was considered statistically significant.

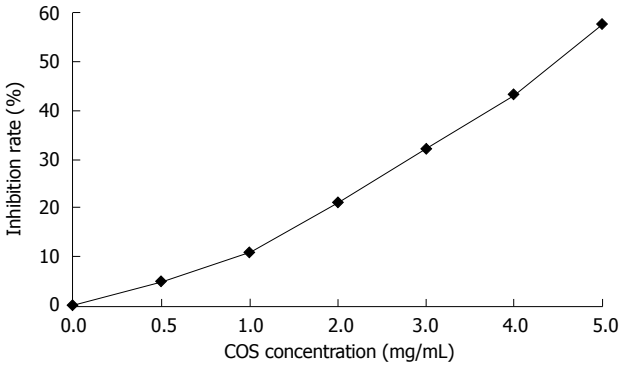

Figure 1 Inhibition curve of chitooligosaccharides for proliferation of SW480 cells.

RESULTS

Inhibitory effect of COS on SW480 cell proliferation

In comparing the COS concentration of the OD value in the 0 mg/mL group, OD values progressively reduced at all levels as COS concentration increased, and the difference was statistically significant ($P < 0.01$). After 48 h of treatment with 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 μmg/mL of COS concentration, SW480 cell survival rates were significantly lower than in the negative control group; and the differences were statistically significant ($P < 0.01$). The inhibition rate and the concentration of COS showed a positive correlation (Table 1, Figure 1).

Comparison of effects of different radiation dose on cell survival rate between groups

With an irradiation dose of 0 Gy as a reference standard, survival rate in the RAY and RAY + COS groups progressively reduced with increased radiation dose, and the difference was statistically significant ($P < 0.01$). The reduction in cell survival rate was greatest in the RAY + COS group. At dose levels 1, 2, 4, 6 and 8 Gy, survival rate in the RAY+COS group was significantly lower than in the RAY group, and the difference was statistically significant ($P < 0.01$). SER was 1.39 for COS in SW480 cells (Table 2, Figure 2).

Cell apoptosis rate between groups

Compared with proliferation in the non-treatment group, apoptosis rate in the RAY and RAY + COS groups increased sharply, the differences were statistically significant ($P < 0.01$). Apoptosis rate in the RAY+COS group also revealed a significant increase compared with the RAY group, and the difference was statistically significant ($P < 0.01$; Table 3, Figure 3).

Comparison of cell cycle distribution between groups

Compared with the non-treatment group, the proportion in G2/M phase in the RAY and RAY + COS

Table 2 Comparison of cells survival rates for different radiation doses between the RAY and RAY + COS group

Irradiation dose (Gy)	Cell survival rate (%)	
	RAY	RAY + COS
0	99.22 ± 3.51	99.17 ± 4.06[1]
1	90.67 ± 3.82	85.30 ± 3.38[2]
2	73.69 ± 3.45	56.11 ± 2.95[3]
4	45.95 ± 3.41	28.64 ± 2.76[4]
6	23.84 ± 2.20	12.53 ± 2.03[5]
8	8.68 ± 1.75	3.81 ± 1.16[6]
P	0	0
F	182.7	243.2

Compared with RAY group: [1]P = 0.978, t = 0.028; [2]P = 0.006, t = 3.158, [3]P = 0.000, t = 11.619; [4]P = 0.000, t = 11.837; [5]P = 0.000, t = 11.335; [6]P = 0.000, t = 6.959. COS: Chitooligosaccharides.

Table 3 Comparison of cell apoptosis rates among the three groups

Groups	Cell apoptosis rate
Non-treatment group	1.79 ± 0.37
RAY group	9.33 ± 1.05[1]
RAY + COS group	22.64 ± 1.27[2,3]

Compared with the control group: [1]P = 0.000, t = -20.318; [2]P = 0.000, t = -47.286; compared with the RAY Group: [3]P = 0.000, t = -24.232. COS: Chitooligosaccharides.

Table 4 Comparison of cell cycle distribution among the three groups

Groups	S (%)	G0/G1 (%)	G2/M (%)
Non-treatment group	30.15 ± 0.82	39.41 ± 1.05	30.44 ± 0.68
RAY group	22.48 ± 0.74[1]	30.51 ± 0.86[2]	47.01 ± 0.52[3]
RAY + COS group	18.89 ± 0.65[4,7]	24.34 ± 0.46[5,8]	56.77 ± 0.28[6,9]

Compared with the control group: [1]P = 0.000, t = 20.832; [2]P = 0.000, t = 19.672; [3]P = 0.000, t = -58.070; [4]P = 0.000, t = 32.283; [5]P = 0.000, t = 39.438; [6]P = 0.000, t = -107.412; compared with the RAY group: [7]P = 0.000, t = 10.935; [8]P = 0.000, t = 18.979; [9]P = 0.000, t = -49.577. COS: Chitooligosaccharides.

groups significantly increased, while the proportions in S and G0/G1 phase all reduced; the differences were statistically significant (P < 0.01). Compared with the RAY group, the proportions in S phase and G0/G1 phase in the RAY + COS group were smaller, while the G2/M phase was significantly longer; the differences were statistically significant (P < 0.01). See Table 4, Figure 4.

DISCUSSION

Rapid economic growth gives rise to the rapid development of science and technology. However, improvements in medical technology have failed in stopping cancer from affecting human health. Modern unhealthy diets and living habits stimulate and mainly cause the continuous rise in colorectal cancer

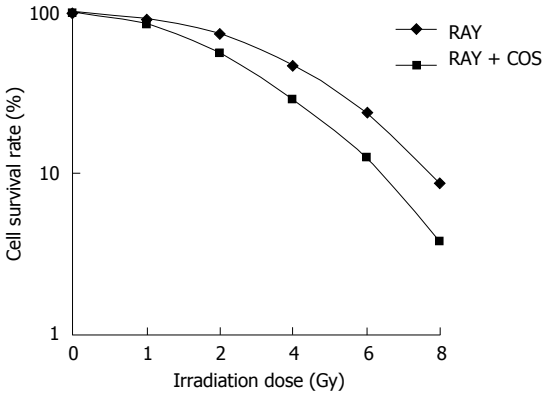

Figure 2 Cells survival rates at different radiation doses between the RAY and RAY + COS groups. COS: Chitooligosaccharides.

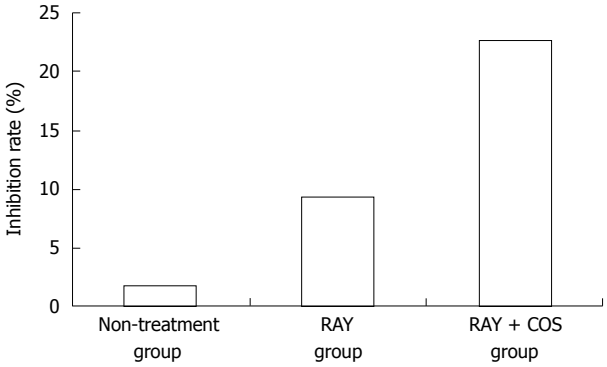

Figure 3 Cell apoptosis comparison between the three groups.

morbidity[15-17]. Statistical data from international cancer research institutions have indicated that China's 2012 annual new-onset cases of colorectal cancer reached 1.47 times that of cases in 2006, and the total incidence grew by nearly 50% in six years[18]. Regarding the incidence of colorectal cancer in China, the annual growth rate rose sharply to more than two times the international average[19]. This situation is not optimistic, and the exploration of more effective drugs and treatment without delay is of great significance.

Inhibitory effect of COS on proliferation of colon cancer SW480 cells

Derived from the depolymerization of chitosan, COS has been considered as the human healthy "almighty" guardian by the biomedical field. It can improve body acid-base balance, activate immune function, remove blood lipids, lower blood sugar, and regulate a variety of physiological activity[20-22]. Particularly, it has anti-tumor effects, which have been a research focus for domestic and foreign scholars in recent years. Based on historical reports, COS has a widespread growth-blocking effect on HL-60, RBL-2H3, SGC-7901 and tumor cell lines of other organs, and there are a variety of ways to achieve this effect[23-25]. In this study, the colorectal homologous colorectal SW480 cell line was the target. This study confirms that COS directly

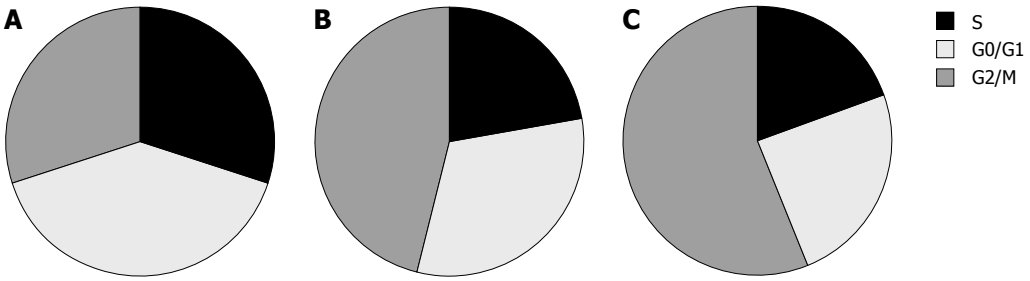

Figure 4 Comparison of cell cycle distribution among the three groups. A: Non-treatment group; B: RAY group; C: RAY + COS group. COS: Chitooligosaccharides.

blocks SW480 cell proliferation, and its inhibitory effect increases with the concentration of COS application showing increase in increments; 5 mg/mL of COS treatment for 48 h induced SW480 cell viability to decrease by 57.51%.

COS enhances SW480 cell sensitivity to radiation

Radiotherapy has an irreplaceable position in the treatment of cancer. It is widely used in various stages of the disease course; preoperatively it shrinks tumors to create conditions for radical enterectomy, it removes residues postoperatively to prevent recurrence, and it cooperates with chemotherapy to prevent metastasis. Early radiotherapy for nasopharyngeal carcinoma, skin cancer and cervical cancer has a possibility of more than 9% cure[26-28]. Early exposure to radiotherapy also can improve the 5-year survival rate from 70% to 80% for esophagus and prostate cancer[29,30]. Although radiotherapy has significantly prevents pain in patients with organ disease, there are also shortcomings. Radiation attack precision is limited, and often implicates normal tissue surrounding lesions, leading to tissue damage, which adds to the double burden of cancer patients both physically and mentally. This study found that X-rays have a mass destruction effect on SW480 cells, and cell death increased with radiation dose; while before radiotherapy, COS application obviously makes the cell survival rate decrease, COS can effectively increase SW480 cell sensitivity to radiation by 1.39 times. Furthermore, this controlled experiment has shown that the RAY + COS group had significantly improved treatment efficacy with the increase in apoptosis rate, and the cell cycle distribution changed significantly.

COS achieves radiosensitization by promoting SW480 cell apoptosis

Apoptosis maintains homeostasis in the body. However, it has an independent regulatory program that takes the initiative to open the "die" mode to conserve limited resources when cells are faced with adverse living conditions. Tumor cells lose this order of regulation and fall into a disordered and uncontrolled proliferation cycle. Radiation-induced apoptosis has been demonstrated for a long time[31-34]. COS combined with radiotherapy reverses the deactivation of the mechanism of apoptosis in a cancer cell to a greater degree, and pulls it back to its normal life trajectory. The complexity of the entire process of cell apoptosis involves cooperation of multiple genes and proteins. Krysko's research has indicated that COS can activate apoptosis promoter Caspase-3[35,36]. Tan believes that COS can damage mitochondrial membrane stability and release Cyt C into the cytosol[37]. Mates has also reported that COS can down-regulate environmental GSH activity and stimulate oxidative damage[38,39]. A number of conclusions have confirmed that COS has a positive effect on the apoptosis of tumor cells.

COS achieves radiosensitization by changing the SW480 cell cycle distribution

The mechanism of action of radiotherapy is to destroy DNA strand integrity including breaking the connection of ester bond sequences and destroying base modifications. From the initial point of its life cycle, radiotherapy blocks various physiological functions of the tumor cell and genetic information delivery[40,41]. Cell cycle distribution has a deep influence on radiotherapy[40,42-45]. Flow cytometry analysis revealed that COS treatment leaves a large number of SW480 cells stranded in the G2/M phase that is very sensitive to radiation, while reducing the G1 phase and S phase that are responsible for DNA damage repair, reducing the resistance of cancer cells to radiotherapy and enhancing its therapeutic effect. Radiation biological research pointed out that in order to ensure a smooth and orderly replication, the whole proliferation process speed is controlled at G1, S and G2 levels, respectively, by three regulatory processes[46-49], speculating that COS control in cell cycle distribution is most likely related to the start-up and expression of these three processes.

In summary, COS not only directly arrests SW480 cell growth, but also helps radiotherapy. COS induces SW480 cells apoptosis accompanied by a large number of proliferation process changes. Thus, this greatly enhances radiation lethality and has a beneficial therapeutic effect. In-depth exploration of COS as radiosensitizer is expected to bring a new dawn for the life of colorectal cancer patients.

COMMENTS

Background

Modern unhealthy diets and living habits are major causes that stimulate the prevalence of colorectal cancer to continue to soar. In China, colorectal cancer has a prevalence of 16.14%, which has clinically already leapt up to fifth place among cancers; and the incidence in males is as high as 18.75%. In 2012, over 250000 cases of patients were added nationwide; this total accounts for 18.6% in the world. China has become a big focus in the forefront of colorectal cancer research, and efficient and low toxicity treatments are needed. Although radiotherapy has great significance for cancer treatment, killing cancer cells could injure healthy tissues, causing malignant complications. This has been a researcher's hurdle that is difficult to bypass. Radiosensitizers have now emerged as a timely aid.

Research frontiers

In order to reduce the toxicity of radiotherapy, current research has focused on primarily two aspects: increase in the accuracy of positioning, and enhancement of radiation radiosensitivity of tumor tissues. Using radiation sensitizers has become popular because it is simple to apply. Previous studies have shown that radiosensitization mechanisms include improved cell hypoxia, increased DNA damage and influence of the cycle phase distribution. In addition to 5-fluorouracil, cisplatin and gemcitabine, the conventional radiotherapy sensitizers, C225, L-778-123 and COX-2 inhibitors and other new sensitizers have gained attention in recent years. Further interdisciplinary approaches have also started to introduce new drugs and new mechanisms of action in the field of radiation sensitizer agents.

Innovations and breakthroughs

Derived from the depolymerization of chitosan, COS has been considered as the human healthy "almighty" guardian by the biomedical field. It can improve body acid-base balance, activate immune function, remove blood lipids, lower blood sugar, and regulate a variety of physiological activity. Particularly, it has anti-tumor effects, which has been a research focus by domestic and foreign scholars in recent years. Based on historical reports, COS has a widespread growth blocking effect on HL-60, RBL-2H3, SGC-7901 and tumor cell lines of other organs, and there are a variety of ways to achieve this effect. Although there are many reports about COS anticancer effects, there are few studies on the applications of radiation sensitization. In this study, human colon cancer SW480 cells were selected and treated with COS application in parallel with radiation, to verify that COS can enhance radiosensitivity in colorectal cancer cell lines.

Applications

This study showed that chitooligosaccharides can effectively enhance the sensitivity of SW480 cancer cells to radiation; chitosan oligosaccharide combined with radiotherapy treatment would be helpful and promising for colorectal cancer. Regarding the effect of chitooligosaccharides on radiosensitization, an in-depth study would be expected to explore this efficient and low-damage anticancer therapeutic breakthrough.

Peer-review

This is a very interesting study about the chitooligosaccharide promotion of radiosensitivity in a colon cancer line. The study is well designed and the manuscript is well written.

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ORIGINAL ARTICLE

Basic Study

Faecalibacterium prausnitzii supernatant ameliorates dextran sulfate sodium induced colitis by regulating Th17 cell differentiation

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Author contributions: Huang XL and Yu CG performed the majority of experiments, analyzed the data, and wrote the paper; Zhang X, Fei XY, and Chen ZG participated equally in treatment of animals; Zhang X and Hao YP cultured cells; Zhang S, Zhang MM, and Yu YQ performed the molecular investigations; and Yu CG designed the research and revised the paper as corresponding author.

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Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Nanjing Drum Tower Hospital, Affiliated Hospital of Nanjing University Medical School.

Conflict-of-interest statement: The authors declare that there is no conflict of interest in this study.

Data sharing statement: No additional data are available.

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Abstract

AIM: To explore the preventive and therapeutic effects of *Faecalibacterium prausnitzii* (*F. prausnitzii*) supernatant on dextran sulfate sodium (DSS) induced colitis in mice.

METHODS: Forty C57BL/6J male mice were randomly

divided into four groups: control group, model group, treatment group, and prevention group. Mice were weighed daily. On day 10, the colon length was measured, the colorectal histopathologic damage score (HDS) was assessed, and plasma interleukin (IL)-17A, IL-6, and IL-4 levels were detected by enzyme-linked immunosorbent assay. The expression of transcription factor retinoic acid-related orphan receptor-γt (RORγt) and IL-17A in colon inflammatory mucosa tissue were determined by immunohistochemical assay, and the expression levels of RORγt mRNA, IL-17A mRNA, and IL-6 mRNA were detected by real-time quantitative polymerase chain reaction (PCR). The proportion of Th17 in mononuclear cells in spleen was assayed by fluorescence activated cell sorter.

RESULTS: When compared with the model group, the colon length ($P < 0.05$) and body weight ($P < 0.01$) in the treatment and prevention groups were significantly increased, and the colon HDS was decreased ($P < 0.05$ and $P < 0.01$). There was no statistical difference between the treatment group and prevention group. After treatment with *F. prausnitzii* supernatant, the plasma levels of IL-17A and IL-6 ($P < 0.05$), the protein and mRNA expression of IL-17A and RORγt, and the Th17 cell ratio of spleen cells ($P < 0.01$) were significantly decreased compared to the model group. Plasma IL-4 level in the prevention group was significantly higher than that in the model group ($P < 0.05$), but there was no significant difference between these two groups in the expression of IL-6 in both the plasma and colon mucosa tissues.

CONCLUSION: *F. prausnitzii* supernatant exerts protective and therapeutic effects on DSS-induced colitis in mice, probably *via* inhibition of Th17 differentiation and IL-17A secretion in the plasma and colon mucosa tissues. It can also improve colitis in mice by downregulating IL-6 and prevent colitis by upregulating IL-4.

Key words: *Faecalibacterium prausnitzii*; Ulcerative colitis; Animal model; Th17 cell; Treatment; Prevention

Core tip: *Faecalibacterium prausnitzii* (*F. prausnitzii*) supernatant has anti-inflammatory and immune regulatory activity. This study showed that the preventive and therapeutic use of *F. prausnitzii* supernatant could ameliorate dextran sulfate sodium (DSS)-induced colitis in mice by inhibiting Th17 cell differentiation and inflammatory cytokines release.

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INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are multifactorial ailments characterized by intestinal inflammation. Although the precise etiology and pathogenesis of IBD are not fully elucidated, multiple factors contribute to IBD, including genetic background, environment, intestinal flora imbalance, and immune disorder[1-4]. It has been hypothesized that an undesired intestinal mucosal immune response to intestinal flora imbalance contributes to the onset of IBD in genetically susceptible individuals.

A T helper (Th)17 cell is defined as a cell producing the cytokine interleukin (IL)-17A, but it also can secrete many other cytokines, such as IL-17F, IL-6, and IL-23, during an inflammatory response[5]. Th17 cells are characterized by the expression of the transcription factor retinoic acid-related orphan receptor (RORγt), and there is growing evidence that Th17 cells are paramount in the development of human autoimmune diseases, including IBD[6-8]. In the intestine of IBD patients, elevated numbers of Th17 cells and increased RORγt and IL-17 levels are found[9]. The differentiation of Th17 cells from naïve CD4+ T cells is known to be affected by multiple cytokines, such as transforming growth factor (TGF)-β, IL-6, IL-4, and IL-23[10,11]. IL-6 plays a key role in cooperating with TGF-β to initiate Th17 differentiation, while IL-4 inhibits Th17 differentiation.

Faecalibacterium prausnitzii (*F. prausnitzii*) is the major bacterium of the Clostridium leptum group, and is one of the most abundant anaerobic bacteria in the human gut[12]. *F. prausnitzii* plays an important role in maintaining the intestinal health and providing energy to the colonocytes[13]. A recent study indicated that *F. prausnitzii* levels were decreased in IBD patients compared with healthy controls[14]. Previously, we confirmed in animals that both the bacteria and its supernatant relieved trinitro-benzene-sulfonic acid induced colitis in rats[15]. Nevertheless, the specific mechanism is largely unclear.

Dextran sulfate sodium (DSS) induced colitis is a well-established animal model for IBD pathogenesis, and it has been used in preclinical studies for over two decades[16,17]. Furthermore, it has been shown that the clinical features and pathological changes of DSS-induced colitis in mice were similar to human UC[18]. Here, we determined whether the *F. prausnitzii* supernatant could relieve DSS-induced colitis in mice by reducing Th17 cells and inflammatory cytokines.

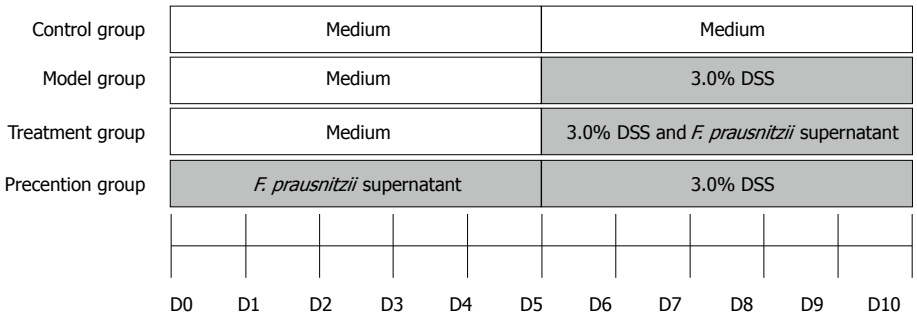


Figure 1 Flow diagram of the study design. DSS: dextran sulfate sodium; *F. prausnitzii*: *Faecalibacterium prausnitzii*.

MATERIALS AND METHODS

Animals

All experiments were approved by the Experimental Animal Ethical Committee of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School. Forty male C57BL/6J mice aged 8-10 wk and weighing 18-22 g were obtained from the Animal Center, Nanjing Drum Tower Hospital (Nanjing, China). The mice were allocated equally and randomly to four groups: control group, model group, treatment group, and prevention group. The group divisible design is shown in Figure 1. The period of observation was 10 d. In the first 5 d, the mice in the prevention group were given supernatant of *F. prausnitzii* (five times concentrated, 0.1 mL/10 g) through gavage once a day, while the other groups received the same dosage of medium. For the next 5 d, all groups, except for the control group, were treated with 3.0% DSS in their drinking water *ad libitum*, the treatment group was fed *F. prausnitzii* supernatant by gavage once a day.

Mice were weighed daily and sacrificed by cervical dislocation at day 10. Colons were dissected, and the distance from cecum to anus was measured. The colon tissues were fixed in 4% formalin for later pathological examination and immunohistochemical study. The peripheral blood and spleen were isolated for testing Th17 cells and cytokines.

F. prausnitzii culture

F. prausnitzii (ATCC 27766, Manassas, VA, United States) was cultured anaerobically at 37 ℃ in LYHBHI medium [main component of brain-heart infusion medium (37 g/L, BD, Franklin Lakes, NJ, United States), yeast extract (5 g/L, Oxoid, Basingstoke, United Kingdom), cellobiose (1 g/L, Sigma, St. Louis, MO, United States), maltose (1 g/L, Amresco, Solon, OH, United States), hemin (5 mg/L, Sigma), and cysteine (0.5 g/L, Sigma)]. The number of live bacteria (colony-forming units, CFU) was calculated according to optical density (OD) at 600 nm. The supernatant was collected from cultures with 109-1010 CFU/mL (OD = 1.9). Sterile culture medium acted as placebo. Bacterial supernatant and sterile culture medium were lyophilized and stored at -80 ℃. They were thawed and diluted to five times concentrated solution with phosphate buffered saline (PBS) before administration.

Colon histopathologic grading

The histopathologic grading of colon damage was scored by two blinded pathologists under microscope based on Neurath Scoring criteria as previously described[19]. In short, 4: transmural leukocyte infiltrations, high vascular density, loss of goblet cells, and thickening of the colon wall; 3: high level of leukocyte infiltration, thickening of the colon wall, high vascular density; 2: low level of leukocyte infiltration; 1: very low level of leukocyte infiltration; and 0: no inflammation.

Isolation of splenic mononuclear cells

Splenic mononuclear cells were isolated from spleens through Ficoll–Isopaue density gradient centrifugation[20]. Fresh spleens were placed in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Carlsbad, NY, United States) and mechanically disrupted by a 2 mL syringe plunger into cell suspensions. Cell suspensions were repeatedly aspirated with a sterile Pasteur pipette and gently filtered through a 200 μm strainer. Splenic single-cell suspensions were layered over an equal volume of Ficoll-Hypaque Solution (Haoyang BioScience Corporation, Tianjin, China) per spleen and centrifuged at 1500 rpm for 20 min. The band of leukocyte enriched fraction at the interface was collected after centrifugation at 1800 rpm for 10 min without brake. The resulting splenic mononuclear cell density was counted in a hemocytometer, and viability was assessed by Trypan blue staining.

Fluorescence activated cell sorter analysis of Th17 in mononuclear cells

Flow cytometry followed routine procedures by using 2 × 10^6 cells per sample. The splenic mononuclear cells were stimulated by phorbol-12-myristate-13-acetate (PMA), ionomycin, and brefeldin A for 5 h at 37 ℃ in a 5% CO2 incubator, then labeled with fluorescein isothiocyanate (FITC) anti-mouse CD4 (eBioscience, San Diego, CA, United States) and APC anti-mouse CD3 (eBioscience). After permeabilization and fixed

Table 1 Polymerase chain reaction primers gene sequences

Target gene	Primer sequence	Product length (bp)
ROR-γt	forward: GACGGCCAACTTACTCTTGG reverse: AGAAACTGGGAATGCAGTGG	109
IL-17A	forward: TCCCTCTGTGATCTGGGAAG reverse: CTCGACCCTGAAAGTGAAGG	154
IL-6	forward: CGGAGAGGAGACTTCACAGAG reverse: CATTTCCACGATTTCCCAGA	105
GAPDH	forward: CATGGCCTTCCGTGTTCCTA reverse: TGTCATCATACTTGGCAGGTTTCT	83

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; ROR-γt: Related orphan receptor-γt.

treatment, cells were labeled with PE anti-mouse IL-17 (eBioscience). The stained cells were tested by flow cytometry (BD, San Jose, CA, United States) and analyzed by the Cell Quest software.

Enzyme-linked immunosorbent assay cytokines in murine plasma

Cytokines (IL-17A, IL-6, IL-4) were measured using a commercially available enzyme-linked immunosorbent assay kit (Yunhan Biological Technology Corporation, Shanghai, China) according to the manufacturers' instructions.

Real-time quantitative polymerase chain reaction

Total RNAs were extracted from mid-colon samples taken from mice in each group using the Trizol reagent (Invitrogen, Carlsbad, CA, United States) with the following procedure. The concentration was determined by NanoDrop TM 1100 (NanoDrop Technologies, Wilmington, DE, United States). Total RNA was reversely transcribed into cDNA using reverse transcription kit. The polymerase chain reaction (PCR) reactions were performed in a 96-well Optical Reaction Plate (Applied Biosystems, Foster City, CA, United States) with the following procedure: degeneration 95 ℃ for 30 s, annealing 95 ℃ for 5 s, 40 cycles of 60 ℃ for 34 s. All primers and probes used in this study are listed in Table 1.

Immunohistochemistry

Paraffin slides of colon were re-hydrated in different concentrations of ethanol and washed in PBS. Sections were microwaved in sodium citrate buffer. After blocking with 10% goat serum for 30 min, sections were incubated with rabbit anti-rat IL-17 antibodies (Abcam, Cambridge, United Kingdom) overnight at 4 ℃. Slides were then incubated with the corresponding secondary antibody (Zsbio, Beijing, China), labelled with horseradish peroxidase, developed using a diaminobenzidine (DAB) reaction, and counterstained with hematoxylin. Cells stained with the antibodies were calculated by random selection of five fields under a microscope at 200 × magnification.

Statistical analysis

The GraphPad Prism version 5.0 (La Jolla, CA, United States) was used for data analysis. Data are presented as mean ± SD and were analyzed using one-way analysis of variance. $P < 0.05$ was considered to be statistically significant.

RESULTS

Symptoms and body weight of mice

Mice became symptomatic (*e.g.*, bloody diarrhea, weight loss, shakes andsloth) by day 3 of drinking 3.0% DSS *ad libitum*. The symptoms worsened with prolonged 3.0% DSS drinking time.

The mice in the model group had obvious weight loss compared to the control group ($P < 0.001$), and the mice from the model group weighed significantly less than those from the treatment and prevention groups. There was no significant difference in weight loss between the treatment group and prevention group (Figure 2).

Colon length and pathological changes

Compared with the control group, the mice in the model group had markedly shorter colon length (7.89 ± 1.536 *vs* 4.92 ± 0.925, $P < 0.001$), more serious colon damage, and higher histopathologic damage scores (0.8 ± 0.632 *vs* 3.7 ± 0.483, $P < 0.01$). Histological examination of model group mice showed that the normal colon mucous membrane structure disappeared, extensive ulceration developed, and a large number of inflammation cells infiltrated. However, culturing supernatant of *F. prausnitzii* in treatment and prevention group mice significantly ameliorated the colon damage by increasing colon length ($P < 0.01$ and $P < 0.05$) and reducing high histopathologic damage scores ($P < 0.05$) as compared with model group (Figure 2).

Th17 cell percentage change in splenic mononuclear cells

The ratio of Th17 cells in splenic mononuclear cells of the model group was significantly higher than that of the control group (4.02 ± 1.111 *vs* 1.34 ± 0.417, $P < 0.001$). It was obviously decreased after preventive and therapeutic application of *F. prausnitzii* supernatant (4.02 ± 1.111 *vs* 2.60 ± 0.839, $P < 0.01$ and 4.02 ± 1.111 *vs* 2.21 ± 1.030, $P < 0.05$), and there was no significant difference between the treatment and prevention groups (Figure 3).

IL-17A, IL–6, and IL-4 levels in peripheral plasma

Plasma IL-17A, IL-6, and IL-4 levels of the control group were significantly different from the model group [15.73 ± 4.382 (pg/mL) *vs* 28.44 ± 4.116 (pg/mL) $P < 0.01$, 81.19 ± 13.609 (pg/mL) *vs* 111.82 ±

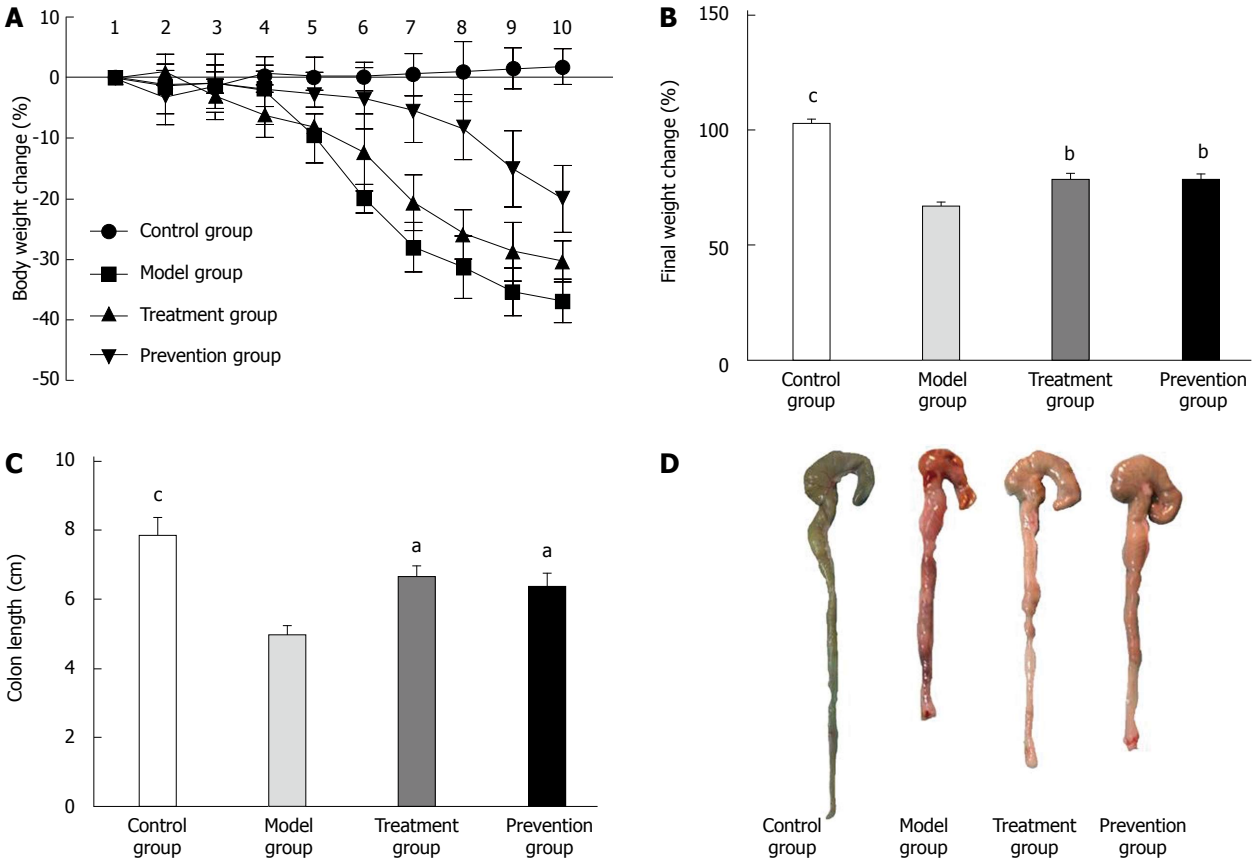


Figure 2 Body weight and colonic length in mice. A, B: Body weight change; C, D: Colon length. Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group.

14.369 (pg/mL) $P < 0.05$, 79.91 ± 12.245 (pg/mL) *vs* 38.16 ± 9.507 (pg/mL) $P < 0.001$]. The plasma levels of IL-17A in the treatment and prevention groups were significantly lower than that in the model group ($P < 0.05$). Plasma IL-6 level in the treatment group was also significantly less than that in the model group ($P < 0.05$), but the difference was not statistically significant between the prevention group and model group. On the contrary, level of plasma IL-4 in the prevention group was obviously higher than that in the model group ($P < 0.05$), while no difference was found between the treatment group and the model group (Figure 3).

Expression of cytokines and RORγt mRNA in colon mucosal tissue

The expression of IL-17A, IL-6, and RORγt mRNA in colon tissue of mice in the model group was significantly higher than that in the control ($P < 0.001$) and treatment groups ($P < 0.05$). When compared with the model group, the expression of IL-17A and RORγt mRNA in colon inflammatory tissue of the treatment and prevention groups was significantly decreased ($P < 0.01$ or $P < 0.05$). There was no difference, however, in IL-6 between the model group and prevention group. As shown in Figure 4, the expression of cytokines and RORγt mRNA in colon mucosal tissue did not significantly differ between the treatment and prevention groups.

Immunohistochemistry

To investigate the effects of IL-17A and RORγt on colon tissue, we conducted immunohistochemical staining of proinflammatory cytokines in tissue sections. Consistent with the results of quantitative real time PCR, the expression of IL-17A and RORγt in the colon tissue of model group mice was significantly increased compared to that in the control group ($P < 0.001$) and treatment group ($P < 0.05$). Although the expression of RORγt in colon tissue was declined after protective use of *F. prausnitzii*, there was no difference between the model and prevention groups (Figure 5).

DISCUSSION

In this study, we found that *F. prausnitzii* supernatant ameliorated colitis in mice by regulating Th17 cell differentiation and inhibiting the excretion of relevant inflammatory cytokines. We also found that *F. prausnitzii* supernatant was effective in the treatment and prevention of DSS-induced mice colitis by inhibiting differentiation of Th17 cell.

Both living *F. prausnitzii* and *F. prausnitzii* supernatant, which contains a mixture of secreted products,

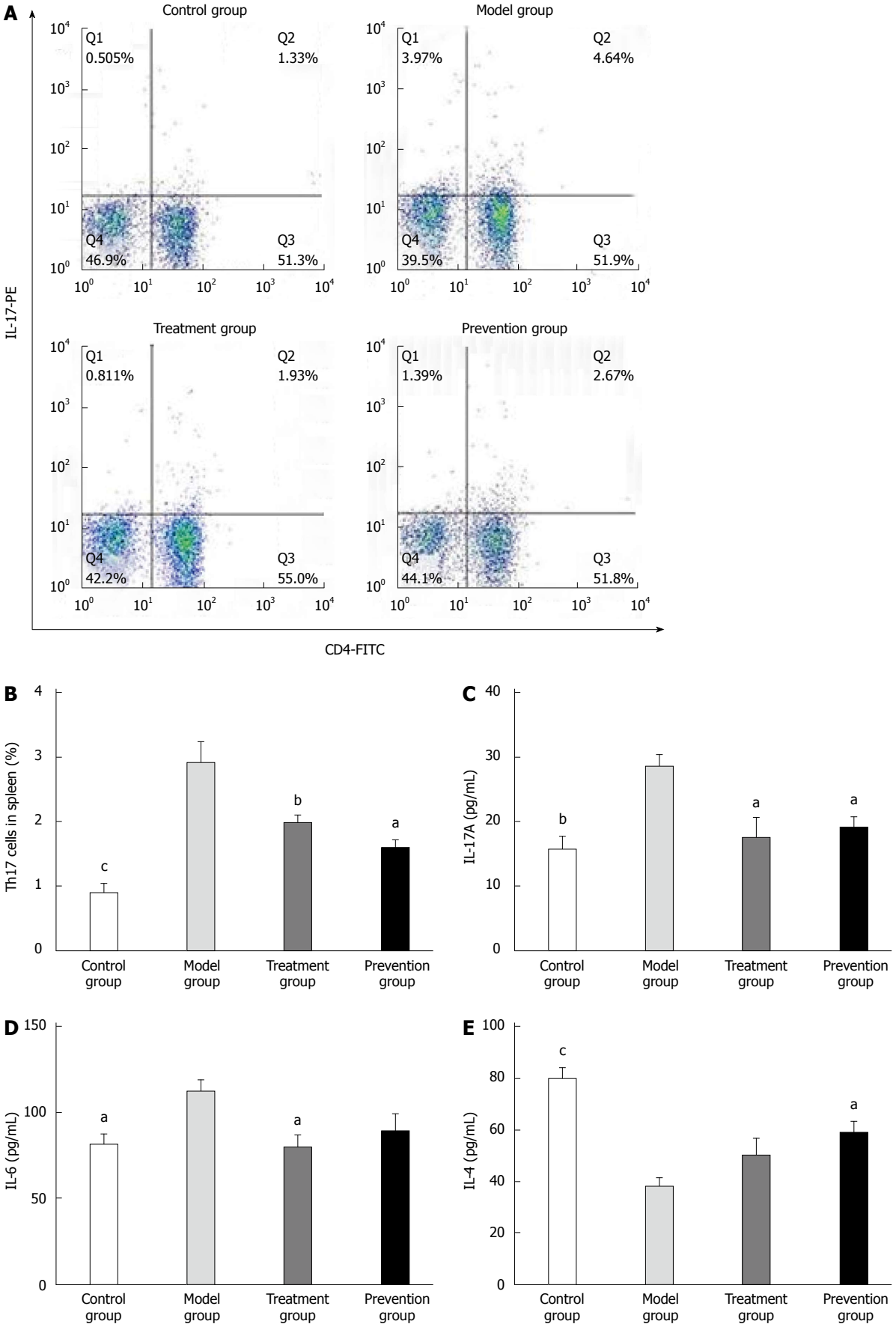


Figure 3 Proportion of Th17 cells in splenic mononuclear cells and plasma cytokines levels. Flow cytometry figures (A) and statistical analysis (B) of Th17 cell in each group of the mice splenic MNC. Plasma IL-17 A (C), IL–6 (D) and IL-4 (E) levels by enzyme-linked immunosorbent assay. Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group. IL: Interleukin; MNC: mononuclear cells.

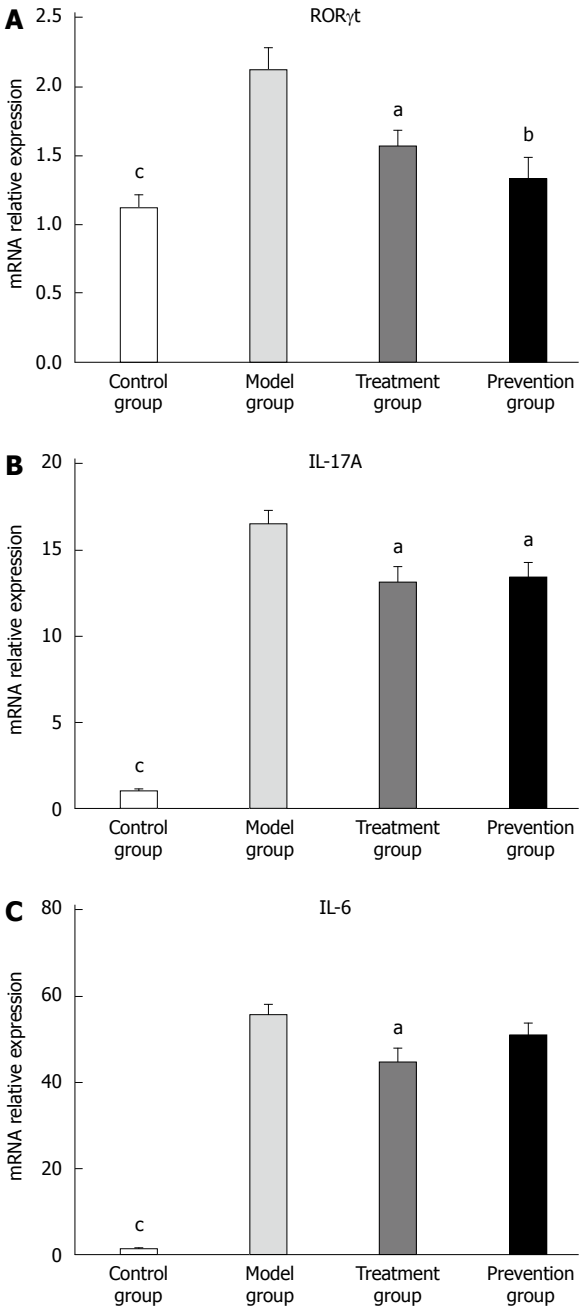


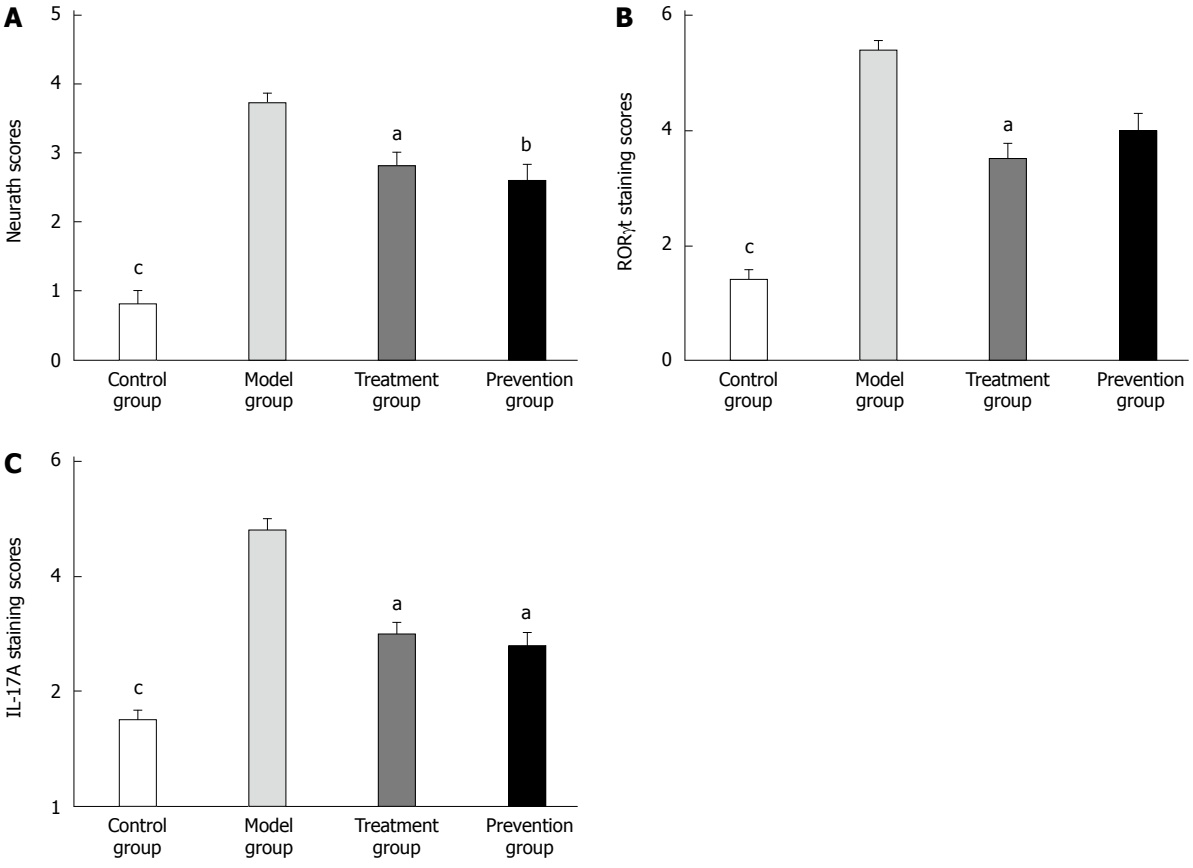
Figure 4 Cytokine mRNA expression in colon mucosal tissue. A: RORγt mRNA; B: IL-17A mRNA; C: IL-6 mRNA. Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group. ROR-γt: Related orphan receptor-γt; IL: Interleukin.

have been shown to have an anti-inflammatory effect[21]. Compared to *F. prausnitzii*, its supernatant could be more effective therapeutically, as it may have a longer shelf-life, which would facilitate delivery, handling, and administration[22]. However, the exact composition and the anti-inflammatory mechanism of *F. prausnitzii* supernatant are currently largely unknown. Therefore, we explored the effects and immune mechanisms of *F. prausnitzii* supernatant on DSS-deduced colitis. Our study showed that the plasma levels of IL-17A and IL-6, the protein and mRNA expression of IL-17A and RORγt in intestinal mucosa, and the Th17 cell ratio of spleen cells ($P < 0.01$) in supernatant treatment group were significantly decreased compared to those in the model group. This finding indicated that the therapeutic use of *F. prausnitzii* supernatant could ameliorate DSS-induced colitis through inhibiting Th17 cells. Carlsson *et al*[23] previously demonstrated that the supernatant of *F. prausnitzii* affected the function of the intestinal barrier.

Th17-related gene polymorphisms are associated with IBD susceptibility[24]. Th17-derived cytokines, such as IL-17A, IL-6, and IL-22, have been shown to be upregulated in the inflamed intestine of IBD patients[25,26]. IL-17A is a strong inflammatory cytokine, which can enhance cell permeability and promote the generation of other pro-inflammatory cytokines and chemokines[27]. Animal experiments, however, have found that neither IL-17A knockout nor neutralization of IL-17 could protect DSS-administrated mice from colitis, suggesting that the role of IL-17 in intestinal inflammation may not be entirely pathogenic[14,28]. Adequate expression of IL-17A plays an important role in maintaining intestinal immune function. Consistent with previous studies, we found that IL-17A levels in the plasma, spleen, and colon tissue were significantly increased in mice with colitis and that these levels were remarkably downregulated in mice treated with *F. prausnitzii* culture supernatant. Therefore, *F. prausnitzii* supernatant could attenuate DSS-induced mice colitis, possibly by inhibiting the expression of IL-17A[15,29].

We also found that levels of IL-6 in plasma and colon tissues of colitis mice were significantly reduced after *F. prausnitzii* supernatant treatment. *F. prausnitzii* supernatant could alleviate mice colitis by downregulating IL-6 levels and inhibiting Th17 cell differentiation, thus leading to reduced secretion of inflammatory cytokines (such as IL-17A and IL-6) and attenuation of the local inflammatory response. However, the regulation of IL-6 expression in the treatment and prevention groups was inconsistent, suggesting that there might be other ways of inhibiting Th17 differentiation. Fu *et al*[29] demonstrated that boosting of Th2 associated cytokines (IL-4, IL-13, and IL-10) can reverse Th17-mediated intestinal inflammation. We also found that plasma IL-4 levels in mice of the prevention group were significantly greater than those in the model group.

In conclusion, *F. prausnitzii* supernatant can prevent DSS-deduced colitis in mice by inhibiting the generation of Th17 cells in the spleen and intestinal mucosa, leading to a reduction of IL-17A and IL-6 levels and attenuation of intestinal inflammation. This study provides the theoretical basis for the application of *F. prausnitzii* supernatant in UC treatment and prevention. However, what specific substances in the supernatant of *F. prausnitzii* possess biological activity needs to be elucidated in future studies. The safety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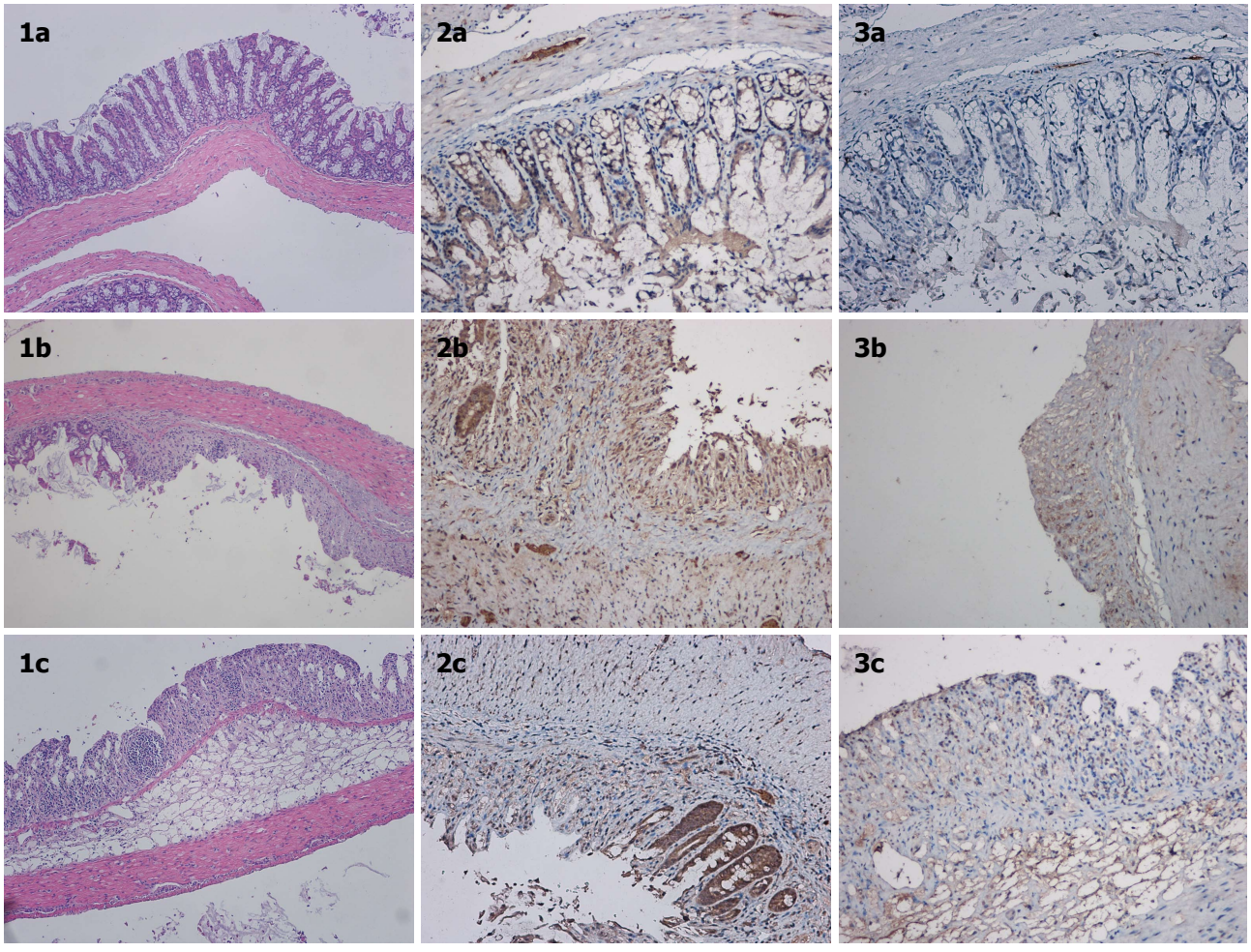
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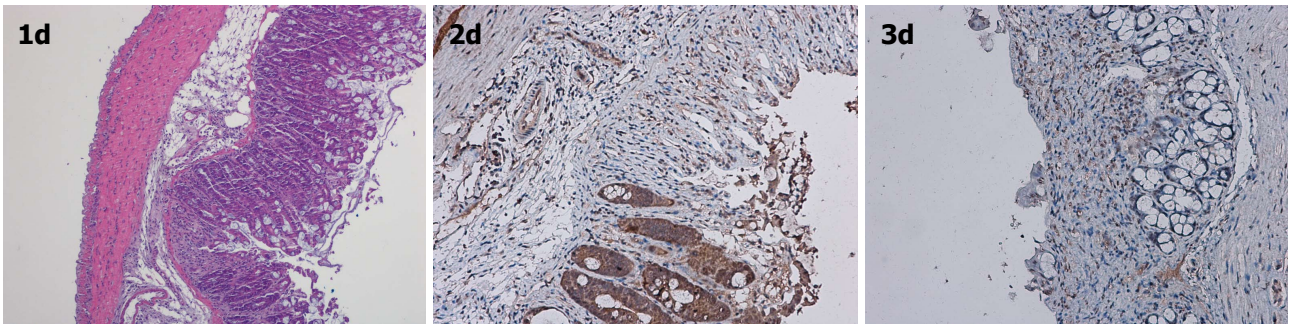

Figure 5 Colon Neurath Scores and related orphan receptor-γt and interleukin-17A protein expression. Colon Neurath Scores (A, 100 magnifications), RORγt (B, 200 magnifications), and IL-17A (C, 200 magnifications) protein expression in mice colon. Representative images of mice colonic mucosa (1a-1d). Representative immunohistochemical staining of RORγt (2a-2d) and IL-17A (3a-3d) in mice colon mucosa. Control group (a); model group (b); treatment group (c); prevention group (d). Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group.

and efficacy of *F. prausnitzii* supernatant also warrant further investigation by more large scale clinical trials.

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COMMENTS

Background

Inflammatory bowel disease (IBD) is a multifactorial ailment characterized by intestinal inflammation, and its etiology is complicated and ambiguous. Factors that contribute to IBD include genetic background, environment, intestinal flora imbalance, and immune disorder as well as the interactions between them.

Research frontiers

Faecalibacterium prausnitzii (*F. prausnitzii*) is a common anaerobic bacteria that colonizes the human gut, and it plays a critical role in IBD. *F. prausnitzii* supernatant has anti-inflammatory and immune regulatory activity. Previously, the authors showed in animals that both the bacteria and its supernatant relieved trinitro-benzene-sulfonic acid-induced colitis in rats. However, the specific mechanism is largely unclear.

Innovations and breakthroughs

This study is the first to show that the preventive and therapeutic use of *F. prausnitzii* supernatant could ameliorate dextran sulfate sodium (DSS) induced mice colitis through inhibiting Th17 cells. The molecular mechanism of proliferation and differentiation of Th17 cells was different. *F. prausnitzii* supernatant may treat colitis in mice by downregulating IL-6 and preventing the upregulation of IL-4.

Applications

This study investigated the molecular mechanism of the preventive and therapeutic use of *F. prausnitzii* supernatant for IBD and provided evidence for the prevention and treatment of the disease.

Terminology

F. prausnitzii is the major bacterium of the Clostridium leptum group and is one of the most abundant anaerobic bacteria in human gut.

Peer-review

The study investigates the preventive and therapeutic role of *F. prausnitzii* supernatant in a mouse model of DSS-induced ulcerative colitis. The topic is interesting, and the design and methods have clear scientific values. The data are clear and well presented.

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ORIGINAL ARTICLE

Basic Study

Visceral hypersensitive rats share common dysbiosis features with irritable bowel syndrome patients

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Abstract

AIM: To evaluate gut microbial dysbiosis in two visceral hypersensitive models in comparison with irritable bowel syndrome (IBS) patients and to explore the extent to which these models capture the dysbiosis of IBS patients.

METHODS: Visceral hypersensitivity was developed using the maternal separation (MS) rat model and post-inflammatory rat model. The visceral sensitivity of the model groups and control group was evaluated using the abdominal withdraw reflex score and electromyography in response to graded colorectal distention. The 16S ribosomal RNA gene from fecal samples was pyrosequenced and analyzed. The correlation between dysbiosis in the microbiota and visceral hypersensitivity was calculated. Positive findings were compared to sequencing data from a published human IBS cohort.

RESULTS: Dysbiosis triggered by neonatal maternal separation was lasting but not static. Both MS and post-inflammatory rat fecal microbiota deviated from that of

the control rats to an extent that was larger than the co-housing effect. Two short chain fatty acid producing genera, *Fusobacterium* and *Clostridium XI*, were shared by the human IBS cohort and by the maternal separation rats and post-inflammatory rats, respectively, to different extents. *Fusobacterium* was significantly increased in the MS group, and its abundance positively correlated with the degree of visceral hypersensitivity. *Porphyromonadaceae* was a protective biomarker for both the rat control group and healthy human controls.

CONCLUSION: The dysbiosis MS rat model and the post-inflammatory rat model captured some of the dysbiosis features of IBS patients. *Fusobacterium*, *Clostridium XI* and *Porphyromonadaceae* were identified as targets for future mechanistic research.

Key words: Animal model; Irritable bowel syndrome; Microbiota; Pyrosequencing; 16S rRNA gene

Core tip: Dysbiosis of the gastrointestinal microbiota and hypersensitivity to colonic distension are critical features of irritable bowel syndrome (IBS). For animal models, the correlation between dysbiosis in the microbiota and visceral hypersensitivity remains unknown. This study identified common biomarkers between the animal models and IBS patients, which may be targets for future mechanistic research.

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INTRODUCTION

The human intestinal tract is home to trillions of bacteria that have co-evolved with their host over millennia[1]. Their combined genomes, called a metagenome, contain 150-fold more genes than do the human hosts, and they provide functions that humans otherwise do not have[2]. Complex interactions exist between the gut microbiota and the host[3]. Irritable bowel syndrome (IBS) is a common gastrointestinal disorder that is characterized by abdominal pain and alterations in bowel habits; statistically, IBS affects 7%-10% of people worldwide[4]. Accumulating evidence has indicated that the gut microbiota may participate in the pathogenesis of IBS[5]. Because collecting fecal samples both before and after a gastrointestinal infection from the same IBS patients is unfeasible for clinics, only gut dysbiosis in standing IBS patients has been evaluated to date[6,7]. However, how gut microbiota abnormalities arise and are maintained over time is unclear. These questions are critical for interventions targeting the microbiota, such as probiotic usage. In this work, we used visceral hypersensitive rat models to investigate the longitudinal changes of gut microbiota.

Currently, both post-infectious/inflammatory models and stress-related models have been frequently used to study the pathophysiology of IBS[8,9]. There are more than 12 major post-infectious/post-inflammatory models to mimic post-infectious IBS, which occurs after an initial episode of acute gastrointestinal infection. Chemicals such as trinitrobenzene sulfonic acid (TNBS)[10], mustard oil[11] and dextran sulfate sodium[12] were used to cause mucosal injury in the post-inflammatory models, and pathogens such as *Trichinella spiralis*[13] and *Campylobacter*[14] were used to infect the gut; both led to visceral hypersensitivity. Stress-related models[15] could also induce the modulation of visceral pain, and this may involve changes in the brain-gut axis[9]. However, one of the unsolved problems is the extent to which these models recapture the characteristics of gut dysbiosis in IBS patients. In this work, we used two visceral hypersensitive models, the TNBS post-inflammatory (pTNBS) model and the maternal separation (MS) model, to investigate: (1) whether and the extent to which these models reproduce the disturbance of gut microbiota in a similar way to that of the IBS patients; and (2) whether microbial dysbiosis, if it exists, is static or shifting in these visceral hypersensitive models. We also hoped to identify targets in the models' gut microbial communities that are suitable for use in developing probiotics to specifically modulate the microbiota.

MATERIALS AND METHODS

Animal maintenance and modeling

Sprague-Dawley rats were purchased from the animal center of Shandong University of Traditional Chinese Medicine. The rats were allowed to habituate for 7 d to the breeding facility prior to mating. They were kept under standardized specific pathogen-free conditions (21-22 ℃, 12:12-h light-dark cycle) with access to pellet food and water *ad libitum*. All experiments were approved by the Ethical Committee and Institutional Animal Care and Use Committee of Qilu Hospital (KYLL-2013-005), and the methods were performed in strict accordance with the Animal Management Rules of the Chinese Ministry of Health. The overall design and co-housing relationship of involved rats are indicated in Figure 1A.

The MS visceral hypersensitive models were developed as previously described[15]. Briefly, rat pups that were randomly assigned to the MS group were stressed by separating them from their mothers for 3 h daily between postnatal days 2-14. The control group (Ctl) received normal breeding during this session. All pups were weaned on postnatal day 22, and only the

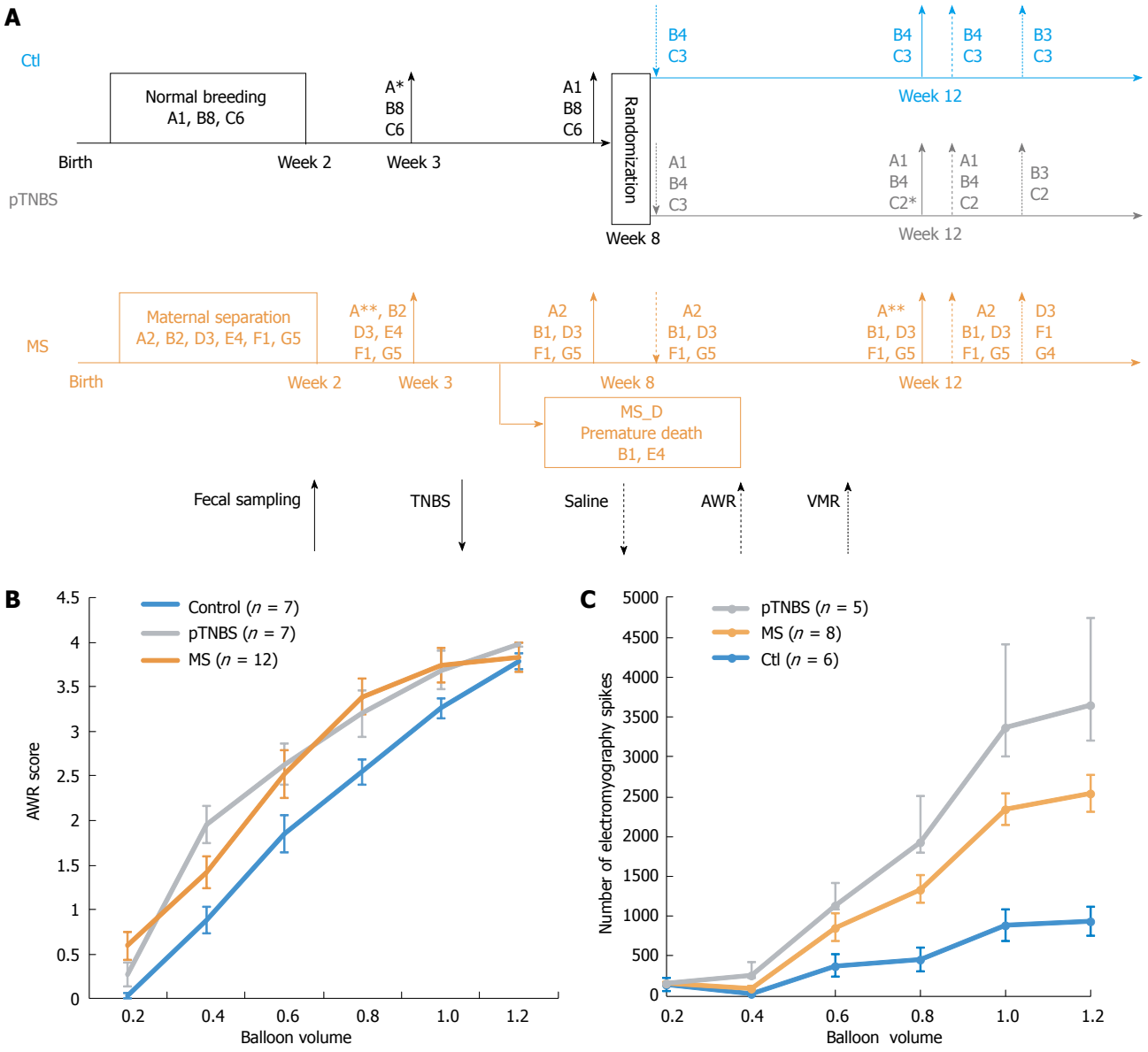


Figure 1 Study design and visceral sensitivity evaluation. A: Schematic flow chart showing the treatment and co-housing relationship of involved rats. For each code, such as "B8", the character "B" indicates the nest and number 8 indicates the number of rats. They were cohoused until "B4", which indicates that four of them were randomly chosen and cohoused together. The asterisk indicates fecal samples that failed to return the sequencing data; B: Abdominal withdrawal reaction (AWR) score in response to the graded colorectal distention (CRD); C: Visceromotor response (VMR) score in response to graded CRD. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

male pups were used for the following study. Because some MS pups naturally died before they aged, 5 male rats were randomly chosen from those that were sampled at week 3 but did not survive to week 8. We indicated this group as the MS early death (MS_D) group. By including the MS_D group, we could test whether the dysbiosis caused by MS stress was more severe in the early dying pups.

After the second fecal collection at week 8, half of the control group was randomly assigned to the post-TNBS inflammation group (pTNBS). The pTNBS group was fasted for 24 h with free access to tap water, and then 0.4 mL of 5% (v/v) TNBS (P2297, Sigma, Shanghai, diluted to 0.8 mL using 50% ethanol) was administered into the colorectum.

Visceral hypersensitivity evaluation

After the last fecal collection at week 12, visceral hypersensitivity was evaluated using both the abdominal withdraw reflex (AWR) score and electromyography in response to graded colorectal distention (CRD). Graded CRD was induced by rapidly injecting (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mL) saline into a urinary catheter balloon placed in the colon over 1 s and maintaining the distention for 20 s. AWR score was recorded according to a previously described method[16]. To represent the overall visceral sensitivity, a visceral hypersensitive index (VHI) for each rat was calculated by summing the rank of the AWR score at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume.

The visceromotor responses (VMRs) to CRD were

quantified through electromyography of the rat obliqus external abdominis. Briefly, 5 d after embedding an electrode in the rat obliqus externus abdominis, the raw electromyography was recorded, rectified and quantified by counting the increased spike bursts during a 20 s window after graded CRD stimulation. The VMR index for each rat was calculated by summing the rank of electromyography spikes at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume.

Fecal sample collection, DNA extraction, and pyrosequencing

Fecal samples were collected 3, 8 and 12 wk after birth. The samples from the pTNBS group at weeks 3 and 8 were indicated as Ctl-pTNBS and were analyzed as the controls because their treatment was the same as that for the Ctl group. A chart illustrating the overall treatment, fecal collection and model evaluation time points is shown in Figure 1A.

The samples were snap-frozen in liquid nitrogen and stored at -80℃. Genomic DNA was extracted with a TIANamp Stool DNA Kit according to the manufacturer's instructions (Cat# DP328, Tiangen, Beijing). DNA purity and concentration were measured using a Nanodrop2000 (Thermo Fisher). The DNA samples were shipped to Majorbio (Shanghai), where the DNA integrity check, PCR amplification, DNA quantification, emPCR (using Roche GS FLX Titanium emPCR Kits) and pyrosequencing of the 16S rRNA gene V3 to V1 region (using Roche Genome Sequencer FLX+) were performed according to their optimized protocols. The sequencing results were archived in the Short Reads Achieve (number pending).

Taxonomy quantification using 16S rRNA gene sequences

Raw sequencing data were prepared using Mothur v 1.33.0 according to their proposed 454 SOP (http://www.mothur.org/wiki/454_SOP)[17]. The raw sff files were decoded, denoised, trimmed and then aligned to Silva references (Release 102) using the default parameters. Chimeras were detected using the chimera.uchime command and were then removed. Distances between sequences were calculated with a cutoff value of 0.15. The sequences were clustered to the same operational taxonomic units (OTUs) if their distances were less than 0.03. The Shannon index and the inverse Simpson index (1/D) were calculated to indicate the diversity in each sample. Both indexes were calculated using Mothur, and the detailed formula can be accessed online (http://www.mothur.org/wiki/Shannon and http://www.mothur.org/wiki/Simpson). The OTU table was converted to biom files and the taxa abundance from domain to genus levels was generated using the summarize_taxa.py command in QIIME v1.8.0.

Statistical analysis

The richness of each taxonomy and the Shannon index between groups were compared using the Kruskal-Wallis test (KW) or a student's *t*-test in SAS V.9.3 statistical software. The heatmap plot with dendrograms was drawn using the heatplot function in the made4 packages in R (version 3.1.1). For primary component analysis (PCA), the axis value of all 80 samples was calculated together using the prcomp function in the stats package in R, and then the samples were plotted by each time point (week 3, 8, and 12). Within each time point, samples were clustered based on the Euclidian distance using the vegdist and hclust in the vegan package. According to the cluster results, the PCA plot points were grouped and connected using the ordispider and ordiellipse functions, where the ellipse was estimated to cover 75% of the dots in this group. The distribution of each group in each cluster was checked using Fisher's exact test in SAS. The community dissimilarity was tested by the weighted and unweighted UniFrac test using Mothur. The specific taxa that were differentially present in each group were identified using the LEfSe [linear discriminant analysis (LDA) coupled with effect size measurements] method with an LDA cut-off value of 2.0[18]. The Spearman correlation between the VHI and the taxonomy richness was calculated using the cor.test function in the stats package in R.

Comparing rat models and human IBS cohort

We downloaded the published 16S rRNA V4 region Miseq sequencing data by Jeffery *et al*[6]. This data set was analyzed by the same pipeline described above. We used LEfSe analysis on this dataset. Each positive finding from the rat experiment was checked against the human cohort. The relationship between human and rat biomarkers was indicated using a Venn plot.

RESULTS

Modeling and visceral hypersensitivity evaluation

The design and co-housing relationship of the rats involved in this study is shown in Figure 1A. Twenty-six of the 27 rats in this study (7 Ctl, 7 pTNBS, and 12 MS, see Figure 1B) were evaluated using AWR. The VHI score was calculated by summing the rank of the AWR score at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume. A significant difference existed in the VHI among the three groups (χ^2 = 9.98, *df* = 2, *P* = 0.0068, KW). The VHI difference in the pTNBS to Ctl comparison was 38.4 (95%CI: 15.7 to 61.0, *P* < 0.05), and the VHI difference in the MS to Ctl comparison was 32.9 (95%CI: 12.7 to 53.0, *P* < 0.05). There was no significant difference in the MS to pTNBS comparison, with a VHI difference of 5.48 (95%CI: -14.7 to 25.7, *P* > 0.05). Nineteen of the 27 rats were evaluated by VMRs (Figure 1C). The VMR index for each rat was calculated by summing the rank of electromyography spikes at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume. The VMR index among groups was insignificant although the control group tended to

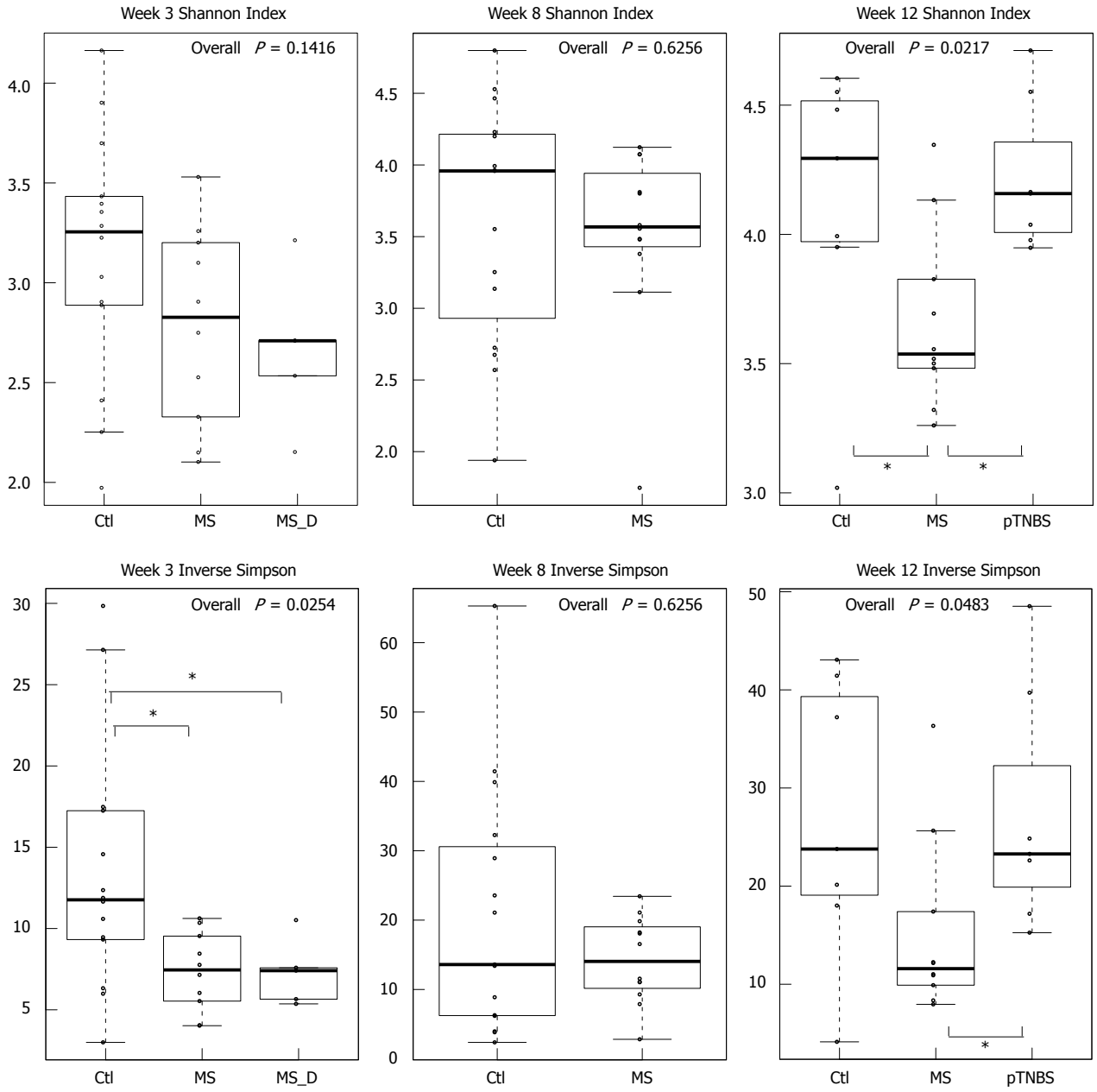


Figure 2 Microbial diversity. Shannon index (upper panel) and inverse Simpson (lower panel) of fecal microbiota at weeks 3, 8 and 12. Asterisk indicates $P < 0.05$ in pairwise comparison. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

be lower than the MS and pTNBS groups (43.5 *vs* 86.5 and 60, respectively, $\chi^2 = 2.235$, $df = 2$, $P = 0.3271$, KW). Overall, these data indicate that both the MS and pTNBS groups developed visceral hypersensitivity at a comparable level.

DNA sequence data and microbial diversity comparison

A total of 489556 valid reads were assigned to 80 sequenced samples after barcode trimming. Sequence length varied between 230 and 327 bp per read. After removing chimeras and non-bacterial reads, 434594 reads remained. Each fecal sample included 3313 to 8161 reads. Based on a 97% species similarity, 2413 OTUs were identified from all of the fecal samples. Good's coverage for each sample varied from 95.57% to 99.72%. The rarefaction curve reached a plateau for most samples, suggesting that the present study captured the dominant phylotypes.

We first compared the microbial diversity among groups using the Shannon index and the inverse Simpson index (Figure 2). By week 3, the inverse Simpson index was significantly higher in the Ctl group ($\chi^2 = 7.34$, $df = 2$, $P = 0.0254$, KW). By week 8, the Shannon index and the inverse Simpson index were similar between the control group and the MS group. By week 12, the MS group has the lowest Shannon index ($\chi^2 = 7.67$, $df = 2$, $P = 0.217$, KW) and inverse Simpson index ($\chi^2 = 6.06$, $df = 2$, $P = 0.0483$, KW) compared with other groups. The pTNBS group had roughly same diversity indexes compared to the Ctl

group. These data indicate that the MS model, but not the pTNBS model, developed fecal microbiota with reduced diversity in a non-static manner.

Dysbiosis of major phyla

We then investigated whether differences in the phylum abundance exist at different time points (Figure 3). *Bacteroidetes* was the dominant phylum across all samples, and *Firmicutes* and *Proteobacteria* were the second and third most abundant phyla. No significance was reached for these three phyla at any of the 3 time points. The *Firmicutes* to *Bacteroidetes* (F/B) ratio was not significantly different ($P > 0.05$, KW).

Fusobacteria was abundant by week 3 (up to 0.25) and dropped to zero in most control rats. No difference in *Fusobacteria* existed by week 3 and week 8; however, by week 12, the MS group had significantly more *Fusobacteria* ($\chi^2 = 6.83$, $df = 2$, $P = 0.0328$, KW, $P < 0.05$ in MS-Ctl comparison). The control group had significantly more *Actinobacteria* than the MS group at week 8 ($P = 0.0034$, $\chi^2 = 8.58$, $df = 1$, KW). However, by week 12, the pTNBS group had significantly more *Actinobacteria* than the Ctl and MS groups ($\chi^2 = 8.07$, $df = 2$, $P = 0.0176$, KW, $P < 0.05$ in the pTNBS-Ctl and pTNBS-MS comparison). These data suggest that the dysbiosis of the major phyla may be phase dependent and different among the visceral hypersensitive rat models.

PCA and cluster analysis

We based the cluster analysis and PCA on the OTU data from the 16S rRNA gene pyrosequencing. The primary components for all 80 samples were calculated from the relative abundance of the 2413 OTUs. The relative importance of the first 20 primary components is plotted in Figure 4A. Primary component 1 and primary component 2 explained 27.7% and 14.6% of the total variance, respectively (Figure 4A). The differences in the primary components between the time points and experimental groups are listed in Table 1. Primary component 1 mainly reflected the effect of time points ($\chi^2 = 37.7$, $df = 2$, $P = 0.0000$, KW). Primary components 2 and 4 reflected the effect of experimental groups on fecal microbiota composition. The other 3rd, 6th, and 9th components were different both among time points and among groups.

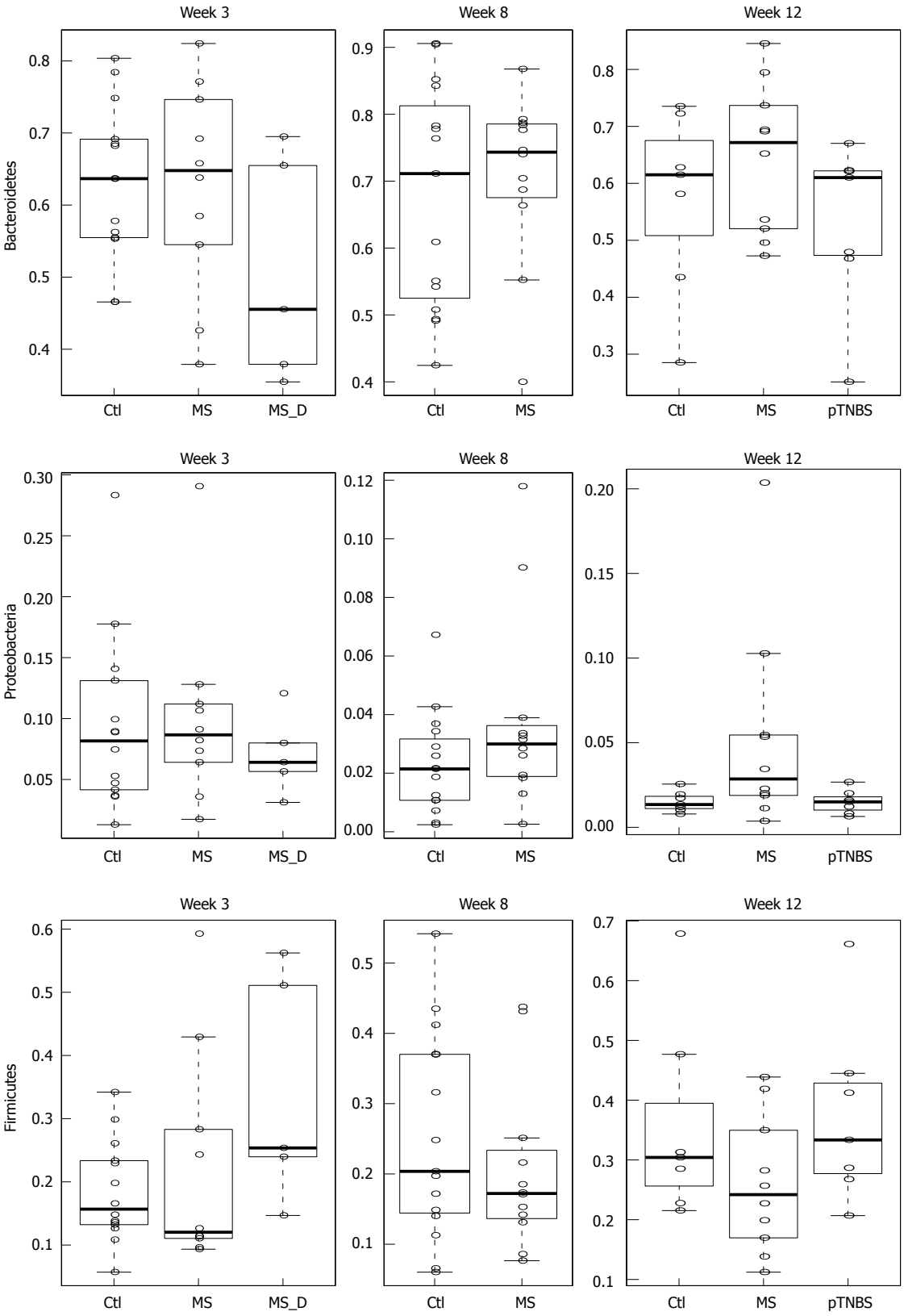
We then used cluster analysis to test whether the groups would fall into the same or different clusters. At each time point, the samples were fitted into the 3 top clusters based on the Euclidean distance. We designated the names of each cluster according to the samples it included. By week 3, the normal cluster included 8 Ctl, and the MS cluster included 4 MS and 4 MS_D samples. The mixed cluster included 6 Ctl, 6 MS and 1 MS_D samples (Figure 4B). The 3 groups' distribution in the clusters was significantly different ($P = 1.702 \times 10^{-4}$, Fisher's test). This result suggests that MS caused dysbiosis in rat models at early ages.

By week 8, the 27 fecal microbiota samples clustered into 3 mixed clusters (Figure 4C). The control group dominated mixed cluster 3 (12/16) while the MS group dominated mixed cluster 2 (7/8). The cluster distribution of the MS and control groups was significantly different ($P = 0.0114$, Fisher's test). By week 12, the 24 fecal microbiota samples formed 3 clusters (Figure 4D). The MS cluster included 5 MS samples, and the mixed cluster included roughly the same number of samples from the Ctl ($n = 6$), MS ($n = 5$), and pTNBS ($n = 7$) groups. Another "orphan" cluster included only one control sample. The difference among groups was significant ($P = 0.0150$, Fisher's test). These data suggest that the dysbiosis triggered by MS during childhood is still substantial in a fraction of adult rats. Four weeks after TNBS administration, the fecal microbiota of the post-inflammatory rat model was more similar to that of the control group as revealed by PCA and cluster analysis.

We tracked the longitudinal dysbiosis of 23 rats whose fecal samples were collected at all 3 time points. We analyzed whether the 10 MS rats clustered to the different or same clusters at week 3 and week 12. Seven out of 10 rats shifted to different clusters (mixed-to-MS or MS-to-mixed) from week 3 to week 12. The agreement Kappa value for cluster classification at week 3 and week 12 was -0.4000 (95%CI: -0.9566 to 0.1566). This result indicates that although MS stress generated an isolated dysbiosis cluster in a fraction of rats, each rat's gut microbiota might shift between the less disturbed (mixed) cluster and the severely disturbed (MS) cluster.

UniFrac test on animal models and co-housing effect

We further tested the effect of modeling and co-housing on fecal microbiota using the weighted and unweighted UniFrac test (Table 2). A phylogenetic tree was built for all samples at each time point, and weighted and unweighted UniFrac scores were calculated to evaluate the community similarity. According to the unweighted UniFrac test, we found that by week 3, the fecal community in Ctl and MS groups was significantly different ($P < 0.001$). The co-housing effect caused community dissimilarity in the 2 houses of control rats (B8 *vs* C6, $P < 0.001$) but not in the 2 houses of MS rats (D3 *vs* G5, $P = 0.507$). By week 8, the co-housing effect was non-significant within the MS and Ctl groups, but the difference was still significant between these two groups. By week 12, a significant community difference existed among the Ctl, MS, and pTNBS groups; the co-housing effect was not obvious within any of the groups. The weighted UniFrac test was significant in all of the above comparisons. Overall, these data suggest that both the MS model and the pTNBS model developed dysbiosis of the fecal microbiota, and the differences were not caused by the co-housing relationship.


Week 3
Week 8
Week 12
Bacteroidetes
Proteobacteria
Firmicutes
Ctl
MS
MS_D
pTNBS

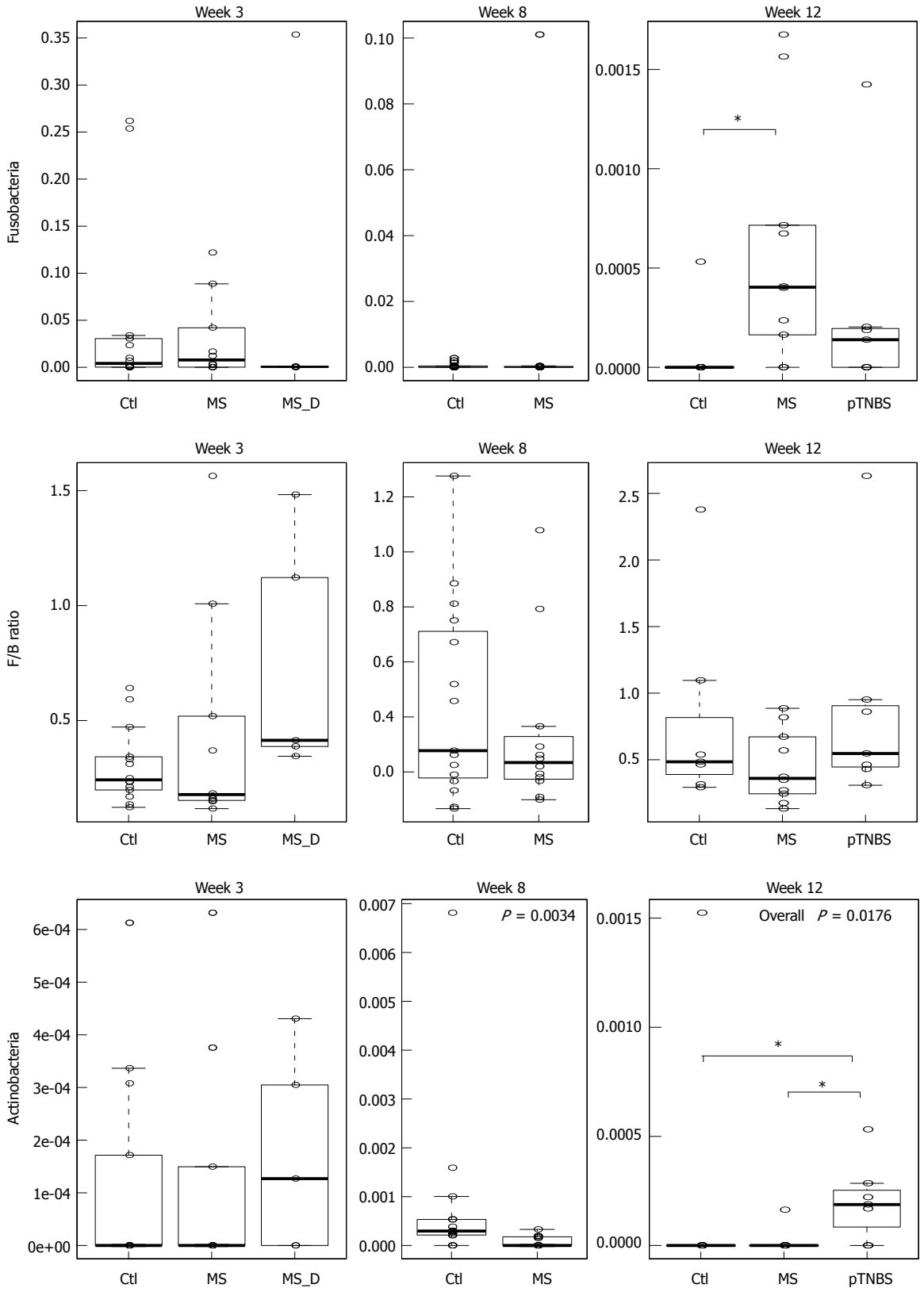


Figure 3 Phyla abundance by weeks 3, 8 and 12. Asterisk indicates $P < 0.05$ in pairwise comparison. $P > 0.05$ (no significant), unless the P-value is drawn in the plot box. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

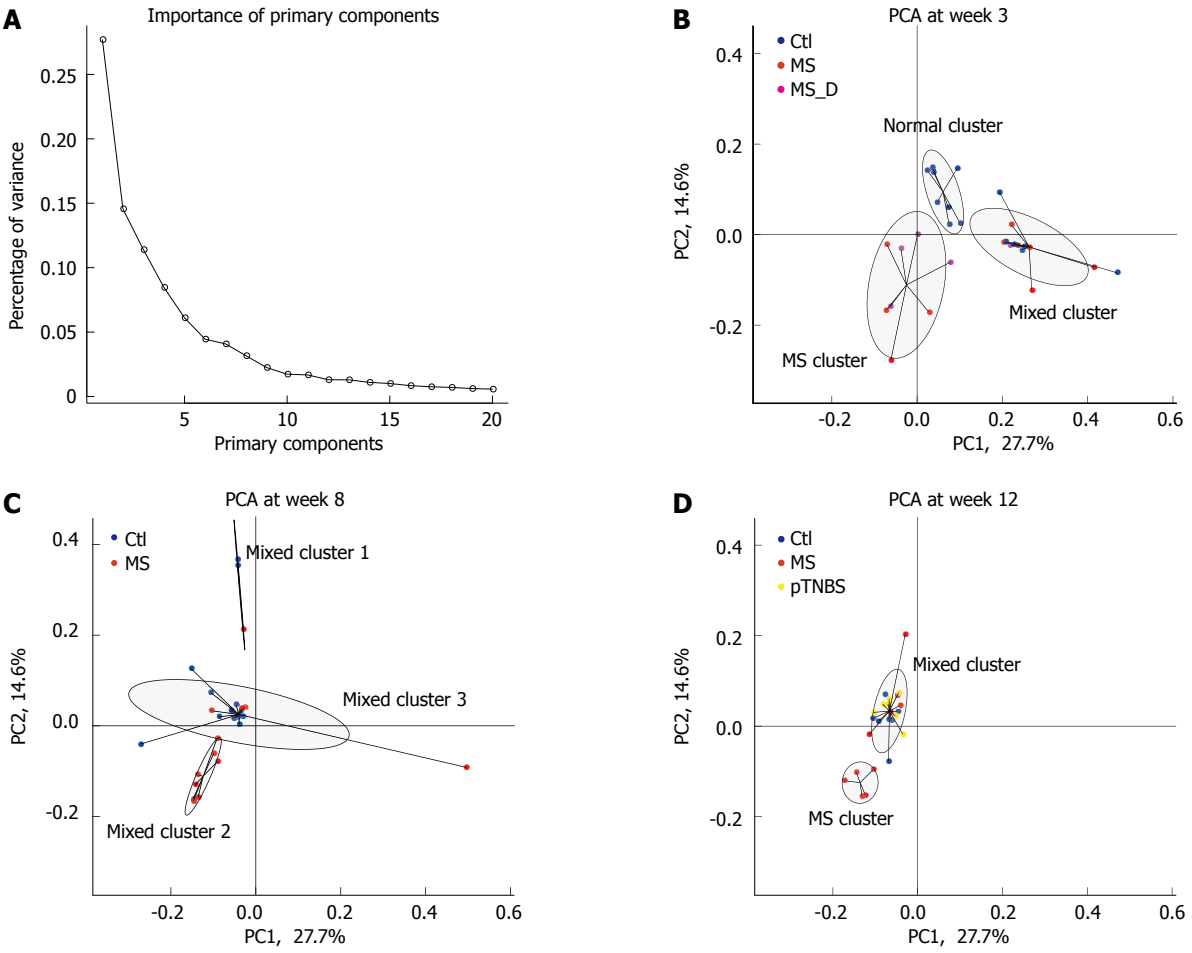


Figure 4 Primary component analysis and cluster analysis by time points. Primary components were calculated from the relative abundance of all 2413 OTUs. A: The percentage of variances explained by the first 20 primary components. The primary component was separately plotted at week 3 (B), week 8 (C), and week 12 (D). The cluster analysis divided the samples into three clusters, and the samples in the same cluster were connected together. The ellipse was estimated to cover 75% of dots in this cluster. Each cluster was named according to the samples involved in this cluster. The MS group formed isolated clusters, indicated as "MS cluster", at week 3 and week 12. MS: Maternal separation; PCA: Primary component analysis.

Table 1 Importance and meaning of the first 10 primary components of fecal microbiota

Primary component	% of variance	Difference among time points (n = 80, df = 2)		Difference among groups (n = 80, df = 3)	
		χ^2	*P*-value	χ^2	*P*-value
1	27.7%	37.67931	0.00000[1]	4.6999109	0.19514
2	14.6%	1.352165	0.50861	20.4278998	0.00014[1]
3	11.4%	6.165561	0.04583[1]	8.6830899	0.03382[1]
4	8.5%	0.719395	0.69789	21.7793862	0.00007[1]
5	6.1%	0.623716	0.73209	4.6400976	0.20013
6	4.5%	8.388254	0.01508[1]	9.3688439	0.02477[1]
7	4.1%	0.844768	0.65548	0.7871023	0.85255
8	3.2%	0.412918	0.81346	6.6021743	0.08572
9	2.2%	8.880049	0.01180[1]	9.9866961	0.01868[1]
10	1.7%	1.762893	0.41418	2.6274074	0.45270

[1]The *P*-value is less than 0.05.

Biomarkers and correlation to visceral hypersensitivity

We performed linear discriminant analysis coupled with effect size measurement (LEfSe) analysis at different time points to screen biomarkers for each group (Figure 5, Figure S1-3). By week 3, 29 samples were analyzed using LEfSe. The control group was strongly associated with higher abundances of unclassified *Bacteroidales*, *Veillonella*, *Treponema*, and unclassified *Clostridiales*. The MS group was associated with higher abundances of unclassified *Burkholderiales*, *Coprobacillus*, and *Clostridium_XIVa*. By week 8, 27 samples were analyzed. The MS group was associated with higher abundances of *Helicobacter*, unclassified *Burkholderiales*, and unclassified *Desulfovibrionaceae*,

Table 2 Weighted and unweighted UniFrac test on experiment groups and co-housing

Time point	Comparison	Number of samples to build phylogenetic tree	Weighted UniFrac test		Unweighted UniFrac test	
			Weighted UniFrac score	*P*-value	Unweighted UniFrac score	*P*-value
Week 3	Ctl-MS	29	0.863902	< 0.0010	0.957176	< 0.0010[1]
	Ctl-MS_D	29	0.926521	< 0.0010	0.963861	0.0130[1]
	MS-MS_D	29	0.833168	< 0.0010	0.909242	0.2790
	Ctl(B8)-Ctl(C6)	29	1.000000	< 0.0010	1.000000	< 0.0010[1]
	MS(D3)-MS(G5)	29	1.000000	0.0070	1.000000	0.5070
Week 8	Ctl-MS	27	0.719683	< 0.0010	0.951668	0.0190[1]
	Ctl(B8)-Ctl(C6)	27	0.83063	< 0.0010	0.955082	0.0870
	MS(D3)-MS(G5)	27	0.617715	< 0.0010	0.874196	0.5220
Week 12	Ctl-MS	24	0.754046	< 0.0010	0.944729	0.0390[1]
	Ctl-pTNBS	24	0.942882	< 0.0010	0.978698	0.0160[1]
	MS-pTNBS	24	0.828407	< 0.0010	0.973938	0.0130[1]
	Ctl(B4)-Ctl(C3)	24	0.928051	< 0.0010	0.976209	0.3200
	MS(D3)-MS(G5)	24	0.771421	< 0.0010	0.912219	0.4550
	pTNBS(B4)-pTNBS(C2)	24	1.000000	< 0.0010	1.000000	0.6390

[1]The *P*-value is less than 0.05. MS: Maternal separation; pTNBS: TNBS post-inflammatory.

which all belong to *Proteobacteria*. The control group was associated with higher abundances of *Barnesiella*, *Actinobacteria*, *Clostridium_XI*, *Allobaculum*, and *Odoribacter*.

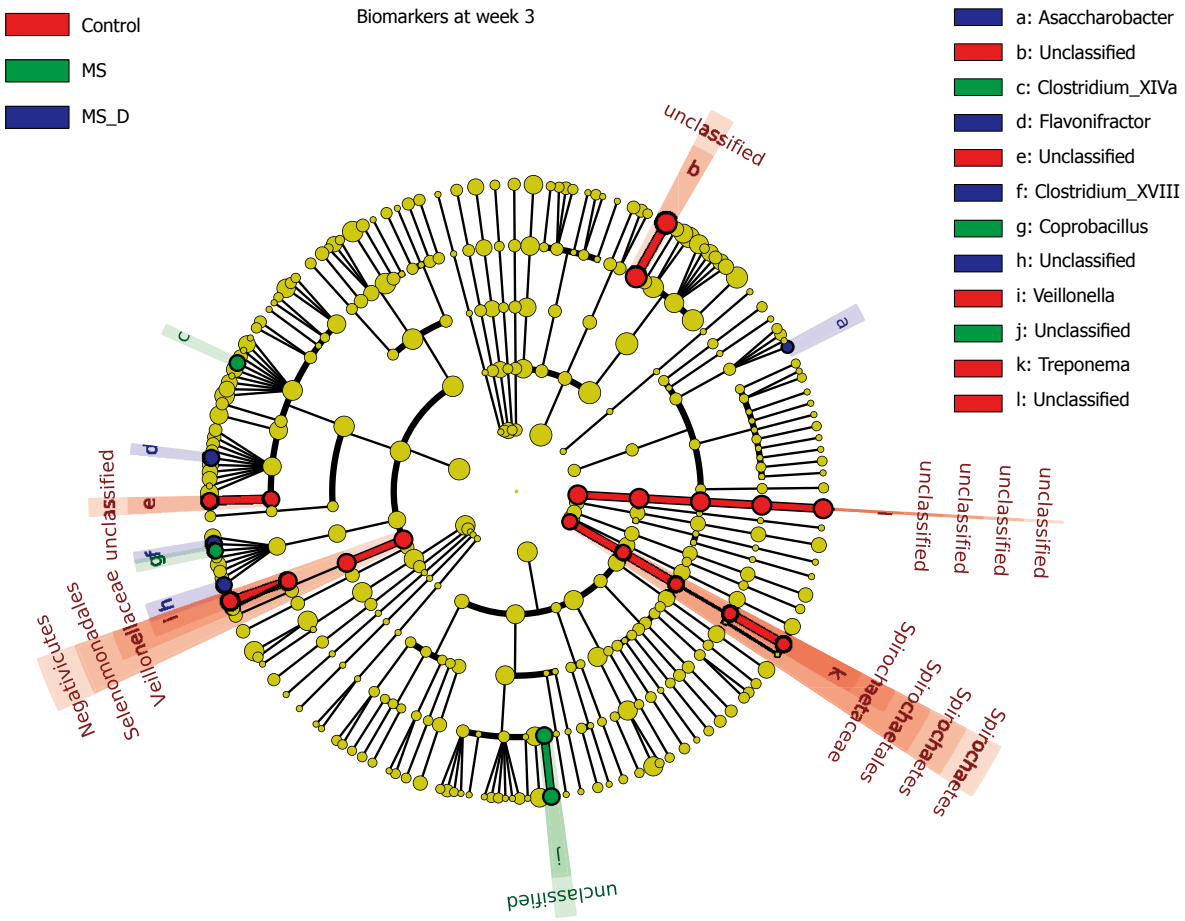
To investigate whether the differentially abundant taxa correlated with the visceral hypersensitivity level, we listed the biomarkers in each group by week 12, and calculated their Spearman correlation to the VHI both within the respective group and across groups (Table 3). By week 12, *Fusobacterium* was associated with the MS group (LDA Score = 2.766). The *Fusobacterium* abundance was also significantly and positively correlated with the VHI across the all 24 samples by week 12 (r = 0.4564, P = 0.0250). Unclassified *Erysipelotrichaceae* was associated with the control group (LDA Score = 3.097) and significantly and negatively correlated with the VHI across groups (r = -0.4944, P = 0.0140). These data suggest that *Fusobacterium* may participate in the pathogenesis of visceral hypersensitivity and that *Erysipelotrichaceae* might protect against the hypersensitivity.

Comparing visceral hypersensitive rat models to IBS patients

Next, we asked to what extent visceral hypersensitive rats' dysbiosis resembles that of IBS patients. We downloaded the pyrosequencing data published by Jeffery *et al*[6], which included 37 IBS patients and 20 controls. We used the LEfSe method to analyze disease and healthy biomarkers in human fecal samples (Figure 6A); this data was further compared to the data from our animal models (Figure 6B). We identified 36 biomarkers of IBS patients and 15 biomarkers of human controls[6] (Figure 6A). The biomarkers of disease were largely different in human IBS and visceral hypersensitive rats, and only a few disease or control biomarkers were shared between the human study[6] and our rat model study. *Fusobacteria* marginally increased in IBS patients compared with human controls[6] (P = 0.063, KW, Figure 6C). The MS rat model did not share increased common biomarkers with human patients. Both control rats and human health controls have fecal microbial markers of *Porphyromonadaceae* and unclassified *Porphyromonadaceae* (Figure 6B). In the IBS cohort published by Jeffery *et al*[6], *Porphyromonadaceae* was significantly lower in the IBS groups (P = 0.0006, KW, Figure 6D), and the MS rats also had lower *Porphyromonadaceae* concentrations (P = 0.0097, KW, Figure 6D). The genus *Clostridium_XI* (belonging to family *Peptostreptococcaceae*, order *Clostridiales*) was a biomarker of both IBS patients and pTNBS rats (Figure 6E). *Clostridium_XI* accounted for up to 6% of the fecal microbiota in the IBS group and was significantly higher than the level in the healthy control group (P = 0.0484, KW). Additionally, *Clostridium XI* also colonized at a higher level in the pTNBS rats (P = 0.0422, KW).

DISCUSSION

In this study, we found that (1) both the MS and the pTNBS rat models developed dysbiosis of fecal microbiota; (2) the fecal microbiota of the MS model was characterized by a lower diversity and a higher level of *Fusobacteria* at week 3 and week 12. A fraction of the MS rats formed an isolated MS cluster that indicated clear-cut dysbiosis in comparison to the controls but the rats in this cluster tended to alternate; (3) the pTNBS model was characterized by higher *Actinobacteria* but did not develop any isolated clusters; (4) among the biomarkers observed by week 12, the *Fusobacterium* positively and unclassified *Erysipelotrichaceae* negatively correlated to visceral hypersensitivity; and (5) in comparison to a previously published fecal microbial profile in human IBS patients[6], *Porphyromonadaceae* was a protective biomarker for both healthy humans and rat controls;


Control
MS
MS_D
Biomarkers at week 3
a: Asaccharobacter
b: Unclassified
c: Clostridium_XIVa
d: Flavonifractor
e: Unclassified
f: Clostridium_XVIII
g: Coprobacillus
h: Unclassified
i: Veillonella
j: Unclassified
k: Treponema
l: Unclassified
unclassified
Veillonellaceae
Selenomonadales
Negativicutes
Spirochaetaceae
Spirochaetales
Spirochaetes
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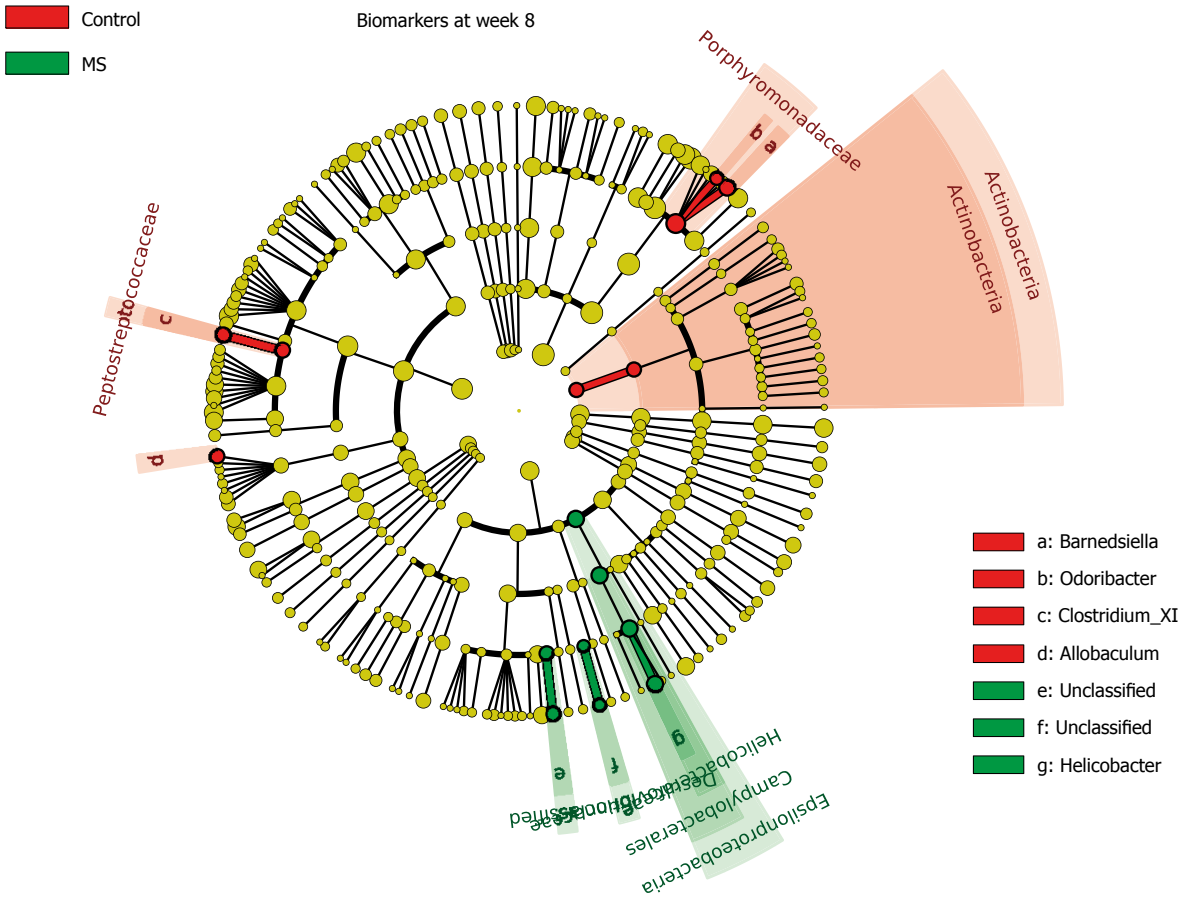
unclassified


Control
MS
Biomarkers at week 8
a: Barnedsiella
b: Odoribacter
c: Clostridium_XI
d: Allobaculum
e: Unclassified
f: Unclassified
g: Helicobacter
Porphyromonadaceae
Actinobacteria
Peptostreptococcaceae
Helicobacteraceae
Campylobacterales
Epsilonproteobacteria

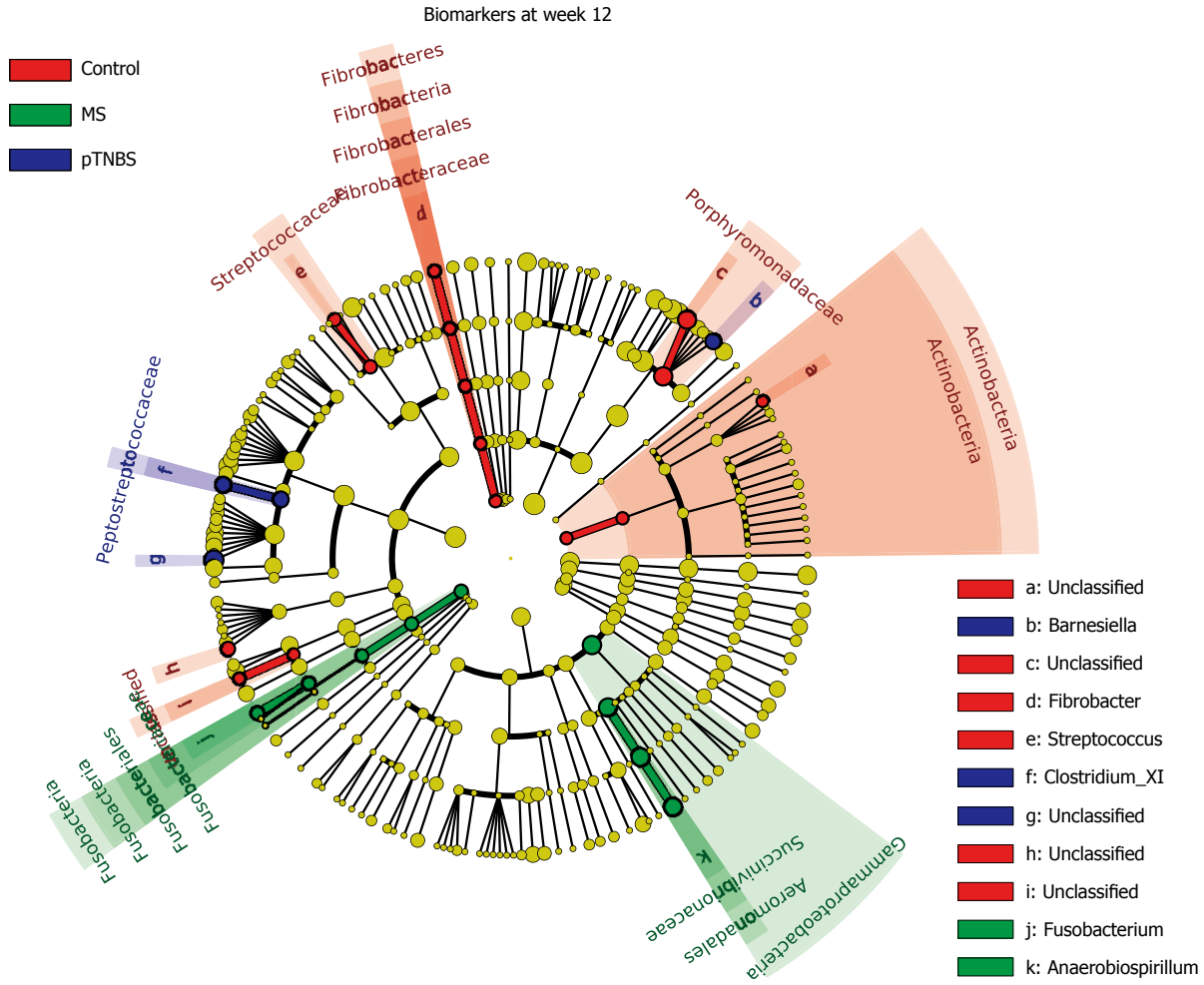


Figure 5 Microbial markers for different groups at weeks 3, 8 and 12. Biomarkers for each time point were calculated using the LEfSe Method. The abundances of taxa at the phylum, class, order, family, and genus levels were compared between the groups. Taxa with different abundances between groups and with an LDA score larger than 2.0 were considered to be a biomarker; biomarkers were indicated with corresponding colors on the cladogram. See also Figure S1, S2, and S3. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

Clostridium_XI was a shared biomarker for human IBS patients and pTNBS rats.

Rodent models have been frequently used to study the pathogenesis and treatment of IBS, where the intestinal microbiota plays an important role. Before this study, dysbiosis in IBS rat models had not been specifically profiled, and whether any members in microbial community were correlated to visceral hypersensitivity was not known. We evaluated the fecal microbiota of MS and control rats at three time points and found that dysbiosis happened shortly after weaning (third week) and at the adult phase (12 wk). The pTNBS rat model did not develop an isolated microbial cluster but had biomarkers of *Barnesiella*, *Clostridium_XI*, and unclassified *Ruminococcaceae*. Through the unweighted UniFrac test, we found that the co-housing effect was no more significant at week 8 or week 12. Thus, both models developed significant dysbiosis of the fecal microbiota.

Approximately 10% of IBS patients believe that their symptoms began with an infectious illness, and prospective studies have shown that 3% to 36% of enteric infections lead to IBS symptoms[19]. Understanding underlying gut microbial dysbiosis associated with PI-IBS is critical for the prevention and management of this disease. *Campylobacter jejuni*, *Campylobacter rodentium*, and *Salmonella enterica* are available bacterial infectious murine models that mimic aspects of the pathogenesis of post-infectious IBS[8]. In this study, two rat models, MS and pTNBS, were not given any specific infector but were colonized more frequently by *Fusobacterium* and *Clostridium XI,* respectively. The latter includes the *Clostridium difficile*, *Clostridium litorale*, and *Clostridium lituseburense*. These two genera were also found to increase or tend to increase in the downloaded Miseq 16S rRNA gene sequencing data from Jeffery's IBS cohort[6]. Thus, these two bacteria may be common dysbiosis features across human and rat models. In a chip-based study by Jalanka-Tuovinen *et al*[7], *C. cellulosi* and its relatives (members from *Clostridium* cluster IV) significantly decreased in IBS-D patients. Thus, whether *Clostridium* plays a mechanistic role in the pathogenesis of IBS warrants further study.

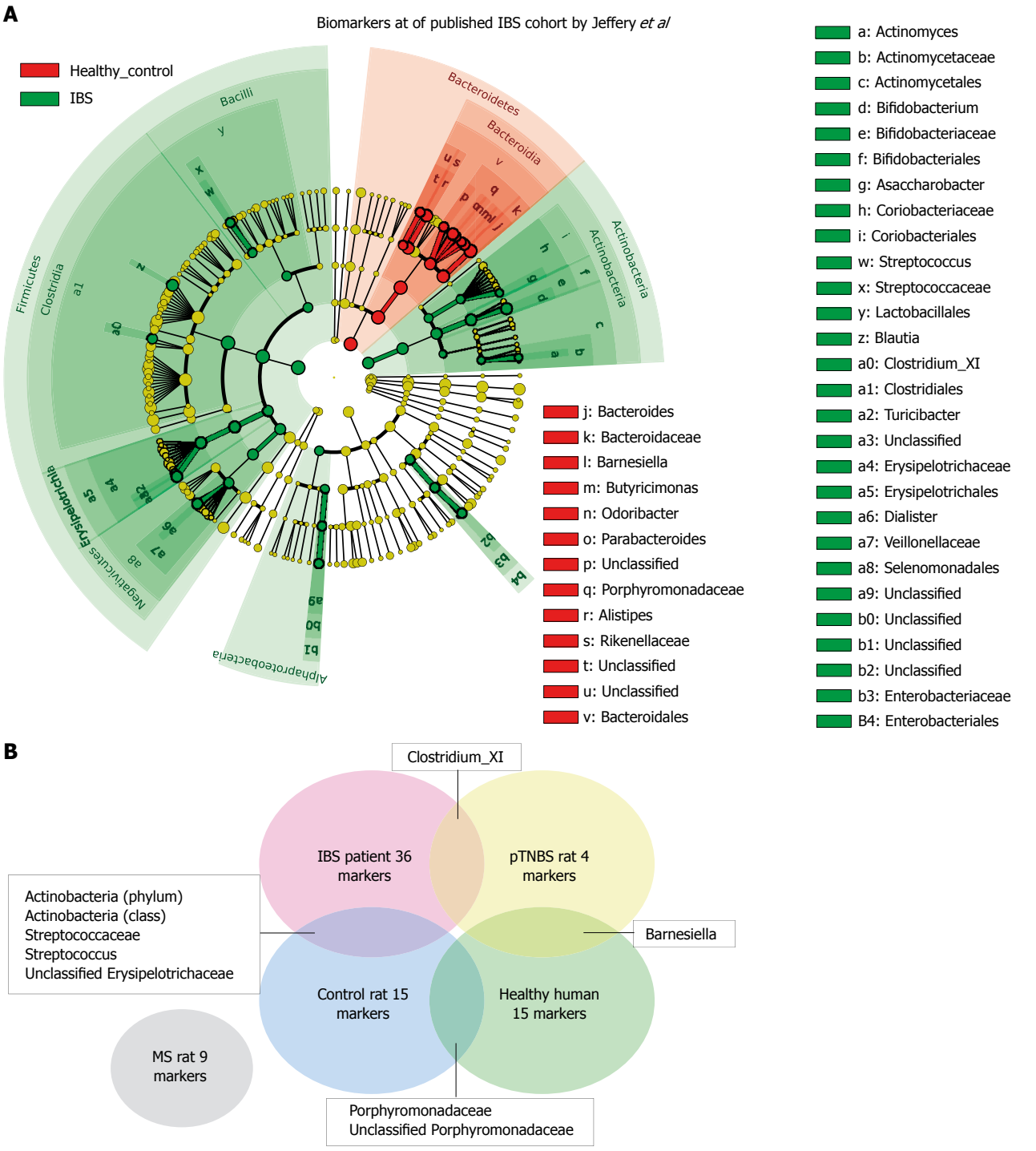
Table 3 Abundance of different taxa among groups by week 12 and correlations to visceral hypersensitivity

Taxonomy	LDA score (Log10)	Group	Across groups (n = 24)		Within group	
			r_all_sample	p_all_sample	r_ingroup	p_ingroup
Actinobacteria	2.338	Control n = 7	0.1835	0.3906	0.2041	0.6606
Actinobacteria | Actinobacteria	2.338		0.1835	0.3906	0.2041	0.6606
Actinobacteria | Actinobacteria | Coriobacteriales | Coriobacteriaceae | Unclassified	2.192		0.2639	0.2127	0.2041	0.6606
Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae	4.610		-0.1005	0.6405	0.1429	0.7825
Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Unclassified	4.543		-0.1344	0.5313	0.1429	0.7825
Fibrobacteres	2.435		-0.3601	0.0839	-0.0741	0.8745
Fibrobacteres | Fibrobacteria	2.435		-0.3601	0.0839	-0.0741	0.8745
Fibrobacteres | Fibrobacteria | Fibrobacterales	2.435		-0.3601	0.0839	-0.0741	0.8745
Fibrobacteres | Fibrobacteria | Fibrobacterales | Fibrobacteraceae	2.435		-0.3601	0.0839	-0.0741	0.8745
Fibrobacteres | Fibrobacteria | Fibrobacterales | Fibrobacteraceae | Fibrobacter	2.435		-0.3601	0.0839	-0.0741	0.8745
Firmicutes | Bacilli | Lactobacillales | Streptococcaceae	2.710		0.1215	0.5717	0.4447	0.3174
Firmicutes | Bacilli | Lactobacillales | Streptococcaceae | Streptococcus	2.710		0.1215	0.5717	0.4447	0.3174
Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | Unclassified	3.097		-0.4944	0.0140[1]	-0.1429	0.7825
Firmicutes | Negativicutes | Selenomonadales | Unclassified	2.396		-0.2047	0.3374	0.7027	0.0782
Firmicutes | Negativicutes | Selenomonadales | Unclassified | Unclassified	2.396		-0.2047	0.3374	0.7027	0.0782
Fusobacteria	2.766		0.4564	0.0250[1]	0.2067	0.5667
Fusobacteria | Fusobacteria	2.766		0.4564	0.0250[1]	0.2067	0.5667
Fusobacteria | Fusobacteria | Fusobacteriales	2.766		0.4564	0.0250[1]	0.2067	0.5667
Fusobacteria | Fusobacteria | Fusobacteriales | Fusobacteriaceae	2.766		0.4564	0.0250[1]	0.2067	0.5667
Fusobacteria | Fusobacteria | Fusobacteriales | Fusobacteriaceae | Fusobacterium	2.766	MS n = 10	0.4564	0.0250[1]	0.2067	0.5667
Proteobacteria | Gammaproteobacteria	4.622		0.1809	0.3976	0.0667	0.8648
Proteobacteria | Gammaproteobacteria | Aeromonadales	4.622		0.1757	0.4115	0.0667	0.8648
Proteobacteria | Gammaproteobacteria | Aeromonadales | Succinivibrionaceae	4.622		0.1757	0.4115	0.0667	0.8648
Proteobacteria | Gammaproteobacteria | Aeromonadales | Succinivibrionaceae | Anaerobiospirillum	4.622		0.1757	0.4115	0.0667	0.8648
Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Barnesiella	3.607		-0.1501	0.4840	-0.0180	0.9694
Firmicutes | Clostridia | Clostridiales | Peptostreptococcaceae	3.776	pTNBS n = 7	0.1946	0.3623	0.0180	0.9694
Firmicutes | Clostridia | Clostridiales | Peptostreptococcaceae | Clostridium_XI	3.776		0.1946	0.3623	0.0180	0.9694
Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Unclassified	5.014		-0.0322	0.8813	-0.1982	0.6701

[1]The P-value is less than 0.05. LDA: Linear discriminant analysis; MS: Maternal separation; pTNBS: TNBS post-inflammatory.

By week 12, *Fusobacterium* colonized in significantly greater abundance in MS rats, and its abundance was positively correlated with higher VHI scores. The *Fusobacteria* phylum also tends to be higher in published IBS cohorts[6]. *Fusobacterium* was invasive to the gut epithelial cells and has already been documented as being involved in the pathogenesis of colorectal adenoma[20-22] and inflammatory bowel disease[23,24]. This represents the first study documenting that *Fusobacterium* was involved in visceral hypersensitivity. Whether the increased colonization of *Fusobacterium* caused low grade inflammation and thus contributed to visceral hypersensitivity is not currently known. Moreover, both *Fusobacterium* and members in *Clostridium* are known short chain fatty acid (SCFA) producers[24-26]. The low fermentable oligo-, di-, and monosaccharides and polyol (FODMAP) diet has been shown to be an efficacious therapy for the reduction of IBS symptoms in randomized controlled trials[27-29]. Supplementing food containing FODMAP to IBS patients and healthy people would trigger gastrointestinal symptoms to a larger extent in the patient group[30]. However, the mechanism of treatment with a low FODMAP diet and why IBS patients are more sensitive to FODMAP remains unknown[31]. In this study, we identified two butyric producing taxa, *Fusobacterium* and *Clostridium*, which significantly correlate to visceral hypersensitivity or are disease biomarkers. Their colonization may render patients more ready to produce SCFA and gas in the presence of FODMAP. It was reported that the butyrate-producing *Clostridium* cluster XIVa significantly increased in IBS patients consuming a typical high FODMAP diet compared with those on a low FODMAP diet[32]. Farmer *et al*[33] showed that caecal intraluminal pH was significantly lower in IBS patients compared to controls. Thus, the detailed mechanistic role of *Fusobacterium* and *Clostridium* in IBS warrants further study.

Porphyromonadaceae is a family belonging to the order of *Bacteroidales* and the class *Bacteroidetes*. Among others, *Barnesiella* and *Butyricimonas* are genera under *Porphyromonadaceae*. Unfortunately, the biological function of *Porphyromonadaceae* has not been characterized in detail, and little attention has been paid to their role in gastrointestinal diseases. A previous study documented that *Barnesiella* was enriched in dextran sulfate sodium-induced colitis[34]. In our study, *Barnesiella* was also enriched 4 weeks after TNBS instillation. Whether *Barnesiella* promoted the inflammation or was passively enriched under inflammatory conditions was not determined in the current study. A recent review paper[35] summarized 29 relevant original research articles concerning microbiota analysis and IBS. Durbán's pyrosequencing


A
Biomarkers at of published IBS cohort by Jeffery et al
Healthy_control
IBS
Bacilli
Bacteroidetes
Bacteroidia
Actinobacteria
Actinobacteria
Firmicutes
Clostridia
Negativicutes
Erysipelotrichia
Alphaproteobacteria
j: Bacteroides
k: Bacteroidaceae
l: Barnesiella
m: Butyricimonas
n: Odoribacter
o: Parabacteroides
p: Unclassified
q: Porphyromonadaceae
r: Alistipes
s: Rikenellaceae
t: Unclassified
u: Unclassified
v: Bacteroidales
a: Actinomyces
b: Actinomycetaceae
c: Actinomycetales
d: Bifidobacterium
e: Bifidobacteriaceae
f: Bifidobacteriales
g: Asaccharobacter
h: Coriobacteriaceae
i: Coriobacteriales
w: Streptococcus
x: Streptococcaceae
y: Lactobacillales
z: Blautia
a0: Clostridium_XI
a1: Clostridiales
a2: Turicibacter
a3: Unclassified
a4: Erysipelotrichaceae
a5: Erysipelotrichales
a6: Dialister
a7: Veillonellaceae
a8: Selenomonadales
a9: Unclassified
b0: Unclassified
b1: Unclassified
b2: Unclassified
b3: Enterobacteriaceae
B4: Enterobacteriales
B
Clostridium_XI
IBS patient 36 markers
pTNBS rat 4 markers
Actinobacteria (phylum)
Actinobacteria (class)
Streptococcaceae
Streptococcus
Unclassified Erysipelotrichaceae
Barnesiella
Control rat 15 markers
Healthy human 15 markers
MS rat 9 markers
Porphyromonadaceae
Unclassified Porphyromonadaceae
C
Fusobacteria
P = 0.063
0.00030
0.00025
0.00020
0.00015
0.00010
0.0005
0.0000
Healthy control
IBS

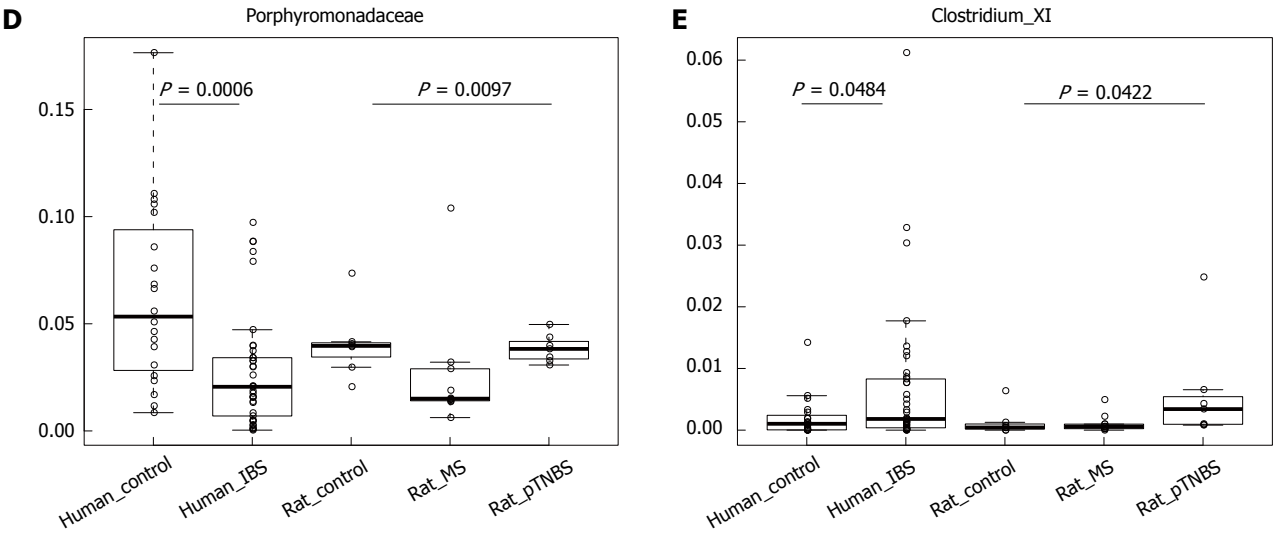


Figure 6 Comparing rat model dysbiosis to that of Jeffery's human irritable bowel syndrome cohort. A: Cladogram indicates the biomarkers of different abundances between groups; B: Venn plot of the positive (increased) biomarkers shared by rat models and Jeffery's human IBS cohort. The overlapped area indicates that the two groups have common biomarkers. The MS rat does not share biomarkers from the human cohort; C: Abundance of the *Fusobacteria* phylum marginally increased in the human IBS cohort; D: Abundance of *Porphyromonadaceae* in fecal samples of healthy human controls, human IBS patients, control rats, MS rats, and pTNBS rats. *Porphyromonadaceae* was depleted in both the human IBS group and the MS rats; E: Abundance of *Clostridium XI* in fecal samples of healthy human controls, healthy IBS patients, control rats, MS rats, and pTNBS rats. *Clostridium XI* increased in both human IBS patients and pTNBS rats. IBS: Irritable bowel syndrome; MS: Maternal separation; pTNBS: TNBS post-inflammatory.

study[36] found that the family *Porphyromonadaceae* was increased in the fecal samples of IBS subjects. In our study, the *Porphyromonadaceae* was highest in the control group by week 12. The discrepancy may be explained by the different nature between human patients and rat models.

IBS is a human disease with multifactorial pathophysiology[37], and the prevalence of IBS is associated with social-economic factors[38]. To date no available model could ideally model the IBS pathogenesis. IBS is heterogeneous and thus unlikely to be modeled in any single model. Although common biomarkers were found between human IBS patients and rat models, the limitations of rat models should also be taken into consideration. The pTNBS model was triggered by a pro-inflammatory molecule (TNBS). Therefore, this model resembles the human inflammatory bowel disease to some extent and can only mimic the post-infectious IBS, which is associated only to a percentage of patients. Furthermore, the causal relationship between visceral hypersensitivity, dysbiosis, and the symptoms of IBS is not clear and remains to be untangled in the future.

In summary, both the MS and the post-inflammation rat models developed dysbiosis in the fecal microbiota, and the models captured parts of the dysbiosis features of human IBS patients. The potential pathogenic role of *Fusobacterium* and *Clostridium XI*, as well as the protective role of *Porphyromonadaceae* warrants further mechanistic study.

ACKNOWLEDGMENTS

We acknowledge Dr. Ian Jeffery and Dr. Paul W. O'Toole for sharing their sequencing data from human IBS patients and healthy controls.

COMMENTS

Background

Previous studies have indicated that the gut microbiota participated in the pathogenesis of irritable bowel syndrome (IBS).

Research frontiers

Dysbiosis of the gastrointestinal microbiota and hypersensitivity to colonic distension are critical features of IBS. For animal models, the correlation between dysbiosis in the microbiota and visceral hypersensitivity remains unknown.

Innovations and breakthroughs

Dysbiosis triggered by neonatal maternal separation (MS) was lasting but not static. Both MS and post-inflammatory rat fecal microbiota deviated from that of the control rats to an extent that was larger than the co-housing effect. *Fusobacterium*, *Clostridium XI* and *Porphyromonadaceae* were identified as targets for future mechanistic research.

Applications

This study indicated that the two animal models could capture part of the dysbiosis features of IBS. Further mechanistic study on the biomarkers' role in the pathogenesis is warranted.

Peer-review

The manuscript is excellent and addresses adequately the relationship between dysbiosis and visceral hypersensitivity in experimental animals. The quality of the study design and experimental investigations are very high.

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ORIGINAL ARTICLE

Case Control Study

Factors affecting occurrence of gastric varioliform lesions: A case-control study

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Author contributions: Zou TH performed telephone interviews of most participants and wrote the manuscript; Zheng RH performed part of the telephone interviews; Gao QY and Kong X provided analytical tools; Chen XY offered the pathological data; Ge ZZ offered the endoscopic data; Chen YX served as scientific advisors; Fang JY designed the study and edited the manuscript; all authors approved the final version of the manuscript.

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Abstract

AIM: To investigate the factors influencing the occurrence of gastric varioliform lesions (GVLs) and their possible link with gastric cancer.

METHODS: A 1:1 matched case-control study was performed to retrospectively analyze data from 1638 chronic gastritis patients who had undergone gastroscopy at one of two Chinese hospitals between 2009 and 2014. Patients with GVLs (cases) were compared to those without such lesions (controls). Endoscopic and pathological findings were recorded, along with interview information on *Helicobacter pylori* (*H. pylori*) infection, medical, drug and family histories, lifestyle and eating habits. The association between each factor and the occurrence of GVLs was estimated, and then multivariate conditional logistic regression was used to evaluate the independent factors.

RESULTS: The frequency and severity of glandular

atrophy, intestinal metaplasia (IM) and low-grade intraepithelial neoplasia were significantly increased in the GVL group ($P < 0.01$). Overall analysis showed that *H. pylori* infection [3.051 (2.157, 4.317), $P < 0.001$], allergic respiratory diseases [3.636 (2.183, 6.055), $P < 0.001$], work-related stress [2.019 (1.568, 2.600), $P < 0.001$], irregular meals [2.300 (1.462, 3.619), $P < 0.001$], high intake of spicy food [1.754 (1.227, 2.507), $P = 0.002$] and high intake of fresh fruit [0.231 (0.101, 0.529), $P = 0.001$] were significantly correlated with the occurrence of GVLs (positively, except for the latter). Stratified analyses indicated that pickled food consumption in patients over 50 years old [7.224 (2.360, 22.115), $P = 0.001$] and excessive smoking in men [2.013 (1.282, 3.163), $P = 0.002$] were also positively correlated, and that, for antral GVLs, vegetable consumption [0.491 (0.311, 0.776), $P = 0.002$] was negatively correlated.

CONCLUSION: Seven risk factors and two protective factors are determined for GVLs, which were found to be associated with premalignant abnormalities.

Key words: Gastric cancer; Gastric varioliform lesions; Precancerous lesion; Risk factor; Varioliform gastritis

Core tip: To our knowledge, this is the first case-control study investigating the factors influencing the formation of gastric varioliform lesions, which were supposed to be associated with gastric neoplasia in previous reports. Our results indicate a potentially increased cancer risk for the affected patients, and that *Helicobacter pylori* infection, allergic respiratory diseases, high work-related stress, irregular meals, high intake of spicy food, pickled food consumption in elder people, excessive smoking in men, consumption of vegetables and high intake of fresh fruit are found to be correlated with the occurrence of gastric varioliform lesions.

Zou TH, Zheng RH, Gao QY, Kong X, Chen XY, Ge ZZ, Chen YX, Zou XP, Fang JY. Factors affecting occurrence of gastric varioliform lesions: A case-control study. *World J Gastroenterol* 2016; 22(22): 5228-5236 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5228.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5228

INTRODUCTION

Varioliform gastritis (VG), or "octopus sucker" gastritis in the foreign literature and verrucous gastritis in the national literature, is a disease with a characteristic endoscopic manifestation but no specific clinical symptoms. The major endoscopic feature is the presence of gastric varioliform lesions (GVLs), namely, widespread small lesions, manifesting as round, oval or irregularly shaped elevations, often possessing a central umbilical-like depression covered in gray-colored secretion or tiny bleeds. In 1947, Moutier and Martin[1] first described two cases of this distinctive gastric mucosal disease, and then in 1978, Lambert *et al*[2] classified the disease, according to its site of occurrence, into "diffuse" VG when spread throughout the stomach, and "antral" VG when restricted to the antrum. These two forms of VG are thought to have different etiopathogenesis and histological manifestations[3]. VG has been recognized as a protruded type of chronic erosive gastritis in the Consensus on Chronic Gastritis in China (2012)[4], but endoscopists more often present the diagnosis as chronic gastritis with varioliform lesions.

Until recently, very little was known about the etiopathogenesis of GVLs. Malfertheiner *et al*[5] reported that the *Helicobacter pylori* (*H. pylori*) infection rate was 89% among 37 patients with GVLs, and their clinical symptoms and mucosal inflammation were substantially improved after effective eradication of the infection. In the national literature, most authors support this point of view and regard *H. pylori* as the main cause of GVLs. On the other hand, several studies have provided compelling evidence that type Ⅰ hypersensitivity may play a role[6]. Andre *et al*[7] found a large number of IgE-containing cells in the affected gastric mucosa and a significantly increased incidence of allergic diseases in patients with GVLs, as compared with the normal population. Furthermore, they performed a randomized double-blind placebo-controlled trial to compare clinical and endoscopic outcomes in patients treated with sodium cromoglycate, cimetidine or placebo[8]. The result stated that treatment with sodium cromoglycate greatly improved both sets of outcomes, whereas treatment with cimetidine or placebo showed no appreciable effect. Other previously reported pathogenic factors include hyperacid[9] and viral infection[10].

Some reports suggest a possible association between GVLs and gastric neoplasia. In 1960, Munoz Monteavaro *et al*[11] observed "*in situ*" carcinomatous transformation in a patient with VG, and other groups have reported similar findings more recently[12,13]. The elevated lesions can persist and transform into sessile polyps and appear as a gastric carcinoma several years later; as a result, the disease was classified as a precursor to gastric cancer at the World Congress of Gastroenterology (WCOG) in 1994. Diverse risk factors are involved in gastric carcinogenesis, including bacterial, environmental, dietary and genetic variables[14]. Numerous epidemiological studies have attempted to shed light on the factors impacting gastric neoplasia and precancerous lesions; these include a history of diabetes[15], aspirin consumption[16], excessive smoking[17] and drinking[18], pickled food consumption[19], tea consumption[20], amongst others. However, the results are somewhat inconsistent due

to the ethnic diversity and limited sample size. A recent systematic review concluded that smoking, drinking, red meat and pickled food were risk factors, and that fresh vegetables and fruit may be protective; there was insufficient evidence to draw conclusions regarding coffee, tea or seafood[21]. GVLs may share some of these risk factors, and clarifying the matter should provide a better understanding of this potentially premalignant condition, allowing physicians to better identify at-risk patients and to devise more effective treatment strategies. Therefore, we carried out a retrospective 1:1 matched bi-center case-control study, analyzing endoscopic and pathological data from 1638 patients with chronic gastritis. The association between potentially relevant variables and the occurrence of GVLs was systematically evaluated, with an aim to find independent risk factors and protective factors.

MATERIALS AND METHODS

Study sample and selection criteria

A 1:1 matched case-control study was conducted, analyzing data from outpatients who had undergone gastroscopy at Renji Hospital, Shanghai Jiao-Tong University School of Medicine or the Nanjing Drum Tower Hospital, Nanjing University School of Medicine between 2009 and 2014. A total of 1638 chronic gastritis patients were enrolled, all of which fell into one of two categories: those with GVLs (cases; n = 819) or those without such lesions (controls; n = 819).

To populate the case group, we searched the electronic databases of the aforementioned hospital endoscopic centers, using the following keywords: "varioliform gastritis" or "with gastric varioliform lesions" or "with erosive elevations"; then we closely examined the corresponding patient images and selected those patients having at least three typical lesions. Any disagreement was discussed by T.H. Zou and R.H. Zheng before reaching a consensus. Control patients, who were diagnosed with chronic gastritis at the same time, but without varioliform lesions, were matched one by one with the case group members, based on gender, age ± 2 years, month of examination and endoscopist. The exclusion criteria were strictly adhered to and were as follows: those who had no biopsy, those who were diagnosed with gastric cancer and those who had undergone partial or total gastrectomy. For those who had repeated examinations, we only recorded data from the first diagnostic gastroscopy.

Data extraction

The endoscopic and pathological findings were recorded. All the patients were required to undergo a gastroscopy with biopsies for the diagnosis. All parts of the upper gastrointestinal tract were carefully examined for any lesions by experienced endoscopists, and at least two biopsies were taken from the antrum. If suspected lesions were found, 2 to 5 more biopsies were taken. Pathological examinations for chronic gastritis were made by experienced pathologists according to the visual analogue scale (VAS) in the updated Sydney System[22,23] that is associated with the Consensus on Chronic Gastritis in China. Histological diagnosis of intraepithelial neoplasia was made based on the World Health Organization (WHO) classification[24]. To concretely differentiate the severity of inflammation, glandular atrophy or IM in the present study, a scheme was introduced using the following calculation: grading index = $(S_1 \times B_1 + S_2 \times B_2 + \ldots + S_n \times B_n)/B_n$, where S is the severity of a particular biopsy specimen, B is the number of the relevant specimen and n is the quantity of specimens[25].

H. pylori infection was detected using a *H. pylori* rapid urease test during endoscopic examination, HE and Giemsa staining of biopsy specimens, and a ^{13}C urea breath test. We defined a positive result as meeting one of the following two criteria: (1) the rapid urease test or HE staining was positive; or (2) if both urease and HE results were negative, yet the specimen was highly inflamed, Giemsa staining was added or a ^{13}C urea breath test was performed, and a positive outcome was considered indication of *H. pylori* infection. A ^{13}C urea breath test was subsequently used when evaluating the effect of eradication on *H. pylori* infection.

A questionnaire was designed by the authors and it was used to conduct telephone interviews with all patients in the study. The investigators were trained to be polite and methodical during interviews and they avoided calling patients at working, or otherwise busy hours. The questionnaire requested information on the patient's gender, age, *H. pylori* infection history, medical history, allergic diseases, drug history, family history, long-term lifestyle and eating habits. *H. pylori* infection history was categorized according to four different conditions: currently infected, but with no previous history of infection; chronic (repeated or persistent) infection; past infection that has been completely eradicated; no current or past infection. Allergic diseases consisted of bronchial asthma, allergic rhinitis, allergic skin disease, drug allergy, *etc*. The presence of allergic diseases was mainly based on the interview data, and the authors made the judgment with reference to the guideline of diagnosis for each disease. Lifestyle variables included sleep quality, work-related stress, tobacco smoking and alcohol consumption. Eating habits comprised irregular meals, intake of spicy food, pickled food, fried food, fresh fruit and vegetables; consumption of a particular food type over four time per week was considered high.

Statistical analysis

Statistical analyses were performed using SPSS Version 20.0 (SPSS Inc., Chicago, IL, United States).

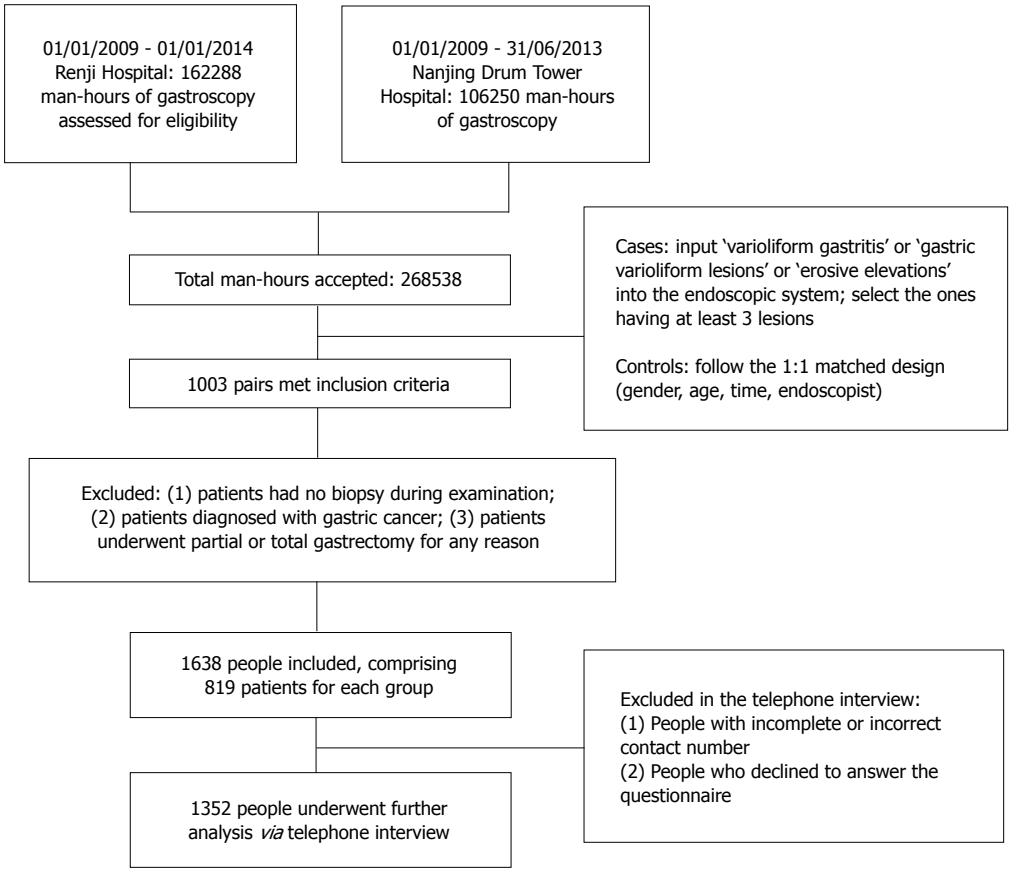

Figure 1 Flow chart of participant selection in the case-control study.

Table 1 Characteristics of the case group *n* (%)

Characteristic	
In total	819 (100)
Hospital	
Renji	491 (60.0)
Drum tower	328 (40.0)
Age (yr)	
< 50	278 (33.9)
⩾ 50, < 60	289 (35.3)
⩾ 60	252 (30.8)
Gender	
Men	448 (54.7)
Women	371 (45.3)
Illness part	
Antral form	806 (98.4)
Diffuse form	13 (1.6)
H. pylori infection	
H. pylori (+)	263 (32.1)
H. pylori (-)	556 (67.9)
Histology	
Glandular atrophy	363 (44.3)
IM	265 (32.4)
Intraepithelial neoplasia	92 (11.2)

H. pylori: *Helicobacter pylori*.

Measurement data were compared between the two groups using a paired *t*-test; where appropriate, numerical data were subjected to a chi-square test, while categorical data using a Mann-Whitney test. Single-factor analysis was used to estimate the association between each potential factor and GVLs, and then multivariate conditional Logistic regression analysis was applied to determine the independent risk and protective factors. Odds ratios (ORs) with 95% confidence intervals (95%CIs) were used to assess the magnitude of the associations. A two-sided *P*-value < 0.05 was considered statistically significant.

RESULTS

The systematic database search yielded 268538 man-hours of gastroscopy over the five-year period of interest. Following the inclusion/exclusion criteria and the 1:1 matched design, 1638 subjects were selected to populate the case and control groups. A flow chart of the selection process is presented in Figure 1. There were 448 men and 371 women in the case group, with a mean age of 53.40 ± 11.41 years (ranging from 18 to 87 years old). The basic, endoscopic and pathological characteristics of the case group are shown in Table 1.

Analysis of histological data

We compared the frequencies of *H. pylori* infection, glandular atrophy, IM and intraepithelial neoplasia between the cases and controls using a χ^2 test. The difference was significant for *H. pylori* infection (OR

Table 2 Overall and stratified analyses of histological data in the case-control study

	Case	Control	OR [95%CI]	*P*-value
Overall analysis				
H. pylori (+)	263	141	2.275 [1.801, 2.872]	< 0.001[b]
Glandular atrophy	363	271	1.610 [1.317, 1.967]	< 0.001[b]
IM	265	182	1.674 [1.343, 2.087]	< 0.001[b]
Intraepithelial neoplasia	92	65	1.468 [1.052, 2.049]	0.023[a]
Antral form				
H. pylori (+)	254	139	2.208 [1.745, 2.794]	< 0.001[b]
Glandular atrophy	341	268	1.472 [1.202, 1.803]	< 0.001[b]
IM	409	179	3.609 [2.908, 4.479]	< 0.001[b]
Intraepithelial neoplasia	79	65	1.239 [0.878, 1.747]	0.222
Diffuse form				
H. pylori (+)	9	2	12.375 [1.828, 83.767]	0.015[a]
Glandular atrophy	13	3		< 0.001
IM	11	3		0.005
Intraepithelial neoplasia	12	0	18.333 [2.522, 133.26]	< 0.001[b]

[a]$P < 0.05$; [b]$P < 0.01$, the case group *vs* the control group. *H. pylori*: *Helicobacter pylori*.

= 2.275, 95%CI: 1.801-2.872, $P < 0.01$), especially in the patients with diffuse GVLs. We also noted statistically significant findings in the pooled analysis for the association between glandular atrophy, with or without IM, and the formation of such lesions. Furthermore, a significantly increased risk of low-grade intraepithelial neoplasia was observed in the case group (OR = 1.468, 95%CI: 1.052-2.049, P = 0.023); this is known to be a premalignant condition. When the patients were further stratified according to VG form, the differences between patients with antral lesions and their matched controls were significant for glandular atrophy and IM, but not for intraepithelial neoplasia. On the other hand, diffuse lesions were strongly associated with all these histological parameters, including intraepithelial neoplasia. The results of the overall and stratified analyses are presented in Table 2.

Indices for grading inflammation, glandular atrophy and IM were calculated separately for the GVL patients and their matched controls. Paired *t*-tests showed that all these indices were significantly increased in the case group ($P < 0.01$).

Analysis of telephone interview data

For 49 patients, the contact number was incomplete or incorrect, and 94 declined to answer the questionnaire. Thus, there was a total of 1352 participants, comprising 676 people from each group; the answering ratio was 82.5%. Single-factor analyses of potential factors demonstrated that current infection with *H. pylori* (OR = 2.203), chronic infection with *H. pylori* (OR = 2.493), bronchial asthma (OR = 6.837), allergic rhinitis (OR = 2.963), family history of gastric cancer (OR = 1.926), high work-related stress (OR = 1.871), irregular meals (OR = 1.703), and high intake of spicy food (OR = 1.540) were positively associated with the occurrence of GVLs, while high intake of fresh fruit was negatively associated (OR = 0.721).

We found a negative correlation between current and chronic infection with *H. pylori* (Pearson coefficient, -0.113), and a positive correlation between bronchial asthma and allergic rhinitis (Pearson coefficient, 0.151). No correlation was found for any other factors. For subsequent analyses, we combined current and chronic infection into a single "*H. pylori* infection" category, and asthma and rhinitis were combined into "allergic respiratory diseases". Based on the results of single-factor analyses, we included the factors with a *P*-value less than 0.05 into the multivariate conditional Logistic regression equation. The adjusted analysis suggested that *H. pylori* infection, allergic respiratory diseases, high work-related stress, irregular meals and high intake of spicy food were independent risk factors for the formation of GVLs; and that high intake of fresh fruit was an independent protective factor. Table 3 shows the overall results of the single-factor and multivariate analyses.

Stratified analysis of telephone interview data

The participants were stratified by age, gender and VG form, and the results of the subsequent analysis are shown in Table 4. For those under 50 years old, high intake of fried food was significantly more common in the GVL group (P = 0.038) under univariate analysis; however, the correlation was not significant in the final multivariate analysis, suggesting that fried food intake may be a confounding factor. In those $\geqslant$ 50 years old, univariate and multivariate analyses indicated that pickled food consumption was a new independent risk factor for GVLs. In males, excessive smoking was also found to be a new independent risk factor, while in females, allergic skin diseases seemed to be a confounding factor. For antral form, single-factor analyses showed significant differences between cases and controls for fried food consumption and intake of vegetable side dishes, but only the latter factor was confirmed as an independent factor by the adjusted multivariate analysis. For diffuse form, current or chronic *H. pylori* infection was found in more than half

Table 3 Overall single-factor and multivariate analyses of impact factors in the case-control study

Impact factor	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P*-value	OR	95%CI	*P*-value
H. pylori infection	2.329	[1.802, 3.011]	< 0.001[b]	3.051	[2.157, 4.317]	< 0.001[b]
Allergic Res. Dis.	3.745	[2.365, 5.930]	< 0.001[b]	3.636	[2.183, 6.055]	< 0.001[b]
Family history of GC	1.926	[1.059, 3.503]	0.029[a]	1.628	[0.801, 3.309]	0.178
Stress ↑	1.871	[1.344, 2.603]	< 0.001[b]	2.019	[1.568, 2.600]	< 0.001[b]
Irregular meals	1.703	[1.184, 2.449]	0.004[b]	2.300	[1.462, 3.619]	< 0.001[b]
Spicy food ↑	1.540	[1.156, 2.052]	0.003[b]	1.754	[1.227, 2.507]	0.002[b]
Fresh fruit ↑	0.721	[0.533, 0.974]	0.033[a]	0.231	[0.101, 0.529]	0.001[b]

[a]$P < 0.05$; [b]$P < 0.01$, the case group *vs* the control group. *H. pylori*: *Helicobacter pylori*.

of the affected patients, whereas only two matched controls had ever been infected. The diffuse form seemed to be more highly correlated with *H. pylori* infection, but with only thirteen pairs of participants making up the sample, no more than a general tendency could be assessed. Allergic respiratory diseases and a family history of gastric cancer were more frequent in patients with diffuse varioliform lesions *vs* matched controls.

DISCUSSION

As is widely accepted, intestinal-type gastric carcinogenesis is a multi-stage process, developing from chronic gastritis through a series of precancerous abnormalities to gastric carcinoma[26,27]. In addition, *H. pylori* infection is thought to be the key promoter[28,29]. These precursor conditions include chronic atrophic gastritis with or without IM, with a reported malignancy rate of 0.5%-1%[30,31], and intraepithelial neoplasia[32], which is classified from low to high grade according to WHO specifications. It is reported that 0%-15% of low-grade intraepithelial neoplasia could progress to high-grade, which has an extremely high malignancy rate of 25%-85%[33]. In the present study, the frequency and severity of glandular atrophy, IM and low-grade intraepithelial neoplasia were significantly elevated in the case group, indicating that the presence of GVLs is a potential risk factor for cancer. Nevertheless, no high-grade intraepithelial neoplasia was observed, and the results were inconsistent when analysis was restricted to antral varioliform lesions. Thus, this malignancy risk should be further investigated *via* a large-scale prospective study.

In view of the association between GVLs and *H. pylori* infection status, the literature is somewhat inconclusive[34]. Our analysis showed a statistically significant difference in infection rates between GVL patients (32.1%) and controls (17.2%). The adjusted analysis of the interview data indicated that *H. pylori* infection, especially chronic persistent infection, was a pathogenic factor. In contrast, no correlation existed where infections had been successfully treated. Thus, *H. pylori* eradication and regular endoscopic follow-ups should be key components of the treatment for GVLs.

European researchers have suggested that there may be an allergic component in the pathogenesis of the disease, specifically that excessive histamine release could play a central role[7,35]; however, no evidence for this has been reported for Chinese patients. Interestingly, we found that the frequency of allergic diseases was increased in patients with varioliform lesions, in particular bronchial asthma and allergic rhinitis. There were 112 GVL patients (16.6%) with at least one allergic disease, and the multivariate analysis confirmed that allergic respiratory disease was an independent risk factor. Family history of gastric carcinoma has been reported as a risk factor for gastric carcinogenesis[36,37], but it was not associated with GVLs in our study. Diffuse form appeared to have a more positive association with allergic diseases and family history of gastric cancer, yet the results were not conclusive owing to the limited sample size, and will thus need to be verified by larger studies in the future.

In the pooled multivariate analysis, the independent risk factors were high work-related stress, irregular meals, and high intake of spicy food, and the one potentially protective factor was high intake of fresh fruit. The stratified analyses indicated that pickled food consumption in people over 50 years old and excessive smoking in men were also risk factors. Intake of vegetable side dishes was found to be negatively correlated with the antral form of GVLs. Indeed, certain habits of daily life could serve as important risk factors for gastric cancer. Previous studies revealed a close association between negative psychological factors like nervousness or anxiety and susceptibility to neoplasia[38,39]. Our participants with high work-related stress could have an increased risk of gastric malignancy, which may be related to constant anxiety-induced stimulation of the sympathetic system. Smoking is also considered a pathogenic factor for multiple cancers. A 50-year observational study of 34439 British doctors indicated that cigarette smoking was a risk factor in the progression of 14 different cancers including gastric carcinoma[40]. In the present study, excessive smoking in men contributed significantly to the risk of GVLs, but not in women, indicating possible male predominance in the morbidity

Table 4 Stratified single-factor and multivariate analyses of impact factors in the case-control study

Factor	Case	Control	Univariate analysis		Multivariate analysis	
			OR [95%CI]	*P*-value	OR [95%CI]	*P*-value
Age < 50 yr						
H. pylori infection	69	37	2.224 [1.419, 3.487]	< 0.001[b]	1.968 [1.222, 3.170]	0.005[b]
Allergic Res. Dis.	34	11	3.445 [1.700, 6.978]	< 0.001[b]	3.784 [1.715, 8.347]	0.001[b]
Stress ↑	41	24	1.858 [1.083, 3.189]	0.023[a]	1.452 [1.076, 1.960]	0.015[a]
Irregular meals	40	25	1.723 [1.008, 2.946]	0.045[a]	2.207 [1.112, 4.381]	0.024[a]
Fried food ↑	56	38	1.622 [1.025, 2.567]	0.038[a]	1.459 [0.846, 2.517]	0.174
Spicy food ↑	50	33	1.654 [1.021, 2.681]	0.040[a]	1.838 [1.011, 3.342]	0.046[a]
Age ≥ 50 yr						
H. pylori infection	151	79	2.386 [1.745, 3.263]	< 0.001[b]	3.402 [2.149, 5.386]	< 0.001[b]
Allergic Res. Dis.	51	14	3.988 [2.173, 7.319]	< 0.001[b]	4.894 [2.164, 11.069]	< 0.001[b]
Stress ↑	68	39	1.879 [1.237, 2.855]	0.003[b]	2.265 [1.594, 3.219]	< 0.001[b]
Irregular meals	44	27	1.699 [1.032, 2.798]	0.035[a]	1.680 [0.918, 3.074]	0.092
Picked-food cons.	149	122	1.334 [1.001, 1.778]	0.049[a]	7.224 [2.360, 22.115]	0.001[b]
Spicy food ↑	86	62	1.481 [1.036, 2.117]	0.031[a]	1.786 [1.114, 2.863]	0.016[a]
Fresh fruit ↑	387	405	0.637 [0.409, 0.993]	0.045[a]	0.422 [0.178, 1.001]	0.050
Male						
H. pylori infection	116	67	2.054 [1.458, 2.893]	< 0.001[b]	3.445 [2.114, 5.612]	< 0.001[b]
Allergic Res. Dis.	42	10	4.599 [2.272, 9.310]	< 0.001[b]	6.563 [2.832, 15.209]	< 0.001[b]
Smoking	99	76	1.410 [1.003, 1.981]	0.047[a]	2.013 [1.282, 3.163]	0.002[b]
Stress ↑	63	34	2.023 [1.298, 3.154]	0.002[b]	2.096 [1.489, 2.950]	< 0.001[b]
Irregular meals	52	34	1.614 [1.021, 2.551]	0.039[a]	2.201 [1.262, 3.839]	0.005[b]
Spicy food ↑	79	57	1.488 [1.022, 2.165]	0.037[a]	2.167 [1.285, 3.653]	0.004[b]
Female						
H. pylori infection	104	49	2.727 [1.850, 4.021]	< 0.001[b]	3.031 [1.897, 4.844]	< 0.001[b]
Allergic Res. Dis.	43	15	3.183 [1.726, 5.867]	< 0.001[b]	3.502 [1.691, 7.255]	0.001[b]
Allergic skin Dis.	15	5	3.106 [1.114, 8.660]	0.023[a]	3.223 [0.966, 10.748]	0.057
Stress ↑	46	29	1.694 [1.032, 2.780]	0.036[a]	1.873 [1.356, 2.589]	< 0.001[b]
Irregular meals	32	18	1.872 [1.026, 3.415]	0.039[a]	2.027 [0.905, 4.538]	0.086
Spicy food ↑	57	38	1.619 [1.036, 2.530]	0.033[a]	2.185 [1.236, 3.861]	0.007[b]
Antral form						
H. pylori infection	211	114	2.248 [1.734, 2.914]	< 0.001[b]	3.124 [2.192, 4.452]	< 0.001[b]
Allergic Res. Dis.	80	25	3.552 [2.237, 5.639]	< 0.001[b]	3.432 [2.062, 5.712]	< 0.001[b]
Stress ↑	107	63	1.833 [1.315, 2.554]	< 0.001[b]	1.984 [1.544, 2.550]	< 0.001[b]
Irregular meals	83	52	1.681 [1.168, 2.422]	0.005[b]	2.191 [1.407, 3.412]	0.001[b]
Fried food cons.	202	169	1.281 [1.007, 1.629]	0.044[a]	1.338 [0.955, 1.876]	0.091
Spicy food ↑	133	95	1.500 [1.124, 2.002]	0.006[b]	1.705 [1.188, 2.447]	0.004[b]
Vegetable Cons.	227	262	0.797 [0.637, 0.996]	0.046[a]	0.491 [0.311, 0.776]	0.002[b]

[a]$P < 0.05$; [b]$P < 0.01$, the case group *vs* the control group. *H. pylori*: *Helicobacter pylori*.

of the disease. Concerning food consumption, pickled foods have been associated with the development of esophageal and gastric cancers, which can damage gastric mucosa and exacerbate the inflammation caused by *H. pylori*[41]. In recent times, Chinese dietary habits have changed dramatically. Pickled food may now be less popular in younger sections of the population, whereas spicy foods have greatly increased in popularity. Although capsaicin in spicy food has been shown to help counter the growth of *H. pylori*[42], we found that high intake of spicy food was a risk factor for varioliform lesions. The reason could be related to oncogene exposure or a chemical process during production. Meanwhile, the present study also provided factors that potentially offer some protection against GVLs. Intake of fresh vegetables and fruit has been reported to beneficial for avoidance of gastric neoplasia[43], which is consistent with the corresponding reduction in the frequency of GVLs seen in our study.

Several limitations of the present study must be taken into account. First, it is a retrospective analysis, for which recall bias and selection bias cannot be completely removed; a prospective study would be required to establish a convincing causal relationship between the factors and the disease. Second, the conclusions of the stratified analyses may be of limited value because of the small sample size, especially in the diffuse form group. Thus, some of the results in our study should be interpreted cautiously. Third, other relevant variables such as body mass index (BMI), hyperlipidemia, ABO blood group, consumption of coffee, carbonated drinks and bean products were not included; in addition, several factors such as the type of cigarette or alcohol consumed, medication dose and professional mental scale were not precisely classified. If the above factors were included in the multivariate regression equation, our results could have been very different. Future studies should therefore use a more complete set of variables.

To the best of our knowledge, this is the first case-control study investigating the factors influencing the formation of GVLs. The results suggest a potentially increased cancer risk for the affected patients, and that *H. pylori* infection, allergic respiratory diseases, high work-related stress, irregular meals, high intake of spicy food, pickled food consumption in older people, and excessive smoking in men were all positively correlated with the occurrence of GVLs. In contrast, consumption of vegetables and high intake of fresh fruit were found to be negatively correlated and therefore potentially protective. In summary, our results suggest that formation of GVLs can be reduced by maintaining a healthy lifestyle and positive attitude, while ensuring that allergic diseases and *H. pylori* infection are treated effectively. We suggest that a prospective study should be carried out in the future to examine the morphological and pathological evolution of GVLs, and thereby clarify their relationship with gastric malignancy. A large-scale, well-designed clinical trial is also warranted to provide more precise and robust conclusions on this matter.

COMMENTS

Background

Researchers discovered the presence of gastric varioliform lesions (GVLs) over 60 years ago, but until now, very little was known about the etiopathogenesis and progression. So the authors try to provide a better understanding of this potentially premalignant disease in the present case-control study.

Research frontiers

Previous reports suggested a possible association between GVLs and gastric cancer. And the disease was classified as a precursor to gastric cancer at the World Congress of Gastroenterology in 1994. More recently, Zhang *et al* performed a proteomic analysis to provide more molecular biological details of GVLs. The important differential proteins could serve as potential biomarkers for the early diagnosis of gastric cancer.

Innovations and breakthroughs

This is the first case-control study investigating the factors influencing the formation of GVLs, and the manuscript provide a better understanding of this potentially premalignant condition, allowing physicians to better identify at-risk patients and to devise more effective treatment strategies.

Applications

A large-scale, well-designed prospective study should be carried out in the future to examine the morphological and pathological evolution of GVLs, and thereby clarify their relationship with gastric malignancy.

Terminology

The term GVLs in the present study is a synonym of varioliform gastritis. The major endoscopic feature of such lesions is widespread small lesions, possessing a central umbilical-like depression covered in gray-colored secretion or tiny bleeds. Patients in China are affected more often by the antral type of the disease, thus endoscopists present the diagnosis as GVLs.

Peer-review

Patients diagnosed in an early stage of gastric cancer present an excellent prognosis, with a five-year survival rate greater than 90%. This well conducted and written retrospective case-control study considers the different risk and protective factors influencing the occurrence of GVLs and their possible link with development of gastric cancer.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Long-term outcomes and prognostic factors of patients with obstructive colorectal cancer: A multicenter retrospective cohort study

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Abstract

AIM: To investigate the long-term oncologic outcomes and prognostic factors in patients with obstructive colorectal cancer (CRC) at multiple Japanese institutions.

METHODS: We identified 362 patients diagnosed with obstructive colorectal cancer from January 1, 2002 to December 31, 2012 in Yokohama Clinical Oncology Group's department of gastroenterological surgery. Among them, 234 patients with stage Ⅱ/Ⅲ disease who had undergone surgical resection of their primary lesions were analyzed, retrospectively. We report the long-term outcomes, the risk factors for recurrence, and the prognostic factors.

RESULTS: The five-year disease free survival and cancer-specific survival were 50.6% and 80.3%, respectively. A multivariate analysis showed the ASA-PS (HR = 2.23, P = 0.026), serum Albumin ⩽ 4.0 g/dL (HR = 2.96, P = 0.007), T4 tumor (HR = 2.73, P = 0.002) and R1 resection (HR = 6.56, P = 0.02) to be independent risk factors for recurrence. Furthermore, poorly differentiated cancers (HR = 6.28, P = 0.009), a T4 tumor (HR = 3.46, P = 0.011) and R1 resection (HR = 6.16, P = 0.006) were independent prognostic factors in patients with obstructive CRC.

CONCLUSION: The outcomes of patients with obstructive CRC was poor. T4 tumor and R1 resection were found to be independent prognostic factors for both recurrence and survival in patients with obstructive CRC.

Key words: Obstructive colorectal cancer; Prognostic factor; Survival

Core tip: Obstructive colorectal cancer (CRC) still have poor prognosis. However, the prognostic factor of obstructive CRC is unclear. The aim of this article is to clarify the long-term outcome and the risk factors for obstructive CRC at multiple institutions. The five-year disease free survival and cancer-specific survival were 50.6% and 80.3%, respectively. T4 tumor and R1 resection were independent prognostic factors for both recurrence and survival.

Atsushi I, Mitsuyoshi O, Kazuya Y, Syuhei K, Noriyuki K, Masashi M, Akira W, Kentaro S, Nobuyuki K, Natsuko S, Jun W, Yasushi I, Chikara K, Itaru E. Long-term outcomes and prognostic factors of patients with obstructive colorectal cancer: A multicenter retrospective cohort study. *World J Gastroenterol* 2016; 22(22): 5237-5245 Available from: URL: http://www.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in Japan, and the incidence of CRC has been increasing rapidly. CRC is difficult to diagnose due to it early atypical symptoms and signs. Around 7%-16% of patients with colorectal malignancy present with acute colorectal obstruction[1-3]. It is generally accepted that right sided obstructive CRC can best be treated by right hemicolectomy with ileocolic anastomosis. On the other hand, the optimal treatment for left-sided obstructive CRC remains controversial[4-7]. The treatment options range from an emergency radical operation, such as Hartmann's procedure, to bowel decompression using metallic stents or transanal tube or proximal diversion with a subsequent staged resection. The choices of surgical intervention for obstructive CRC vary greatly, according to the tumor location, general condition of the patients, and the experience level of the surgeons[8]. Therefore, It has been reported that CRC patients with obstruction have an advanced stage and worse long-term survival compared to non-obstructive CRC[3,9-11]. Although the negative impact of obstruction on the postoperative outcomes has been well documented, few studies have examined the outcomes of obstructive CRC patients in Japan[11-13]. Furthermore, the risk factors for recurrence and the prognostic factors are unclear owing to the small number of patients in previous study.

The aim of this study is to investigate the long-term oncologic outcomes and prognostic factors in patients with obstructive CRC at multiple Japanese institutions.

MATERIALS AND METHODS

Three hundred and sixty-two patients who were diagnosed to have obstructive colorectal cancer from January 2002 to December 2012 at the Yokohama Clinical Oncology Group's Department of Gastroenterological Surgery (10 institutions) were enrolled. Obstructive CRC was diagnosed based on medical history, physical examination, abdominal computed tomography (CT), and colonoscopy, and the surgical findings. We first performed emergency decompression of bowel obstruction by ileostomy/colostomy or the insertion of a decompression tube, or emergency resection of the primary lesion. The type of decompression method was chosen according to the surgeon's judgment and preference. Patients with distant metastatic lesions (n = 103), who only underwent stoma creation and best supportive care (n = 23), stage Ⅰ (n = 2) were excluded from this study. As a result, 234 patients who underwent surgical resection were analyzed retrospectively (Figure 1). The prognostic factors

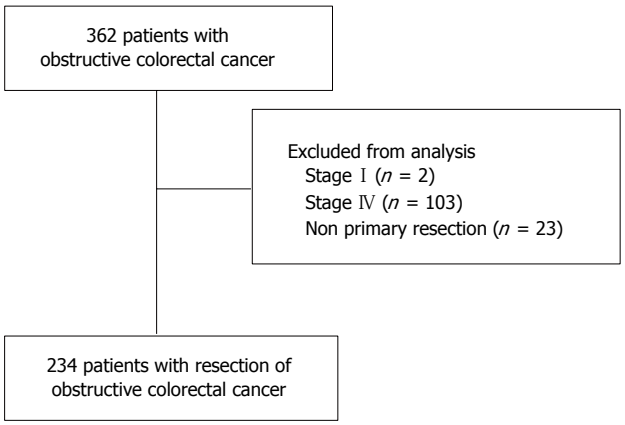

Figure 1 Study flowchart.

influencing survival and risk factors for recurrence were analyzed.

Clinicopathological information was obtained from the medical records of the patients including gender, age, The American Society of Anesthesiologists (ASA)-physical status (PS), serum albumin, CEA, preoperative decompression, location of the tumor, tumor size, differentiation of the tumor, depth of the tumor, intramural lymphatic invasion, intramural vascular invasion, lymph node dissection, number of lymph nodes harvested, lymph node involvement, postoperative complication, anastomotic leakage, curability, and adjuvant chemotherapy. There were missing values for BMI in 13 patients, for serum albumin in 14 patients, for CEA in 19 patients, for tumor size in 4 patients, for lymphatic invasion in one patient and for harvested lymph nodes in 10 patients because this was a retrospective study.

Japanese D3 lymphadenectomy is equivalent to complete mesocolic excision (CME) with central vascular ligation (CVL)[14]. D2 lymphadectomy includes pericolic and intermediate nodes region, and D0-1 includes only pericolic nodes region.

Statistical analysis

The disease-free survival (DFS) and cancer-specific survival (CSS) were estimated using the Kaplan-Meier method, and statistical significance was determined by the log-rank test. A multivariate analysis was performed using the Cox proportional hazard model to examine the independent prognostic factors and risk factors of recurrence. A *P* value of < 0.05 indicated statistical significance. All analyses were performed using the IBM SPSS, version 21 (SPSS Inc., Chicago, IL, United States).

This study received approval from the institutional review board of Yokohama City University.

RESULTS

Characteristic of the patients

The clinicopathological characteristics of the patients are summarized in Table 1. There were 234 patients who underwent surgical resection for obstructive CRC. The median age of the patients was 71 years (range 35-96) and there were 141 (60.3%) men and 93 (39.7%) women. Of these patients, 183 patients (72.2%) received preoperative decompression by colostomy/ileostomy (*n* = 56) or transanal tube insertion (*n* = 127)[15]. The most common tumor site was the sigmoid colon (*n* = 95). Other primary tumors were located in the descending colon (*n* = 36), ascending colon (*n* = 33), transverse colon (*n* = 33), rectum (*n* = 27), and cecum (*n* = 10). Among the 234 patients in this study, 165 patients (70.5%) had obstructing cancers at a site distal to the splenic flexure. T4 tumors were found in 125 patients (53.4%). There were 113 stage Ⅱ patients and 121 stage Ⅲ patients. In the stage Ⅲ cases, 90 patients had N1 disease and 31 patients had N2.

Table 1 Clinicopathological characteristic of patients with obstructive colorectal cancer *n* (%)

Variable	Category	*n* = 234
Gender	Male	141(60.3)
	Female	93 (39.7)
Age (yr)[1]		71 (35-96)
ASA	Ⅰ	70 (30)
	Ⅱ	128 (54.7)
	Ⅲ	36 (15.3)
Location of tumor	Cecum	10 (4.3)
	Ascending colon	33 (14.1)
	Transverse colon	33 (14.1)
	Descending colon	36 (15.4)
	Sigmoid colon	95 (40.6)
	Rectum	15 (10.5)
Decompression	+	183 (78.2)
	-	51 (21.8)
CEA (mg/dL)[1]		4.9 (0.3-2470)
Serum albumin[1]		3.4 (1.4-4.9)
Tumor size(mm)		48 (10-140)
Depth of tumor	pT3	109 (46.6)
	pT4a	93 (39.7)
	pT4b	32 (13.7)
Lymph node involvement	N0	113 (48.3)
	N1	90 (38.5)
	N2	31 (13.2)
R0 resection	+	219 (93.6)
	-	15 (6.4)
Adjuvant chemotherapy	+	91 (38.9)
	-	143 (61.1)

[1]Median (range).

A total of 219 patients (93.6%) underwent R0 resection of the primary lesion. The reasons of R1 resection (*n* = 15) were positive surgical margins in 11 patients, other organ involvement in 3 patients and residual lymph node metastasis in 1 patient.

Ninety-one of the 234 patients underwent adjuvant chemotherapy. The chemotherapeutic regimen was oral 5-fluorouracil (5-FU) in 47 patients, oral 5-FU plus leucovorin in 36, oxaliplatin-based chemotherapy in 5, and a Roswell Park Memorial Institute (RPMI) regimen in 3[16].

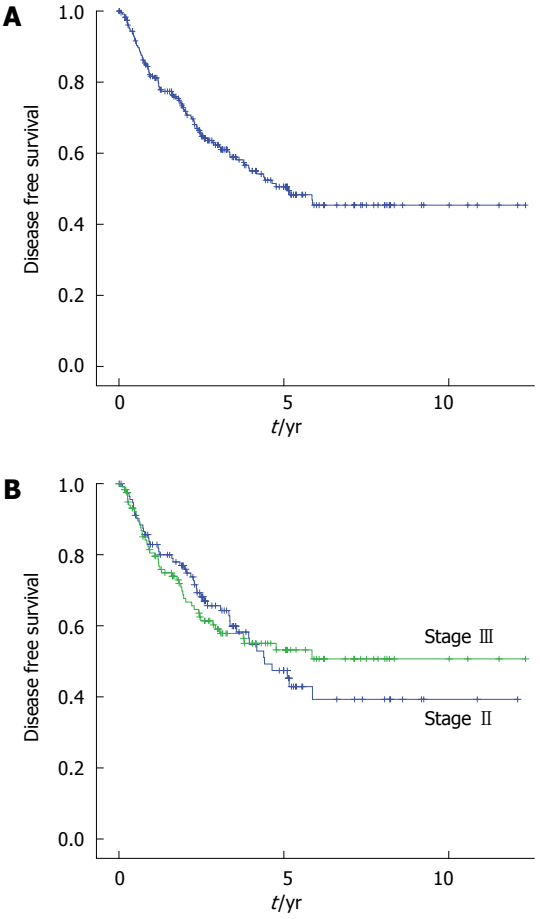

Figure 2 Kaplan-Meier curves showing the disease free survival after primary tumor resection in patients with obstructive colorectal cancer. A: All stage (n = 234); B: Stage Ⅱ (n = 114, blue line), Stage Ⅲ (n = 120, green line).

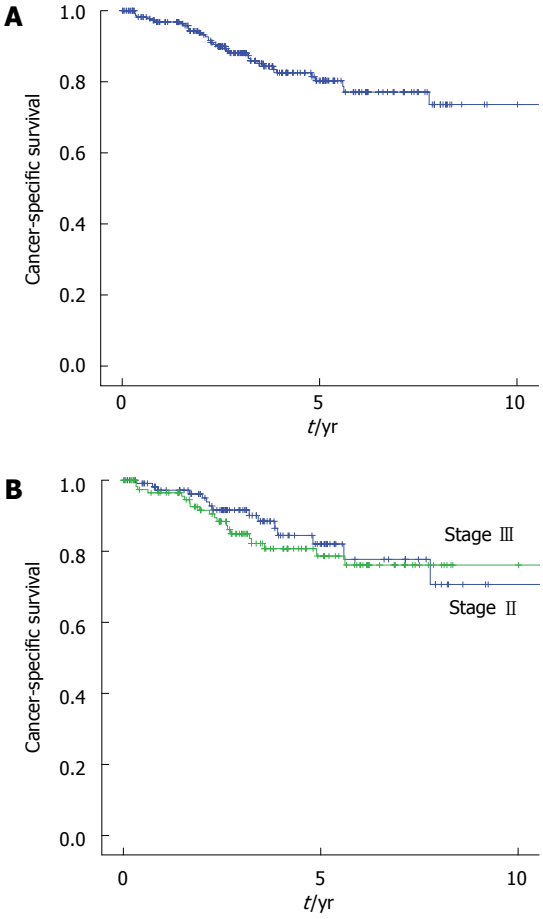

Figure 3 Kaplan-Meier curves showing the cancer-specific survival rates after primary tumor resection in patients with obstructive colorectal cancer. A: All stage (n = 234); B: Stage Ⅱ (n = 114, blue line), Stage Ⅲ (n = 120, green line).

Long-term outcomes

The median follow-up interval was 39 mo. The five-year DFS and CSS for all patients were 50.6% and 80.3%, respectively. The 5-year DFS of the patients with stage Ⅱ and stage Ⅲ disease were 47.4% and 55.1%, respectively. The 5-year CSS were 78.7% and 82.0%, respectively. There were no significantly differences in both the DFS and CSS between stage Ⅱ and stage Ⅲ disease (P = 0.856, P = 0.560) (Figures 2 and 3).

Recurrence site

A total 71 patients (30.3%) experienced recurrence during the study follow-up (Table 2). The most common site of recurrence was the liver (n = 27, 11.5%), followed by the lung (n = 22, 9.4%), peritoneum (n = 21, 9.0%), and local recurrence (n = 9, 3.8%). Other sites of recurrence included the non-regional lymph nodes (n = 6), anastomosis (n = 3), abdominal wall (n = 2), and pleural dissemination (n = 2). The rate of the recurrence sites did not differ substantially between stage Ⅱ and stage Ⅲ disease (data not shown).

Table 2 Patterns of recurrence following colorectal resection of obstructive colorectal cancer (n = 71)

Site of recurrence	n (%)
Liver	27 (11.5)
Lung	22 (9.4)
Peritoneal dissemination	21 (9.0)
Local recurrence	9 (3.8)
Lymph node	6 (2.6)
Anastomosis	3 (1.3)
Abdominal wall	2 (0.9)
Pleural dissemination	2 (0.9)

Risk factors for recurrence

The risk factors for recurrence according to our analysis are shown in Table 3. According to a univariate analysis, the factors associated with recurrence were age ⩾ 75 years (P = 0.011), ASA-PS (P = 0.017), serum albumin ⩽ 4.0 g/dL (P = 0.001), T4 tumor (P = 0.001), and R1 resection (P < 0.001). A multivariate analysis of these factors confirmed significant differences for ASA-PS (HR = 2.234, P = 0.026) serum albumin (HR = 2.967, P = 0.007), depth of tumor

Table 3 Result of the univariate and multivariate analysis of risk factors for recurrence

Factor		*n*	Univariate analysis			Multivariate analysis	
			3-yr RFS	5-yr RFS	*P* value	HR (95%CI)	*P* value
Gender	M	141	59.2%	47.6%	0.409		
	F	93	66.8%	54.7%			
Age (yr)	⩾ 75	96	55.0%	42.4%	0.011	1.228 (0.659-2.290)	0.518
	< 75	138	67.3%	55.9%			
ASA-PS	1	71	75.7%	64.8%	0.017	2.234 (1.101-4.535)	0.026
	2-3	163	56.6%	44.6%			
BMI (kg/m^2)	⩾ 25	28	66.0%	49.7%	0.951		
	< 25	193	62.7%	52.7%			
Serum albumin (g/dL)	⩽ 4.0	172	54.9%	41.3%	0.001	2.967 (1.342-6.560)	0.007
	> 4.0	48	79.7%	73.1%			
CEA (mg/dL)	⩾ 5.0	102	52.4%	44.8%	0.052		
	< 5.0	113	74.1%	54.2%			
Decompression	+	183	62.9%	50.7%	0.572		
	-	51	60.5%	50.4%			
Location of tumor	Right side	69	62.5%	55.5%	0.738		
	Left side	165	62.1%	48.7%			
Tumor size (mm)	⩾ 50	99	61.0%	48.8%	0.384		
	< 50	121	63.9%	54.1%			
Differentiation of tumor	tub1,tub2	222	63.7%	51.2%	0.080		
	por, muc	12	36.7%	36.7%			
Depth of tumor	T3	108	73.5%	64.7%	0.001	2.728 (1.467-5.072)	0.002
	T4	126	53.0%	38.7%			
Intramural lymphatic invasion	+	157	61.4%	53.2%	0.600		
	-	73	64.4%	45.2%			
Intramural vascular invasion	+	164	60.3%	49.3%	0.683		
	-	70	68.1%	57.4%			
Lymph node involvement	+	120	65.7%	47.4%	0.856		
	-	114	59.1%	53.2%			
Lymph node dissection	D0,D1	31	44.3%	44.3%	0.067		
	D2,D3	199	64.7%	50.8%			
No. of lymph nodes harvested	< 12	73	54.8%	44.5%	0.085		
	⩾ 12	151	66.4%	52.5%			
Postoperative complication	+	78	57.3%	44.7%	0.071		
(≥ Grade 2)	-	156	64.7%	53.5%			
Anastomotic leakage	+	18	71.1%	56.9%	0.562		
	-	191	61.7%	50.0%			
Curability	R0	219	66.4%	54.8%	< 0.001	6.555 (1.344-31.970)	0.020
	R1	15	7.6%	0.0%			
Adjuvant chemotherapy	+	91	66.2%	55.6%	0.069		
	-	143	59.8%	47.2%			

(HR = 2.728, *P* = 0.002) and curability (HR = 6.555, *P* = 0.02). There were no differences in the relapse rate according to whether the patients underwent preoperative decompression or not. Furthermore, lymph node involvement was also not associated with recurrence.

Prognostic factors for CSS

The prognostic factors for CSS are shown in Table 4. A univariate analysis identified age ⩾ 75 (*P* = 0.027), poorly or mucinous differentiation(*P* = 0.001), T4 tumor (*P* = 0.001), D0 or D1 lymph node dissection(*P* = 0.0014), R1 resection (*P* < 0.001) as poor prognostic factors. According to a multivariate analysis, poorly differentiated cancers or mucinous differentiation (HR = 6.282, *P* = 0.009), T4 tumor (HR = 3.458, *P* = 0.011) and R1 resection (HR = 6.162, *P* = 0.006) were independent prognostic factors in patients with obstructive CRC (Figure 4).

DISCUSSION

In the present study, we evaluated the long-term oncologic outcomes and prognostic factors in patients with obstructive CRC in multiple Japanese institutions. Most previous studies have reported that patients with obstructive CRC have significantly poorer oncologic outcomes than patients with nonobstructive CRC[9,11,12,17]. Obstructive tumors have been reported to have a more advanced stage than nonobstructive tumors[11,18]. The reported 5-year survival ranges between 36% to 64.6% in patients with obstructive CRC[11,17,19,20]. Our retrospective data showed 5-year CSS to be 80.3%, which was higher than the previously reported findings. One reason for the good outcomes might be that Japanese standard surgical procedures include complete tumor resection and extended D2/D3 lymph node dissection, including the pericolic, intermediate and most central lymph

Table 4 Result of the univariate and multivariate analysis of prognostic factors for cancer-specific survival

Factor		*n*	Univariate analysis			Multivariate analysis	
			3-yr CSS	5-yr CSS	*P* value	HR(95%CI)	*P* value
Gender	M	141	88.3%	80.8%	0.924		
	F	93	87.8%	79.7%			
Age (yr)	⩾ 75	96	82.2%	71.6%	0.027	1.464 (0.647-3.310)	0.360
	< 75	138	91.9%	85.4%			
ASA-PS	1	71	86.0%	83.5%	0.710		
	2-3	163	89.0%	78.7%			
BMI (kg/m^2)	⩾ 25	28	92.0%	85.8%	0.389		
	< 25	193	87.3%	80.0%			
Serum albumin (g/dL)	⩽ 4.0	172	85.6%	82.6%	0.536		
	> 4.0	48	87.7%	76.2%			
CEA (mg/dL)	⩾ 5.0	102	86.7%	80.0%	0.715		
	< 5.0	113	90.5%	82.0%			
Decompression	+	183	88.4%	81.7%	0.514		
	-	51	87.1%	74.8%			
Location of tumor	Right side	69	82.6%	70.5%	0.103		
	Left side	165	90.4%	84.3%			
Tumor size (mm)	⩾ 50	99	84.1%	76.9%	0.291		
	< 50	121	90.4%	85%			
Differentiation of tumor	tub1, tub2	222	89.9%	82.5%	0.001	6.282 (1.584-24.909)	0.009
	por, muc	12	50.0%	50.0%			
Depth of tumor	T3	108	95.7%	92.3%	0.001	3.458 (1.324-9.031)	0.011
	T4	126	81.1%	69.2%			
Intramural lymphatic invasion	+	157	90.8%	82.7%	0.449		
	-	73	86.9%	79.4%			
Intramural vascular invasion	+	164	94.2%	90.1%	0.152		
	-	70	85.9%	76.7%			
Lymph node involvement	+	120	92.5%	82.9%	0.332		
	-	114	84.0%	77.7%			
Lymph node dissection	D0, D1	31	60.7%	60.7%	0.014	0.958 (0.300-3.056)	0.942
	D2, D3	199	90.4%	83.0%			
No. of lymph nodes harvested	< 12	73	83.3%	78.3%	0.314		
	⩾ 12	151	91.1%	81.0%			
Postoperative complication	+	78	85.9%	75.6%	0.644		
(⩾ Grade 2)	-	156	89%	82.5%			
Anastomotic leakage	+	18	100%	100%	0.069		
	-	191	87.1%	78.7%			
Curability	R0	219	91.3%	84.7%	< 0.001	6.162 (1.692-22.445)	0.006
	R1	15	39.7%	19.9%			
Adjuvant chemotherapy	+	91	87.7%	81.8%	0.800		
	-	143	88.8%	79.4%			

nodes. West *et al* reported that the Japanese surgical procedures as well as CME with CVL eradicates tumors more effectively than the conventional procedures[14]. In our study, D2/D3 lymph node dissection was performed in about 70% of all patients.

Several authors have suggested preoperative obstruction to be a prognostic factor in CRC[12,17]. However, there are few data concerning the prognostic factors associated with obstructive CRC patients[4,19]. Jiang *et al*[4] reported a delayed resection to provide a better oncologic outcome than a primary resection for obstructive left-sided colorectal cancer. Other authors have showed that decompression followed surgery is better than emergency surgery in terms of the primary anastomosis rate, the stoma rate, the morbidity rate, the successful treatment of the patient's comorbidities, and preparation for elective surgery[21,22]. According to our results, however, no difference in the prognosis was found in regard to whether patients underwent preoperative decompression or not, It therefore remains inconclusive as to which approach may be superior to the other.

Malignant obstruction can occur in any part of the colon and rectum, however, the risk varies at different locations. In present study, 70.5% of the obstructive CRC occurred in the left-sided colon and most of them occurred in the sigmoid colon. This tumor distribution is similar to what has been reported by other series[11,19]. Our results showed that the prognosis was not different between right-sided and left-sided obstructive CRC.

Obstructive tumors are reported to be associated with a more advanced stage than nonobstruction[11,18]. Our data showed 53.4% to have T4 tumors, and 51.7% had positive lymph nodes. This is one of the reasons why obstructive CRC has a worse prognosis. In present study, especially, a T4 tumor was found to be a risk factor for recurrence in patients with

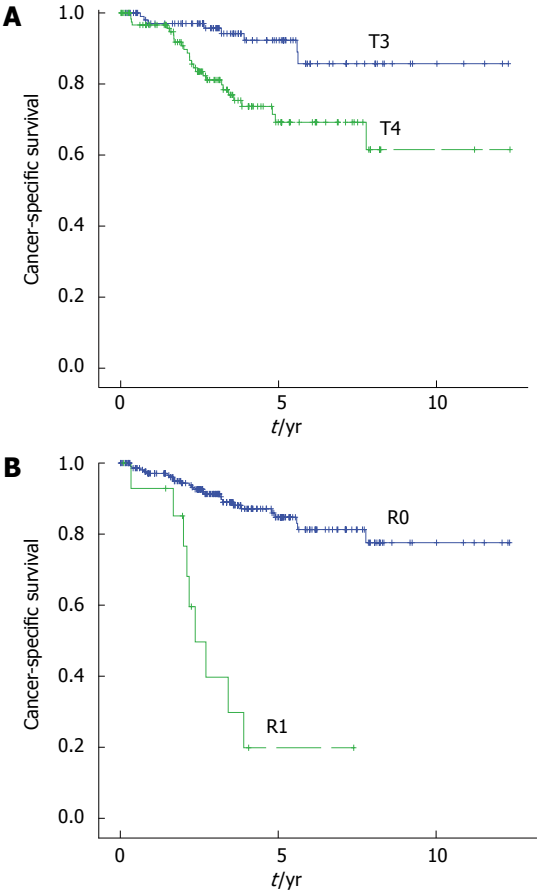

Figure 4 Kaplan-Meier curves showing the cancer-specific survival rates in patients with T4 tumor and R1 resection. A: T3 (*n* = 108, blue line); T4 (*n* = 126, green line); B: R0 (*n* = 219, blue line), R1 (*n* = 15, green line).

obstructive CRC.

Seventy-one patients had a recurrence of the disease as the first event in our study. The distant metastasis rate is significantly higher in obstructed patients when compared with nonobstructed patients[17]. The common sites of recurrence were the liver (11.5%) and lung (9.4%), which were similar to previous reports[23]. An interesting finding in the present study is that patients with obstructive CRC showed a higher rate of peritoneal dissemination (9.0%) than previously reported (1.9%-3.5%) in nonobstructive CRC[24,25]. These findings suggested that obstructive CRC was locally advanced cancer consisting of T4 tumors and which may be unexpectedly exposed during R1 resection. Therefore, reducing the rate of performing R1 resection might be a key to achieving improved surgical results.

In our study, patients with stage Ⅱ disease and those with stage Ⅲ disease had similar poor outcomes in terms of the 5-year DFS and CSS. This finding suggests that lymph node involvement, which is a well-known prognostic factor, does not have any significant impact on the outcomes in patients with obstructive CRC. One of the reasons for this finding is due to the fact that Japanese standard lymph node dissection procedures for advanced colorectal cancer include D2/D3 lymph node dissection, which is nearly the same method as that performed for CME and CVL[14]. In our clinical oncology group, lymphadenectomy for colorectal cancer was routinely performed during the study period. Therefore, a T4 tumor was identified as the most important prognostic factor, in which the 5-year DFS and OS were 38.7% and 60%, respectively.

In the present study, a lower level of albumin was also a predictive factor for survival. The serum albumin levels have recently been studied as the Glasgow Prognostic Score (GPS), based on a combination of albumin and C-reactive protein (CRP). Several authors have revealed the GPS to have prognostic value in patients with advanced colorectal cancer[26,27]. However, we failed to collect data of CRP because this was a retrospective analysis. It is estimated that low albumin levels are associated with a decreased survival time because a low albumin level likely reflects some type of systemic compromise[28].

Our results demonstrated that poorly differentiated tumors or mucinous differentiated tumors are also predictive factors for survival. Histologically, poorly differentiated CRC represents from 4.8% to 23.2% of all colorectal cancers[29]. The rate of poorly differentiated tumors was not higher than that described in previous reports. Poorly differentiated cancers have been linked to adverse prognoses in many studies[30].

Recently, several authors have suggested the feasibility of performing preoperative chemotherapy without the routine use of radiation therapy for locally advanced rectal cancer and a high R0 resection rate[31,32]. Furthermore, the FOxTROT Collaborative Group demonstrated the feasibility of performing preoperative chemotherapy for locally advanced colon cancer[33]. Our result suggested that obstructive colorectal cancer is also locally advanced cancer. Therefore, preoperative chemotherapy after the decompression of bowel obstruction may also be useful for the management of obstructive colorectal cancer.

Our retrospective study had several important limitations. First, there were several missing data and we could not obtain the clinical course related to the treatment of patients after recurrence. Second, the adjuvant therapy, which affects the outcome, was not uniform.

In conclusion, in addition to generally accepted knowledge, we found that T4 tumor and R1 resection were prognostic factors for both recurrence and survival. These results suggested that a curative resection of the tumor is very important and that systemic treatment for preventing distant metastasis, such as peritoneal dissemination associated with T4 tumors, is necessary in patients with obstructive colorectal cancer.

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COMMENTS

Background

Colorectal cancer (CRC) is one of most common cancer in the world. Around 7%-16% of patients with colorectal cancer present with acute colorectal obstruction. It has been reported that CRC patients with obstruction have an advanced stage and poor long-term survival compared to non-obstructive CRC. However, the risk factors for recurrence and the prognostic factors of patients with obstructive CRC are unclear.

Research frontiers

The authors often treat the obstructive CRC. However, there are few literatures concerning survival and prognostic factor of obstructive CRC. The research hotspot is to introduce long-term outcome of patients with obstructive colorectal cancer and prognostic factors in Japan.

Innovations and breakthroughs

The present study represents the characteristics and the long-term outcome of obstructive CRC patients in Japan and revealed that T4 tumor and R1 resection are risk factors of recurrence and prognostic factors. These results suggested that a curative resection of the tumor is very important and systemic treatment for preventing distant metastasis, such as peritoneal dissemination associated with T4 tumors, is necessary in patients with obstructive colorectal cancer.

Applications

This study showed the poor survival for obstructive colorectal cancer patients and prognostic factor. The present study provided readers the important information of treatment for patients with obstructive CRC.

Peer-review

The authors demonstrated that T4 tumor status and R1 resection are independent prognostic factors in patients with obstructive colorectal cancer.

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ORIGINAL ARTICLE

Retrospective Study

Post-discharge complications after esophagectomy account for high readmission rates

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Author contributions: Chen SY, Molena D, Stem M, Mungo B and Lidor AO designed the study, wrote the manuscript and made the decision to submit; Chen SY, Stem M and Lidor AO contributed to the data collection, analysis, interpretation.

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Conflict-of-interest statement: Daniela Molena is a speaker for Novadaq Corporation; the remaining authors have no conflicts of interest.

Data sharing statement: No additional data are available. American College of Surgeons National Surgical Quality Improvement Program and the hospitals participating in the ACS-NSQIP are the source of the data used herein; they have not verified and are not responsible for the statistical validity of the data analysis or the conclusions derived by the authors.

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Abstract

AIM: To identify rates of post-discharge complications (PDC), associated risk factors, and their influence on early hospital outcomes after esophagectomy.

METHODS: We used the 2005-2013 American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP) database to identify patients ⩾ 18 years of age who underwent an esophagectomy. These procedures were categorized into four operative approaches: transhiatal, Ivor-Lewis, 3-holes, and non-gastric conduit. We selected patient data based on clinical relevance to patients undergoing esophagectomy and compared demographic and clinical characteristics. The primary outcome was PDC, and secondary outcomes were hospital readmission and reoperation. The patients were then divided in 3 groups: no complication (Group 1), only pre-discharge complication (Group 2), and PDC patients (Group 3). A modified Poisson regression analysis was used to identify risk factors associated with developing post-discharge complication, and risk ratios were estimated.

RESULTS: 4483 total patients were identified, with

8.9% developing PDC within 30-d after esophagectomy. Patients who experienced complications post-discharge had a median initial hospital length of stay (LOS) of 9 d; however, PDC occurred on average 14 d following surgery. Patients with PDC had greater rates of wound infection (41.0% *vs* 19.3%, $P < 0.001$), venous thromboembolism (16.3% *vs* 8.9%, $P < 0.001$), and organ space surgical site infection (17.1% *vs* 11.0%, $P = 0.001$) than patients with pre-discharge complication. The readmission rate in our entire population was 12.8%. PDC patients were overwhelmingly more likely to have a reoperation (39.5% *vs* 22.4%, $P < 0.001$) and readmission (66.9% *vs* 6.6%, $P < 0.001$). BMI 25-29.9 and BMI $\geqslant$ 30 were associated with increased risk of PDC compared to normal BMI (18.5-25).

CONCLUSION: PDC after esophagectomy account for significant number of reoperations and readmissions. Efforts should be directed towards optimizing patient's health pre-discharge, with possible prevention programs at discharge.

Key words: Reoperation; Hospital readmission; Post-discharge complications; Esophagectomy; Outcomes research

Core tip: In this study, we used the 2005-2013 ACS-NSQIP database to identify the rate of post-discharge complications, their associated risk factors, and their influence on early hospital readmission after esophagectomy. This report demonstrates that post-discharge complications after esophagectomy account for a significant number of reoperations and readmissions. We believe that implementing prevention strategies to decrease common post-discharge complications like venous thromboembolism and infection should be considered, and that directing our energies toward optimizing patient health prior to discharge may improve overall surgical outcomes.

Chen SY, Molena D, Stem M, Mungo B, Lidor AO. Post-discharge complications after esophagectomy account for high readmission rates. *World J Gastroenterol* 2016; 22(22): 5246-5253 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5246.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5246

INTRODUCTION

Esophagectomy is the mainstay treatment for localized esophageal carcinoma without medical contraindications[1]. It can also be indicated for certain benign conditions, including high-grade dysplasia in Barrett's esophagus, caustic ingestion, reflux esophagitis complications, esophageal stricture, and esophageal neuromotor dysfunction (achalasia, spasm, scleroderma)[2]. Over 5000 esophagectomies are performed in the United States and United Kingdom annually[3]. Despite improved surgical techniques and intensive care unit therapy[4], post-esophagectomy mortality and morbidity rates remain suboptimal, ranging from 7%-28%[5,6] and 10%-27%[7], respectively. Moreover, readmission rates after esophagectomy range from 5%-25%[8,9], and the overall 5-year survival rate following esophagectomy range from 15%-40%[10,11].

These dismal outcomes after esophagectomy, compounded with national health policy changes by the Centers for Medicare and Medicaid Services (CMS) and the Affordable Care Act that emphasize reducing hospital readmission rates to improve health care quality, have spurred growing interest in investigating quality measures related to esophagectomy. Although several studies have explored esophagectomy complications and readmission[12-16] none to date have delved into risk factors associated specifically with post-discharge complications (PDC) after esophagectomy. Given that approximately a third of post-operative surgical complications occur post-discharge, and that PDC may differ from pre-discharge complications[17], we hypothesize that several clinical factors may increase risk for developing PDC after esophagectomy.

Using 2005-2013 data from the American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP), we sought to identify the rate of PDC, their associated risk factors, and their influence on early hospital outcomes including readmission and reoperation. We believe that understanding these risk factors will provide additional insights to improve esophagectomy quality outcomes.

MATERIALS AND METHODS

Data source

This study is a retrospective analysis using the 2005-2013 ACS-NSQIP database. ACS-NSQIP is a nationally-validated, risk-adjusted, and outcomes-based program created for the purpose of measuring and improving surgical quality care[18,19]. The program collects data on patients undergoing surgery from over 650 participating hospitals of varying size and academic affiliation[20]. Eligibility criteria for hospital participation include: hiring a surgical clinical reviewer who uses a standardized format to capture and review data from clinical records, identifying a Surgeon Champion to lead the program at the hospital, agreeing to program protocols, meeting minimum case standards, and paying an annual participation fee to ACS. Prospective, systematic data collection is performed on 150 preoperative and intraoperative variables, in addition to 30-d postoperative morbidity and mortality. This study was reviewed and approved by the Institutional Review Board of the Johns Hopkins University School of Medicine.

Study population

Patients ⩾ 18 years of age who underwent an esophagectomy (defined as current procedural terminology codes 43107, 43108, 43112, 43113, 43117, 43118, 43121, 43122, or 43123) were included. These procedures were then categorized into four operative approaches: transhiatal (if the chest was not entered and the stomach was used as a conduit), Ivor-Lewis (if the anastomosis was done in the chest and the stomach was used as a conduit), 3-holes (if the anastomosis was done in the neck, the chest was entered and the stomach was used as a conduit), and intestinal conduit (if any of the above approaches were used but intestine, either large or small, was used as a conduit). Patients who had missing data for days from operation to discharge, and days from operation to complication (for patients who had complication) were excluded. Patients who were not discharged within 30-d of operation were also excluded. Lastly, patients who died during initial hospitalization were excluded from the analysis that aimed to identify risk factors associated with PDC (univariate logistic regression), as these patients were not at risk for PDC.

Baseline characteristics of patients

We selected patient data based on clinical relevance to patients undergoing esophagectomy and compared demographic and clinical characteristics. Three groups of patients were defined: no complication (Group 1), only pre-discharge complication (Group 2), and PDC patients (Group 3). Patients with esophageal/gastric cancer were defined with a diagnosis (International Classification of Diseases, 9th Revision, codes of 150, 150.1, 150.2, 150.3, 150.4, 150.5, 150.8, 150.9, 151, 151.0).

Outcomes

The primary outcome was PDC, which we defined as an event for which the time interval (days) between the initial operation and a complication was greater than the interval from operation to discharge. The secondary outcomes included hospital readmission (2011-2013) and reoperation (2012-2013). Complication types included from ACS-NSQIP were investigated. Prolonged length of stay and prolonged operative time, defined as stay or time greater than the 75th percentile, respectively, were also investigated.

Statistical analysis

Categorical variables were compared using Pearson's χ^2 test or Fisher's exact test when appropriate. Student's *t*-test or ANOVA were used to compare continuous variables. Modified Poisson regression analysis was performed to identify factors associated with developing a PDC, and risk ratios (RR) were estimated. A *P*-value of $P < 0.05$ was determined statistically significant. We performed all data analyses and management using Stata/MP version 14 (StataCorp LP, College Station, TX, United States).

RESULTS

Study population

A total of 4872 patients underwent esophagectomy between 2005 and 2013. However, 389 (7.98%) patients were not discharged before the 30-day period and were excluded for this very reason. 4483 patients represented our study population, including 2497 (55.7%) patients who had no postoperative complications, 1588 (35.4%) who had at least one pre-discharge complication, and 398 (8.9%) who had at least one PDC. The mean age was 63.1 years, with 80.0% male and 85.9% whites. The mean BMI was 27.9 kg/m^2. PDC patients tended to be slightly older with greater ASA class, BMI, and more comorbidities including diabetes and, dyspnea compared to no complication group (Table 1).

Unadjusted outcomes

The overall PDC rate was 8.9%. Patients who experienced PDC had a median initial length of hospital stay of 9 d; however, PDC occurred on average 14 d after surgery. Among procedure types, PDC rates were 35.7% for transhiatal, 34.1% for Ivor Lewis, 36.9% for 3-holes, and 44.3% for intestinal conduit. Interestingly, PDC rates remained similar over the studied years (8.3% in 2005-2006 *vs* 8.9% in 2013) (Figure 1). Of the patients who experienced PDC, 127 of them (31.9%) also had pre-discharge complications. The overall readmission rate (2011-2013) after esophagectomy was 12.8%. Only from 2012 NSQIP identified if readmissions were likely related to the principle surgical procedure. There were 253 readmissions (253/1989, 12.72%) between 2012 and 2013 and 83.8% (212/253) of these readmissions were related to the initial surgical procedure. PDC patients were overwhelmingly more likely to have a reoperation (39.5% *vs* 22.4% for 2012-2013, $P < 0.001$) and to be readmitted (66.9% *vs* 6.6% for 2011-2013, $P < 0.001$). Moreover, pre-discharge and PDC differed by complication types (Figure 2). PDC patients had greater rates of wound infection (41.0% *vs* 19.3%), VTE (16.3% *vs* 8.9%), and organ space SSI (17.1% *vs* 11.0%) ($P \leqslant 0.001$ for each) than pre-discharge complication patients (Table 2). Although 30-day mortality rates were greater for patients who experienced PDC, this finding was not statistically significant.

Risk factors associated with post-discharge complications

Univariate modified Poisson regression analysis revealed that greater BMI, specifically BMI 25-29.9 (RR = 1.37, 95%CI: 1.08-1.74) and BMI ⩾ 30 (RR = 1.34, 95%CI: 1.04-1.72), were associated with increased risk of PDC, compared to normal BMI (18.5-25) (Table 3).

Table 1 Baseline demographic and clinical characteristics of patients undergoing esophagectomy *n* (%)

Characteristic	Group 1 No complication *n* = 2497 (55.70)	Group 2 Pre-discharge complication *n* = 1588 (35.42)	Group 3 Post-discharge complication *n* = 398 (8.88)	*P* value
Age, mean (median)	62.5 ± 11.2 (63)	63.9 ± 10.8 (65)	63.4 ± 10.9 (64)	0.001
Age group (yr)				0.048
< 60	921 (36.88)	513 (32.30)	143 (35.93)	
60-69	862 (34.52)	579 (36.46)	126 (31.66)	
70-79	602 (24.11)	407 (25.63)	109 (27.39)	
⩾ 80	112 (4.49)	89 (5.60)	20 (5.03)	
Male[1]	2042 (81.88)	1220 (76.83)	325 (81.66)	< 0.001
Race				0.088
White	2135 (85.50)	1363 (85.83)	352 (88.44)	
Black	76 (3.04)	62 (3.90)	6 (1.51)	
Other/unknown	286 (11.45)	168 (10.26)	40 (10.05)	
ASA classification[1]				< 0.001
No disturb/mild disturb	611 (24.50)	237 (14.92)	88 (22.17)	
Serious disturb	1746 (70.01)	1161 (73.11)	269 (67.76)	
Life threat/moribund	137 (5.49)	190 (11.96)	40 (10.08)	
Body mass index, mean (median)	27.9 ± 6.4 (27)	27.7 ± 6.3 (26.9)	28.4 ± 6.2 (27.6)	0.132
Body mass index group[1] (kg/m^2)				0.013
< 18.5	68 (2.75)	59 (3.75)	9 (2.26)	
18.5-24.9	761 (30.72)	522 (33.14)	100 (25.13)	
25-29.9	905 (36.54)	532 (33.78)	159 (39.95)	
⩾ 30	743 (30.00)	462 (29.33)	130 (32.66)	
Diabetes	341 (13.66)	300 (18.89)	75 (18.84)	< 0.001
Current smoker	616 (24.67)	434 (27.33)	86 (21.61)	0.033
Dyspnea	209 (8.37)	205 (12.91)	48 (12.06)	< 0.001
History of COPD	131 (5.25)	156 (9.82)	23 (5.78)	< 0.001
Weight loss	468 (18.74)	301 (18.95)	73 (18.34)	0.959
Steroid use	74 (2.96)	42 (2.64)	12 (3.02)	0.820
Emergency case	18 (0.72)	39 (2.46)	3 (0.75)	< 0.001
Diagnosis				0.002
Benign disease	355 (14.22)	289 (18.20)	56 (14.07)	
Esophageal/gastric cancer	2142 (85.78)	1299 (81.80)	342 (85.93)	
Year of operation				0.012
2005-2007	293 (11.73)	223 (14.04)	41 (10.30)	
2008-2010	746 (29.88)	409 (25.76)	109 (27.39)	
2011-2013	1458 (58.39)	956 (60.20)	248 (62.31)	

[1]Different denominator due to missing data: gender (Total *n* = 4480; Group 1 *n* = 2494; Group 2 *n* = 1588; Group 3 *n* = 398); functional status (*n* = 4481; 2497; 1586; 398); ASA class (*n* = 4479; 2494; 1588; 397); BMI (*n* = 4450; 2477; 1575; 398). ASA: American Society of Anesthesiology; COPD: Chronic obstructive pulmonary disease; BMI: Body mass index.

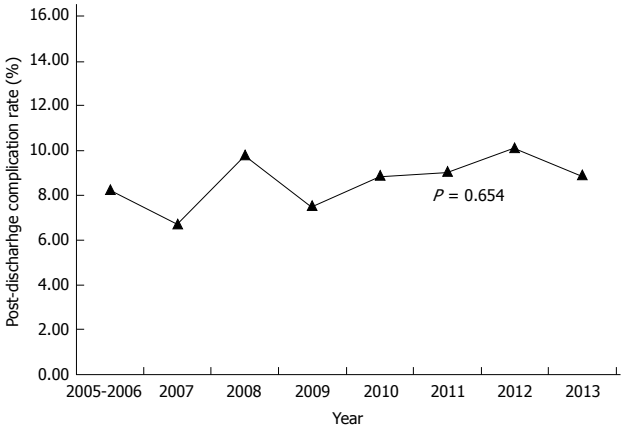

Figure 1 Post-discharge complications rates after esophagectomy by year.

DISCUSSION

Although several studies have investigated post-operative complications and readmissions following esophagectomy, none to our knowledge have explored the distinct role of PDC on early hospital outcomes. This is the first study to use ACS-NSQIP to examine the rate of PDC, their associated risk factors, and their influence on early hospital outcomes after esophagectomy. ACS-NSQIP offers a unique opportunity to assess at a national, multi-institutional level these specific health quality measures that may be unavailable in other large, population-level databases. Our retrospective analysis demonstrates that PDC occur at a low rate but account for a significant number of reoperations and readmissions.

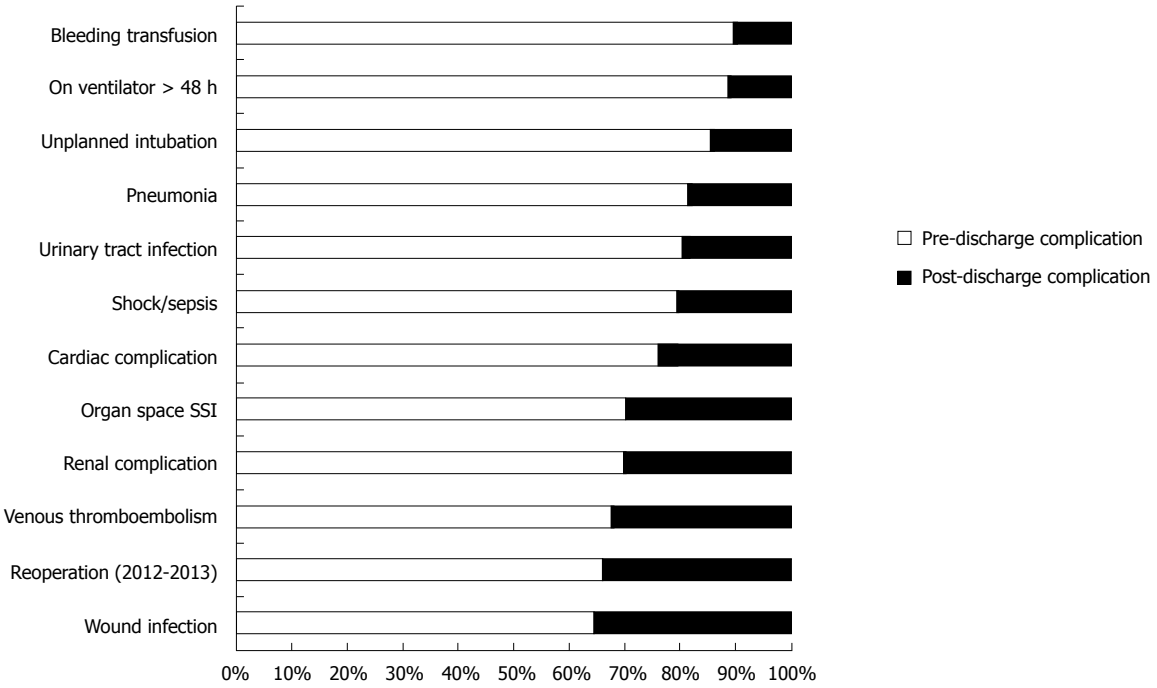

Figure 2 Proportion of pre-discharge *vs* post-discharge complications after esophagectomy for each morbidity.

Table 2 Observed unadjusted rates of pre- and post-discharge outcomes after esophagectomy *n* (%)

Outcome	Total (*n* = 1986)	Group 2 Pre-discharge complication *n* = 1588 (79.96)	Group 3 Post-discharge complication *n* = 398 (20.04)	*P* value
30-d mortality[1]	140 (7.05)	104 (6.55)	36 (9.05)	0.082
Overall morbidity				
Wound infection	470 (23.67)	307 (19.33)	163 (40.95)	< 0.001
Pneumonia	535 (26.94)	444 (27.96)	91 (22.86)	0.040
Urinary tract infection	112 (5.64)	91 (5.73)	21 (5.28)	0.725
Venous thromboembolism	207 (10.42)	142 (8.94)	65 (16.33)	< 0.001
Cardiac complication	108 (5.44)	92 (5.79)	16 (4.02)	0.163
Shock/sepsis	518 (26.08)	422 (26.57)	96 (24.12)	0.319
Unplanned intubation	420 (21.15)	368 (23.17)	52 (13.07)	< 0.001
Bleeding transfusion	584 (29.41)	526 (33.12)	58 (14.57)	< 0.001
Renal complication	59 (2.97)	50 (3.15)	9 (2.26)	0.351
On ventilator > 48 h	439 (22.10)	396 (24.94)	43 (10.80)	< 0.001
Organ space SSI	242 (12.19)	174 (10.96)	68 (17.09)	0.001
Reoperation 12-13	235 (25.97)	160 (22.38)	75 (39.47)	< 0.001
Serious Morbidity[2]	1102 (55.49)	901 (56.74)	201 (50.50)	0.025
Year of operation				0.142
2005-2007	264 (13.29)	223 (14.04)	41 (10.30)	
2008-2010	518 (26.08)	409 (25.76)	109 (27.39)	
2011-2013	1204 (60.62)	956 (60.20)	248 (62.31)	
Length of stay, d (median)	11.7 ± 5.4 (10)	15.0 ± 6.3 (14)	10.3 ± 4.3 (9)	< 0.001
Prolonged length of stay[3]	765 (38.52)	707 (44.52)	58 (14.57)	< 0.001
Operative time, min (median)	342.3 ± 134.7 (328)	358.6 ± 145.6 (343)	350.4 ± 133.1 (335.5)	0.308
Prolonged operative time[4]	570 (28.70)	467 (29.41)	103 (25.88)	0.164
Readmission 2011-2013	226 (19.07)	62 (6.60)	164 (66.94)	< 0.001

[1]In-hospital and post-discharge mortality among patients with pre or post-discharge complications. The overall mortality rate was 3.12% (140/4483); [2]Serious morbidity: cardiac complication, shock/sepsis, unplanned intubation, on ventilator > 48 h, organ space SSI, and reoperation 2012-2013; [3]Defined as length of stay > 75th percentile; [4]Defined as operative time > 75th percentile. SSI: Surgical site infection.

Higher BMIs were associated with increased risk of PDC. PDC were different than pre-discharge complications and potentially preventable.

Of the patients with PDC, wound infection, pneumonia, and VTE were among common PDC that could serve as targeted areas for quality improvement. These PDC are consistent with prior studies examining perioperative complications after esophagectomy[21,22].

Table 3 Unadjusted risk and risk ratio for post-discharge complications after esophagectomy

Risk factor	PDC risk (*n*/total)	RR (95%CI)
Overall PDC risk	398/4379 (9.09)	-
Procedure type		
Ivor-Lewis	196/2219 (8.83)	Ref
Transhiatal	115/1261 (9.12)	1.03 (0.83-1.29)
3-holes	66/730 (9.04)	1.02 (0.78-1.34)
Intestinal conduit	21/169 (12.43)	1.41 (0.92-2.15)
Age group (%)		
< 60	143/1562 (9.15)	Ref
60-69	126/1529 (8.24)	0.90 (0.72-1.13)
70-79	109/1082 (10.07)	1.10 (0.87-1.39)
⩾ 80	20/206 (9.71)	1.06 (0.68-1.65)
Male (%)	325/3505 (9.27)	1.11 (0.87-1.41)
Race (%)		
White	352/3763 (9.35)	Ref
Black	6/137 (4.38)	0.46 (0.21-1.00)
Other/unknown	40/479 (8.35)	0.89 (0.65-1.22)
ASA classification (%)		
No disturb/mild disturb	88/930 (9.46)	Ref
Serious disturb	269/3104 (8.67)	0.92 (0.73-1.15)
Life threat/moribund	40/341 (11.73)	1.24 (0.87-1.76)
Body mass index (%)		
18.5-24.9	100/1347 (7.42)	Ref
< 18.5	9/129 (6.98)	0.94 (0.49-1.81)
25-29.9	159/1561 (10.19)	1.37 (1.08-1.74)
⩾ 30	130/1310 (9.92)	1.34 (1.04-1.72)
Diabetes (%)	75/692 (10.84)	1.24 (0.98-1.57)
Current smoker (%)	86/1111 (7.74)	0.81 (0.64-1.02)
Dyspnea (%)	48/436 (11.01)	1.24 (0.93-1.65)
History of COPD (%)	23/297 (7.74)	0.84 (0.56-1.26)
Weight loss (%)	73/812 (8.99)	0.99 (0.77-1.26)
Steroid use (%)	12/124 (9.68)	1.07 (0.62-1.84)
Emergency case	3/52 (5.77)	0.63 (0.21-1.90)
Esophageal/gastric cancer (%)	342/3707 (9.23)	1.11 (0.84-1.45)
Prolonged length of stay[1] (%)	58/938 (6.18)	0.63 (0.48-0.82)
Prolonged operative time[2] (%)	103/1087 (9.48)	1.06 (0.85-1.31)

[1]Defined as length of stay > 75th percentile; [2]Defined as operative time > 75th percentile. PDC: Post-discharge complication; RR: Risk ratio; ASA: American Society of Anesthesiology; COPD: Chronic obstructive pulmonary disease.

Interventions to reduce PDC, such as adopting best practices to prevent wound infection and VTE, may improve esophagectomy outcomes. In one study, selective anticoagulant thromboprophylaxis using a DVT risk factor index significantly decreased DVT rates after esophageal cancer surgery[23]. Another study showed that low molecular weight heparin prophylaxis resulted in a 72% reduction in DVT risk after general surgery[24]. These suggest that efforts aimed to reduce common complications after surgery may also be effective and applicable to esophagectomy and should be continued after discharge. Interestingly, PDC rates have remained similar over the studied years, revealing likely no significant changes in practice or indications.

Our modified Poisson regression analysis demonstrates several factors that may increase the risk of PDC and therefore enable us to stratify higher-risk patients for perioperative risk assessment. These include patients with BMI 25.0-29.9 and BMI ⩾ 30, compared to normal BMI 18.5-25.0. Although obesity is associated with higher incidence of medical comorbidities like hypertension, diabetes, and cardiovascular disease[25], the association of BMI and complications after esophagectomy is conflicting. Several studies have suggested that overweight and obese patients may have increased risk for complications after esophagectomy, such as longer operative times[26] and greater risks for anastomotic leaks[27], respiratory complications[27], and surgical site infections[28]. However, other studies have shown that obese patients do not have more postoperative complications and longer hospital stays compared to non-obese patients[14,29,30]. Several routine measures implemented in the hospital setting to decrease the most common post-operative complications (*i.e.,* early mobilization, incentive spirometry and pulmonary toilet, DVT prophylaxis, daily dressing changes) are not continued after discharge, and the lack of preventive actions may impact obese patients more than non-obese. As such, appropriate interventions for higher BMI patients may be beneficial and may include enhanced perioperative management of patient comorbidities, preoperative risk stratification, additional patient education, continuation of pulmonary toilet, exercise programs, and DVT prophylaxis after discharge and earlier follow-up. For example, one study has shown that performing preoperative risk analysis on pulmonary function and general status when selecting patients for transthoracic esophagectomy reduced postoperative morbidity rates[31]. Interventions targeting higher BMI patients undergoing esophagectomy may therefore reduce PDC rates.

We decided to separate the patients with pre-discharge complications to keep our groups as homogeneous as possible for fair comparison. Since these patients have a longer hospital stay (median LOS 14 d), a 30-d follow-up may not be long enough to record PDC. It was interesting, however, to see that the most common types of PDC are different than those occurring in the initial post-operative period. This information is helpful to the physician to identify patients at risk of PDC and to plan preventative measures.

The overall readmission rate in our study is 12.8%, consistent with another study's rate of 18.6%[12]. From our study, 39.5% of PDC patients underwent reoperation (2012-2013) compared to 22.4% of pre-discharge complication patients. This suggests that reoperation may have been indicated due to complications that developed after discharge and subsequent delays in management. Even more striking is our finding that 66.9% of PDC patients were readmitted, compared to 6.6% of pre-discharge complication patients. Recent changes in health policy and reimbursements have brought issues of health care costs to the forefront, resulting in some hospital

administrations advocating for earlier discharge of patients. Readmission has become a major focus from health care quality and cost-savings standpoints. However, the use of readmission as a quality metric is still debatable[32,33]. In the case of esophagectomy, efforts to reduce costs by promoting earlier discharge may increase readmission[16], reoperation, and PDC rates; hospital readmission after esophagectomy for esophageal cancer is also associated with poor survival[12]. One study has shown that postoperative complications lead to greater readmission rates after colon resection[34]. In bariatric surgery patients, PDC account for a substantial source of patient morbidity and readmissions[17]. Rather than emphasizing shorter hospital lengths of stay, putting more energy into preventing complications after esophagectomy, and optimizing patient health prior to discharge instead may lead to improved surgical quality outcomes and reduced hospital spending.

Despite the advantages of ACS-NSQIP, the database does pose some limitations to our study. ACS-NSQIP does not contain consistent information on tumor histology, margins, stage, surgical history, neoadjuvant chemotherapy (within 30 d preoperatively), and neoadjuvant radiation (within the last 90 d) that could otherwise provide greater context in interpreting our results. Several complications common to esophagectomy, such as anastomotic leaks, chyle leak, and delayed gastric emptying, are not captured in the database. ACS-NSQIP also identifies 30-d postoperative readmission rather than 30-d post-discharge readmissions, which may introduce immortal person-time bias and lead to shorter number of follow-up days for patients with longer hospital stays. Although PDC could lead to greater mortality, because ACS-NSQIP does not capture data beyond 30-d postoperatively, further studies examining long-term outcomes including mortality is warranted. Readmission data is available only from 2011-2013, but the large patient sample population within that cohort should prevent significant alterations in our conclusions. It is also uncertain whether our findings are applicable to all hospital settings, as hospital participation is voluntary and comprised primarily of academic, tertiary-care centers. Hospital size, setting, patient volume, teaching status, and individual surgeon experience also cannot be adjusted for.

In summary, PDC after esophagectomy account for a significant number of reoperations and readmissions. Adopting best practices to reduce common PDC like VTE and infection, and performing interventions for higher-risk individuals such as those with high BMI, should therefore be considered.

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COMMENTS

Background

Esophagectomy is the mainstay treatment for esophageal carcinoma and can also be indicated for benign conditions with end-stage organ dysfunction or perforation. Despite improved surgical and medical care, post-esophagectomy mortality and morbidity rates remain suboptimal. Such disappointing outcomes after esophagectomy, compounded with national health policy changes that emphasize reducing hospital readmission rates and improving health care quality, have spurred growing interest in investigating quality measures related to esophagectomy. Using 2005-2013 data from the American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP), we sought to identify the rate of post-discharge complications, their associated risk factors, and their influence on early hospital outcomes including readmission and reoperation.

Research frontiers

Although several studies have explored post-esophagectomy complications and readmission, none to date have delved into specifics of post-discharge complications and risk factors associated with them. The results from this study offer additional insights to improve esophagectomy quality outcomes.

Innovations and breakthroughs

No other studies have investigated the specific outcomes of post-discharge complications after esophagectomy. In this study, The authors demonstrated that post-discharge complications after esophagectomy occur a median of 14 d postoperatively and account for a significant number of reoperations and readmissions. Moreover, pre- and post-discharge complications differed by type, with venous thromboembolism and infection occurring more commonly after discharge.

Applications

These research findings can be applied to predict, identify, and prevent adverse outcomes in patients who have undergone esophagectomies. Implementing strategies to decrease common post-discharge complications like venous thromboembolism and infection should be considered, and directing our energies toward optimizing patient health prior to discharge may improve overall surgical outcomes.

Peer-review

This is a review of the ACS-NSQIP esophagectomy database, meant to identify post-discharge complications. It has a huge sample, a clear presentation and analysis, and an interesting discussion. It is without doubt an important topic deserving of evaluation.

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ORIGINAL ARTICLE

Retrospective Study

Clinical significance of HOTAIR expression in colon cancer

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Abstract

AIM: To detect the expression of the long noncoding RNA HOTAIR in colon cancer and analyze its relationship with clinicopathological parameters of colon cancer.

METHODS: Total RNA was extracted from 80 colon cancer tissues and matched tumor-adjacent normal colon tissues and reverse transcribed. Quantitative polymerase chain reaction was used to detect the expression of HOTAIR. The relationship between the expression of HOTAIR and clinicopathological parameters of colon cancer was analyzed.

RESULTS: The expression of HOTAIR was significantly higher in colon cancer tissues than in matched tumor-adjacent normal colon tissues ($P < 0.05$). HOTAIR expression was significantly higher in cases with lymph node metastasis than in those without metastasis; in lowly differentiated and undifferentiated cases than in highly and moderately differentiated cases; and in stages Ⅲ + Ⅳ cases than in stages Ⅰ + Ⅱ cases ($P < 0.05$).

CONCLUSION: HOTAIR expression is upregulated in colon cancer, suggesting that HOTAIR plays an important role in the tumorigenesis, development and metastasis of colon cancer. HOTAIR may act as an oncogene and represents a new molecular target for the treatment of colon cancer.

Key words: HOTAIR; Long non-coding RNA; Oncogene; Colon tumor

Core tip: This study aimed to detect the expression of HOTAIR in colon cancer and analyze its relationship with clinicopathological parameters of colon cancer. Total RNA was extracted from 80 colon cancer tissues and matched tumor-adjacent normal colon tissues and reverse transcribed. HOTAIR expression was upregulated in colon cancer, suggesting that it may play an important role in the tumorigenesis, development and metastasis of colon cancer. HOTAIR might acts as an oncogene and could be a new molecular target for the treatment of colon cancer.

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INTRODUCTION

Colon cancer is a clinically common, highly malignant tumor of the digestive tract. Although drugs targeting epidermal growth factor receptor (EGFR) and KRAS mutations have significantly extended the survival of some colon cancer patients[1-3], only a small number of patients can benefit from these drugs because of the complex etiology of this malignancy. Overall, the effects of current therapies for colon cancer are not satisfactory[4,5].

Long noncoding RNAs (lncRNAs) are non-protein coding transcripts of around 200 nucleotides, which exist widely in the genome and can regulate gene expression[6]. HOTAIR is one of the extensively studied lncRNAs in recent years. Many studies have indicated that HOTAIR plays an important role in breast cancer, pancreatic cancer, liver cancer, gastric cancer, esophageal cancer and non-small cell lung cancer[7-10]. Studies in colon cancer suggest that HOTAIR is an important oncogene that affects the biological behavior of colon cancer[11] and can serve as an independent risk factor[12]. The latest research suggests that the expression of HOTAIR is associated with tumor metastasis[13].

In the present study, we detected the expression of HOTAIR in 80 colon cancer tissue samples by quantitative polymerase chain reaction (qPCR). Based on the clinical and pathological parameters of colon cancer patients, we analyzed the possible role of HOTAIR in colon cancer development, metastasis and sensitivity to treatment, with an emphasis on the role of HOTAIR in colon cancer treatment. The findings will provide a theoretical basis for developing a new, targeted therapy for colon cancer.

MATERIALS AND METHODS

Clinical materials and reagents

Eighty patients with pathologically proven colon cancer who underwent surgery at our hospital from September 2011 to September 2013, and had complete clinical records, were included. All patients provided written informed consent, and the study protocol was approved by the Medical Ethics Committee of Zhengzhou University. The mean age of the patients was 64 ± 16 years. There were 46 patients with stage Ⅰ or Ⅱ disease, and 34 patients with stage Ⅲ or Ⅳ disease. Forty-one patients had well or moderately differentiated tumors, and 34 patients had poorly differentiated or undifferentiated tumors. No patients had undergone radiotherapy or chemotherapy before surgery. Tumor tissues and normal colon tissues at least 7 cm away from the tumor were taken, frozen in liquid nitrogen within 30 min and preserved for further use.

Trizol was purchased from Invitrogen. The reverse transcription kit and DNA ladder were purchased from Takara. Primers for qPCR were designed and synthesized by Shanghai GenePharma. The qPCR kit was purchased from Thermo.

RNA preparation and reverse transcription

Tissue samples preserved in liquid nitrogen were put into an RNase-free mortar with liquid nitrogen and pulverized. For each 100 mg of tissue, 1 mL of Trizol was added. RNA preparation was then performed following the manufacturer's instructions. The obtained RNA was dissolved in DEPC-treated water, and the RNA concentration was measured using a micro UV-Vis fluorescence spectrophotometer (e-spect, Malcom, Japan). The obtained RNA was preserved at -80 ℃ for further use.

RNA reverse transcription was performed using a reverse transcription kit in a 20-μL system, containing 11 μL of DEPC-treated water, 1 μL of total RNA, 4 μL of 5 × buffer, 1 μL of RNase inhibitor, 2 μL of dNTPs, and 1 μL of reverse transcriptase. Reaction parameters were 42 ℃ for 60 min and 95 ℃ for 5 min. The obtained cDNA was preserved at -80 ℃ for further use.

qPCR: qPCR was performed in a 20-μL system containing 1 μL of cDNA, 10 μL of 2 × Master Mix with 0.03 × ROX added, 1 μL of forward primer (final concentration of 0.5 μmol/L), 1 μL of reverse primer, and 8 μL of DEPC-treated water on a Mx3005p cycler. PCR amplification was performed in triplicate. Cycling parameters were 95 ℃ for 7 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 30 s.

Statistical analysis

The expression levels of HOTAIR in tissues are expressed as mean ± SD and were compared using a

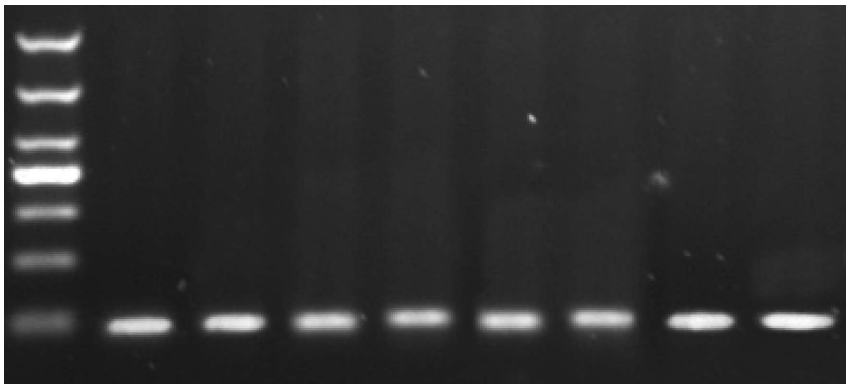

Figure 1 Quantitative polymerase chain reaction products of HOTAIR.

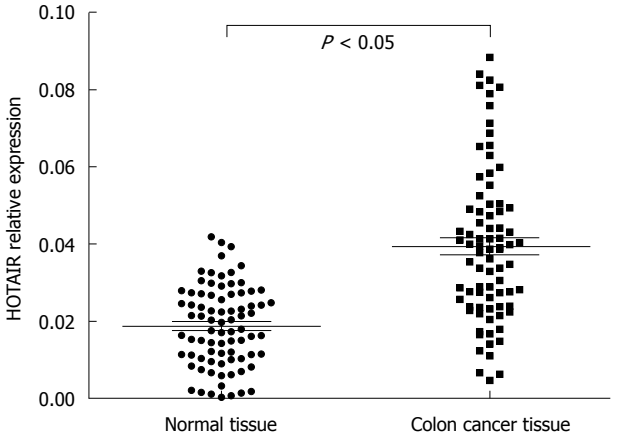

Figure 2 Expression of HOTAIR in colon cancer. The expression of HOTAIR in colon cancer is significantly higher in colon cancer tissues than in tumor-adjacent normal colonic tissues.

two-sample *t*-test. Statistical analyses were performed using SPSS13.0. *P*-values < 0.05 were considered statistically significant.

RESULTS

Agarose gel electrophoresis of qPCR products

The length of the expected PCR product for HOTAIR is 91 bp, and agarose gel electrophoresis showed that qPCR yielded PCR products of expected size (Figure 1).

Expression of HOTAIR is higher in colon cancer tissues than in tumor-adjacent normal colonic tissues

QPCR analysis showed that, although the expression of GAPDH showed no significant differences, the Ct value of HOTAIR was significantly lower in colon cancer tissues than in tumor-adjacent normal colonic tissues, suggesting that HOTAIR expression is upregulated in colon cancer. When the relative expression level is expressed as N ($N = 2^{-\Delta Ct}$, $\Delta Ct = Ct_{HOTAIR} - Ct_{GAPDH}$[14]), the relative expression level of HOTAIR was significantly higher in colon cancer tissues than in tumor-adjacent normal colonic tissues ($P < 0.05$, Figure 2).

Relationship between HOTAIR expression and clinicopathological parameters in colon cancer

HOTAIR expression was significantly correlated with lymph node metastasis, tumor differentiation and TNM stage ($P < 0.05$). HOTAIR expression was significantly higher in cases with lymph node metastasis than in those without metastasis, in lowly differentiated and undifferentiated cases than in highly and moderately differentiated cases, and in stages Ⅲ + Ⅳ cases than in stages Ⅰ + Ⅱ cases. By contrast, HOTAIR expression had no significant correlation with patient gender, age or tumor size ($P > 0.05$) (Tables 1-3).

Relationship between HOTAIR expression and survival in colon cancer

The Kaplan-Meier method was used to assess the impact of HOTAIR expression on survival of patients with colon cancer. The cumulative survival rate was significantly higher in patients with low HOTAIR expression than in those with high HOTAIR expression ($P < 0.05$) (Figure 3).

Risk factors for prognosis of colon cancer patients

Using prognosis of colon cancer patients as the dependent variable and factors possibly influencing the prognosis as independent variables, Cox multiple regression analysis was performed. The results showed that TNM stage, lymph node metastasis and HOTAIR expression were independent risk factors for prognosis of colon cancer patients.

Relationship between HOTAIR expression and prognosis in colon cancer

The relationship between prognosis of colon cancer patients after chemotherapy and HOTAIR expression was analyzed. The results showed that high HOTAIR expression was associated with poorer prognosis (Figure 4).

DISCUSSION

Colon cancer is a common malignancy[15,16]. With the

Table 1 Primers used for quantitative polymerase chain reaction

Primer	Sequence
HOTAIR	
Forward	5'-CAGTGGGGAACTCTGACTCG-3'
Reverse	5'-GTGCCTGGTGCTCTCTTACC-3'
GAPDH	
Forward	5'-GTCAACGGATTTGGTCTGTATT-3'
Reverse	5'-AGTCTTCTGGGTGGCAGTGAT-3'

Table 2 Relationship between HOTAIR expression and clinicopathological parameters in colon cancer

Clinicopathological parameter	No. of cases	HOTAIR expression	*P* value
Age (yr)			
< 6	49	3.69 ± 1.94	0.188
⩾ 60	31	3.45 ± 1.55	
Gender			
Male	43	3.91 ± 1.85	0.761
Female	37	3.23 ± 1.68	
Tumor size (cm)			
< 7	38	3.59 ± 1.59	0.599
⩾ 7	42	3.60 ± 1.81	
Lymph node metastasis			
Yes	48	4.27 ± 1.54	0.024
No	32	3.11 ± 1.92	
Tumor differentiation			
High and moderate	41	2.93 ± 1.62	0.019
Low and undifferentiated	39	4.35 ± 1.82	
TNM stage			
Ⅰ + Ⅱ	46	3.17 ± 1.77	0.034
Ⅲ + Ⅳ	34	3.87 ± 1.66	

Table 3 Cox multiple regression analysis of risk factors for prognosis of colon cancer patients

Variable	Regression coefficient	SE	χ^2	*P* value	OR	95%CI for OR	
						Lower	Upper
TNM stage	0.732	0.345	4.489	0.034	2.0790	1.056	4.090
Lymph node metastasis	-2.512	1.088	5.325	0.021	0.0081	0.010	0.685
HOTAIR expression	-2.048	0.785	6.806	0.090	0.1290	0.028	0.601

development of diagnostic technology, advances in endoscopy and imaging techniques, as well as the clinical application of the carcinoembryonic antigen assay, have greatly improved the early diagnosis and treatment effect of colon cancer[17,18]. Patients with early colon cancer have localized lesions, and surgery with adjuvant radiochemotherapy is the preferred treatment, which is often associated with a good prognosis. However, because of the unbalanced regional development in China, many patients with colon cancer, especially those in rural regions, are diagnosed at advanced stages, and some patients even present with metastases as the first manifestation. Although drugs targeting EGFR and

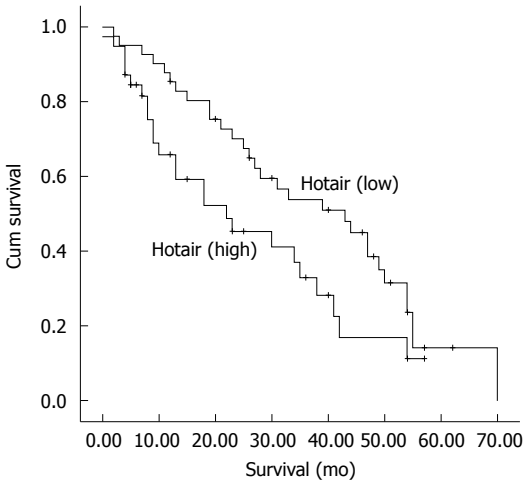

Figure 3 Relationship between HOTAIR expression and survival in colon cancer. The cumulative survival rate was significantly higher in patients with low HOTAIR expression than in those with high HOTAIR expression ($P < 0.05$).

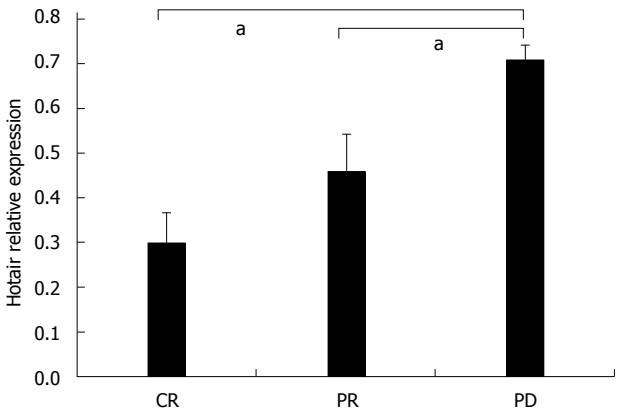

Figure 4 Relationship between HOTAIR expression and prognosis in colon cancer. [a]$P < 0.05$ *vs* PD group.

KRAS mutations have been effective in some patients with colon cancer[1-3], molecular targeted drugs, which often target only one or several molecules, are not suitable for all patients because of the complexity etiology of colon cancer. Therefore, there is an urgent need to find new therapeutic targets.

LncRNAs are non-protein coding transcripts of around 200 nucleotides that are widely distributed in the genome. Many lncRNAs can bind to DNA binding proteins and alter the chromosome state to participate in the regulation of many genes[6,19]. HOTAIR is an lncRNA located in the *HOXC* locus, and it can interact with polycomb repressive complex 2 and mediate the histone H3 lysine 27 methylation and lysine 4 demethylation in the *HOXD* locus, in which EZH2 also plays an important role[9,20,21]. HOTAIR can alter the state of chromosomes, thus affecting the expression of many genes. Researchers have found that HOTAIR expression is upregulated in cancer tissue samples from patients with breast cancer, pancreatic cancer, liver cancer, gastric cancer, or non-small cell lung cancer, and the expression is even higher in metastatic tissue.

Both *in vivo* and *in vitro* studies have confirmed that upregulated expression of HOTAIR enhances the ability of tumors to invade and metastasize[7-9]. The aim of this study was to detect the expression of HOTAIR in tissue samples from patients with colon cancer, analyze the relationship between HOTAIR expression and clinicopathological parameters and explore the role of HOTAIR in colon cancer development and metastasis.

The results showed that the expression of HOTAIR is upregulated in colon cancer, suggesting that HOTAIR may act as an oncogene in the development of colon cancer. We also discovered that HOTAIR expression was significantly higher in lowly differentiated and undifferentiated cases compared with highly and moderately differentiated cases; in stages Ⅲ + Ⅳ cases compared with stages Ⅰ + Ⅱ cases; and in cases with lymph node metastasis compared with those without. These results are similar to the findings of a previous study[22]; however, that study found that the expression of HOTAIR did not differ significantly between cases with and without lymph node metastasis, but was significantly higher in cases with liver metastasis compared with those without. The present study did not compare the HOTAIR expression between cases with and without liver metastasis. Low differentiation, late stage or lymph node metastasis in colon cancer are often associated with poor prognosis; therefore, our findings need to be validated by studies with a larger sample size.

Although HOTAIR might affect response to therapy in some tumors; for example, HOTAIR is associated with resistance to chemotherapy in ovarian cancer and sarcoma[23,24], there have been no reports in colon cancer. Our study, together with previous research, found that HOTAIR has an impact on the biological behavior of colon cancer[13], and detecting the level HOTAIR in blood could be used to predict prognosis of colon cancer. We speculated that this finding may be related to the role of HOTAIR in chemotherapy resistance, and this, therefore, was the focus of this study. We found that tumors with high expression of HOTAIR tended to develop resistance to chemotherapy, which may be the reason that high expression of HOTAIR is associated with a poor prognosis.

This finding also suggested that it is essential to explore the relationship between HOTAIR and resistance to chemotherapy *in vitro*, as well as the impact of HOTAIR on the biological behavior of tumor cells. Several studies have revealed that HOTAIR has an important role in tumor metastasis. On the basis of these findings, our subsequent follow-up study will expand the sample size to conduct prognostic and survival analyses to further define the relationship between HOTAIR and tumor metastasis in colon cancer, to explore the mechanism of pathogenesis of this malignancy and provide new targets for molecular therapy for colon cancer patients.

COMMENTS

Background

Long noncoding RNAs are non-protein coding transcripts of around 200 nucleotides that are widely distributed in the genome.

Research frontiers

The expression of HOTAIR, a long noncoding RNA, is upregulated in cancer tissue samples from patients with breast cancer, pancreatic cancer, liver cancer, gastric cancer, or non-small cell lung cancer, and the expression is even higher in metastatic tissue.

Innovations and breakthroughs

High expression of HOTAIR tended to develop resistance to chemotherapy, which may be the reason that high expression of HOTAIR is associated with a poor prognosis.

Applications

By exploration of the mechanism of HOTAIR expression in colon cancer, the authors might identify new targets for molecular therapy for colon cancer patients.

Terminology

HOTAIR might acts as an oncogene and represents a new molecular target for the treatment of colon cancer.

Peer-review

It is a very good and interesting study. The authors found that the expression of HOTAIR was significantly higher in colon cancer tissues than in matched tumor-adjacent normal colon tissues. HOTAIR expression was significantly higher in cases with lymph node metastasis than in those without metastasis, in lowly differentiated and undifferentiated cases than in highly and moderately differentiated cases. HOTAIR expression is upregulated in colon cancer, which may plays an important role in tumorigenesis, development and metastasis of colon cancer.

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ORIGINAL ARTICLE

Clinical Trials Study

Beneficial effects of antidepressant mirtazapine in functional dyspepsia patients with weight loss

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Author contributions: Jiang SM and Jia L designed the research; Liu J, Shi MM and Xu MZ performed the research; Jiang SM, Jia L and Liu J analyzed the data; Jiang SM and Jia L wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Guangzhou First People's Hospital and Guangzhou Nansha Central Hospital.

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

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Abstract

AIM: To explore the effects and mechanism of action of antidepressant mirtazapine in functional dyspepsia (FD) patients with weight loss.

METHODS: Sixty depressive FD patients with weight loss were randomly divided into a mirtazapine group (MG), a paroxetine group (PG) or a conventional therapy group (CG) for an 8-wk clinical trial. Adverse effects and treatment response were recorded. The Nepean Dyspepsia Index-symptom (NDSI) checklist and the 17-item Hamilton Rating Scale of Depression (HAMD-17) were used to evaluate dyspepsia and depressive symptoms, respectively. The body composition analyzer was used to measure body weight and fat. Serum hormone levels were measured by ELISA.

RESULTS: (1) After 2 wk of treatment, NDSI scores were significantly lower for the MG than for the PG and CG; (2) After 4 or 8 wk of treatment, HAMD-17 scores were significantly lower for the MG and PG than for the CG; (3) After 8 wk of treatment, patients in the MG experienced a weight gain of 3.58 ± 1.57 kg, which was significantly higher than that observed for patients in the PG and CG. Body fat increased by 2.77 ± 0.14

kg, the body fat ratio rose by 4%, and the visceral fat area increased by 7.56 ± 2.25 cm^2; and (4) For the MG, serum hormone levels of ghrelin, neuropeptide Y (NPY), motilin (MTL) and gastrin (GAS) were significantly upregulated; in contrast, those of leptin, 5-hydroxytryptamine (5-HT) and cholecystokinin (CCK) were significantly downregulated.

CONCLUSION: Mirtazapine not only alleviates symptoms associated with dyspepsia and depression linked to FD in patients with weight loss but also significantly increases body weight (mainly the visceral fat in body fat). The likely mechanism of mirtazapine action is regulation of brain-gut or gastrointestinal hormone levels.

Key words: Mirtazapine; Functional dyspepsia; Weight loss; Depression

Core tip: A part of functional dyspepsia (FD) patients were found with weight loss in recent studies. As an antidepressant, mirtazapine was found not only to alleviate symptoms associated with dyspepsia and depression linked to FD with weight loss, but also to significantly increase body weight (mainly the visceral fat in body fat). Moreover, the likely mechanism of mirtazapine action is the regulation of brain-gut or gastrointestinal hormone levels.

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INTRODUCTION

Functional dyspepsia (FD) is a common clinical syndrome characterized by chronic and recurrent symptoms in the gastroduodenal region in the absence of any organic or metabolic disease that explains the symptoms[1]. It impairs the patient's quality of life and work efficiency, and increases the utilization of medical resources[2-4]. Psychosocial factors may play an important role in FD and lead to the use of antidepressant and anxiolytic agents in FD management[5].

Weight loss is a common symptom of digestive diseases, and may indicate an organic disease[6]. However, the indicators of weight loss for the diagnosis of an organic disease are limited[7-9]. Tack *et al*[10] found that of 40 FD patients, 55% had a weight loss that was > 5% of initial body weight. In a study investigating laboratory parameters and the nutritional status of 180 patients who were diagnosed with FD, 16.67% of patients had a weight loss from 5% to 10% of their initial body weight, and 4.44% had a weight loss of > 10% of their initial body weight[11]. Our previous multi-center research study of 1057 FD patients showed that with the onset of dyspepsia symptoms, 19.58% had lost ⩾ 5% of their initial body weight during the previous 12 mo or less. FD patients with weight loss had lower body mass index, more frequent physician visits, higher psychological disorders, poorer appetite and lower quality of life[12].

Antidepressant mirtazapine is clinically used in the treatment of depression or anxiety disorders. In recent years, many clinical trials associated with antidepressants for FD have indicated that antidepressants are effective in treating FD patients[13-15]. A case report about mirtazapine in the treatment of an FD patient with depression reported that the patient's indigestive symptoms, appetite, depression, and quality of life were improved after taking mirtazapine for 4 wk[16]. However, studies that have focused on the parts of the body that are involved in weight gain and the underlying mechanisms have been rare.

Clinical observations have shown that increases in appetite and food intake, and consequent weight gain occur in some patients undergoing mirtazapine treatment. Whereas such side effects may limit the general application of mirtazapine in antidepressant therapy, these very same effects proved to be beneficial in treating FD patients with weight loss.

Therefore, expanding upon previous work[12,17], in this study we comprehensively explored the effect of mirtazapine on depressive FD patients with weight loss by dynamic observation not only of the changes in dyspepsia and depressive symptoms but also the modifications of body weight and fat distribution and the levels of serum hormones.

MATERIALS AND METHODS

Ethics statement

This study was a prospective, randomized, controlled trial in depressive FD patients with weight loss and was approved by the hospital ethics committee (Clinical trial registration number: ChiCTR-TRC-13003161). Written informed consent was obtained from the patients according to the Declaration of Helsinki.

Patients

In this prospective study, 60 patients were recruited between September 2011 and June 2013 from the gastroenterology outpatient clinic of Guangzhou Nansha Central Hospital. All the patients fulfilled the following criteria[12]: (1) diagnosed with FD according to Rome Ⅲ criteria; (2) with a weight loss of ⩾ 5% of initial body weight since the onset of symptoms; (3) diagnosed with depression by psychiatrists according to the Chinese Classification of Mental Disorders (CCMD-3)

and scores of the Hamilton Rating Scale of Depression (HAMD) over 18; and (4) ranged in age from 18 to 65 years; and (5) signed informed consent statements.

The following exclusion criteria were adopted: (1) organic diseases such as peptic ulcers, atrophy or erosive gastroduodenal lesions, tumors, and esophagitis by gastroscopic examination; (2) liver, gallbladder, pancreas, spleen and bowel organic disease by laboratory, B ultrasonic or X-ray examination; (3) dyspepsia symptoms and weight loss that were explained by metabolic or infectious diseases such as diabetes, hyperthyroidism, or tuberculosis; (4) anorexia nervosa and patients with body weight management problems; (5) age < 18 or > 65 years; (6) pregnancy or breast feeding; (7) disabilities; (8) current use of other drugs in clinical research or use of similar drugs in the last half-month; (9) in a severe anxiety or depressive state, or with suicidal tendencies; (10) current use of non-steroidal anti-inflammatory drugs, steroids, or drugs affecting gastric acid secretion; and (11) contraindications for paroxetine or mirtazapine use including hypersensitivity, liver dysfunction or renal failure.

Grouping

Sixty depressive FD patients with weight loss were randomly divided into a mirtazapine group (MG), a paroxetine group (PG) or a conventional therapy group (CG) with 20 patients in each. The trial period spanned 8 wk. The CG was treated with histamine type 2 receptor antagonists or proton pump inhibitors or prokinetic agents; MG was treated with mirtazapine (Remeron®, N.V. Organon, Holland, 30 mg/d); and PG was treated with paroxetine (Seroxat®, SK&F, China, 20 mg/d). All protocols were based on conventional therapy.

Assessments

Adverse effects and treatment response were recorded and data collected at specific time points. These were before treatment (for baseline determination), 2 wk, 4 wk, 6 wk and 8 wk of treatment for the following assessments: dyspepsia symptoms were evaluated with NDSI; depressive symptoms, with HAMD-17; and the change in body weight and the distribution of body fat with the body composition analyzer (InBody720, Biospace, South Korea). Serum hormone levels were measured at baseline, 4 wk and 8 wk; expression levels of ghrelin, leptin, neuropeptide Y (NPY), 5-hydroxy tryptamine (5-HT), cholecystokinin (CCK), motilin (MTL) and gastrin (GAS) were assayed by ELISA.

NDSI: The NDSI evaluated the frequency, intensity, and practical impediments of 15 GI symptoms (including epigastric pain, epigastric burning, postprandial fullness, and early satiety) over a 2-wk period. We recorded each subscale score concerning daily activities/work (13 items), knowledge and control (7 items), eating/drinking (3 items), and sleep disturbance (2 items)[18]. We added each item score within each subscale to produce a subscale score. Low scores indicate mild symptoms.

HAMD-17: The rating standards of the HAMD[19] were as follows: no depression (0-6), mild depression (7-17), moderate depression (18-24), and severe depression (> 25). A higher score indicates worse depression.

Body composition analyzer (InBody720): Patient requirements included: empty stomach, empty bladder, light clothes and no shoes for measurement in the early morning on the body composition analyzer. Patients with a pacemaker or with metal in the body were excluded from measurement on this instrument.

Treatment response: Treatment response was defined as a > 50% reduction in the NDSI score. The response was calculated as: [(score at treatment - score at baseline)/score at baseline] × 100. The treatment responses of the three groups were calculated independently.

Statistical analysis

Data analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago IL, United States), and measurement data are reported as the mean ± SD, and were compared across groups using one-way ANOVA and the Student-Newman-Keuls test for multiple comparisons. Count data were compared across groups using a χ^2 test. All tests were two-tailed and $P < 0.05$ was considered statistically significant.

RESULTS

Study participants

A total of 60 depressive FD patients with weight loss were enrolled in the study. All patients were randomized to receive mirtazapine, paroxetine or conventional treatment. No patient was lost to follow-up. The baseline characteristics of the patients are shown in Table 1. No differences were observed among the three groups in gender, age, height, weight, body mass index (BMI) or body loss when diagnosed.

Improvement of dyspepsia symptoms

As shown in Figure 1A, the patients' dyspepsia symptoms gradually improved over the course of treatment in the three groups. After 2 wk, the NDSI score was significantly lower in the MG than in the PG and CG ($P < 0.05$ for all), and this trend continued until the end of the study. Since 6 wk, NDSI score of the PG was significantly lower than that of the CG ($P < 0.05$).

Improvement of depressive symptoms

Patients' depressive symptoms were improved in the

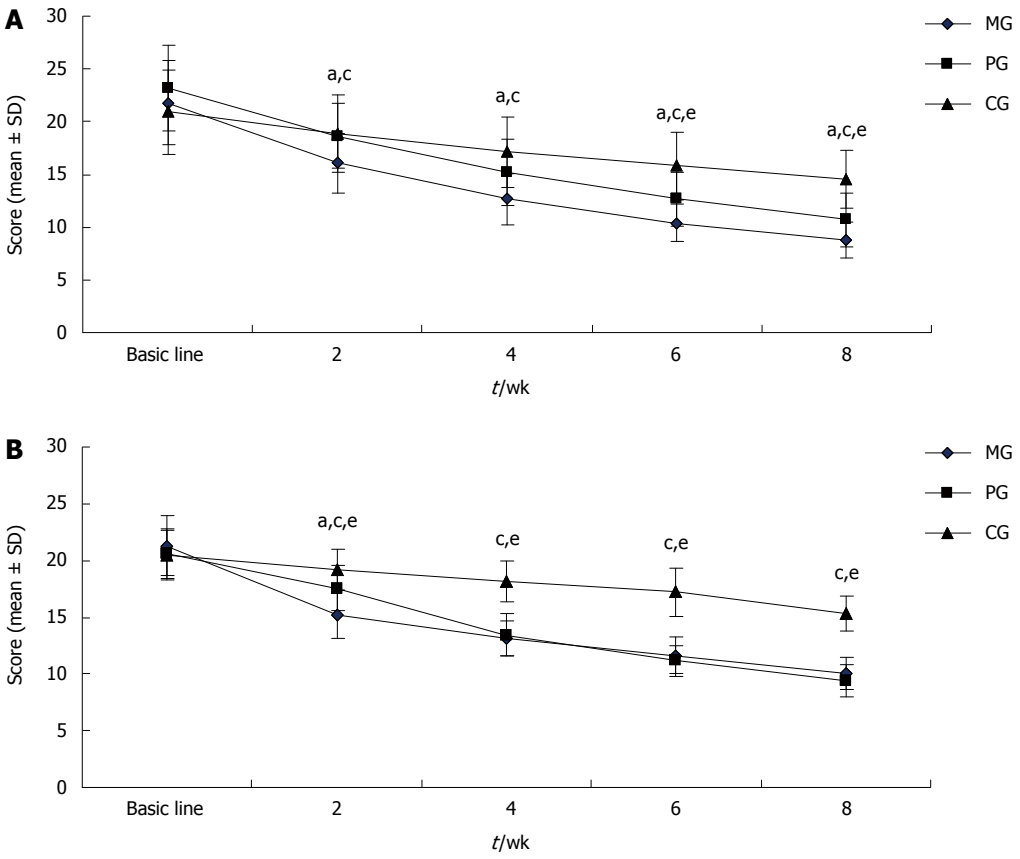

Figure 1 Comparison of Nepean Dyspepsia Index-symptom scores (A) or Hamilton Rating Scale of Depression-17 scores (B). [a]*P* < 0.05, MG *vs* PG; [c]*P* < 0.05, MG *vs* CG; [e]*P* < 0.05, PG *vs* CG. MG: Mirtazapine group; PG: Paroxetine group; CG: Conventional group.

Table 1 General characteristics of study patients

Variable	MG (*n* = 20)	PG (*n* = 20)	CG (*n* = 20)	*P* value
Gender (M/F)	8/12	11/9	7/13	0.725
Age (yr)	43.45 ± 11.50	37.75 ± 10.78	39.95 ± 6.84	0.914
Height (cm)	163.81 ± 12.36	162.36 ± 10.06	160.72 ± 10.63	0.834
Weight (kg)	49.77 ± 6.79	48.93 ± 5.89	48.22 ± 5.57	0.973
BMI (kg/m^2)	18.73 ± 5.62	18.65 ± 4.73	18.84 ± 6.38	1.005
Body loss when diagnosed	3.42 ± 0.54	3.72 ± 0.64	3.69 ± 0.71	0.872

MG: Mirtazapine group; PG: Paroxetine group; CG: Conventional group.

three treatment groups (Figure 1B). At all time points, the HAMD-17 score was significantly lower in the MG and PG than in the CG ($P < 0.05$). After 2 wk of treatment, the HAMD-17 score was sharply lower in the MG than in the PG ($P < 0.05$); however, at 4 wk, the score of PG became very close to that of the MG and remained so until the end of the study.

Change of body weight and its composition

As shown in Figure 2, the patients' body weights were not significantly different before treatment among the three groups. As treatment progressed, body weight of patients in the MG gradually increased. At 6 wk and 8 wk, patients' body weights were significantly heavier in the MG than those in the PG and CG ($P < 0.05$ for all); thus, there was no significant body weight change over the test period in either the PG or CG.

Further analysis of body weight and its composition is shown in Table 2. After 8 wk of treatment, 19 patients in the MG presented an increase in body weight and BMI. The patients in the MG gained 3.58 ± 1.57 kg, which was significantly higher than that gained in either the PG, at 0.53 ± 0.44 kg or the CG, at 0.56 ± 0.45 kg ($P < 0.05$). However, no obvious change of body weight was observed in the PG or CG throughout treatment. Body fat is one of the main components contributing to body weight. Over the course of treatment, body fat increased by 2.77 ± 0.14 kg; the body fat ratio rose by 4%; and visceral fat area was increased by 7.56 ± 2.25 cm^2 ($P < 0.05$). No significant change in muscle volume was detected over the treatment period.

Changes in expression levels of serum hormones

After 4 wk and 8 wk of treatment in MG, the levels of ghrelin, NPY, MTL, and GAS were significantly upregulated, while the levels of leptin, 5-HT and CCK were significantly downregulated ($P < 0.05$). After 8 wk of treatment, significant differences appeared between the levels in the MG and those in the PG and CG ($P < 0.05$). Moreover, at 4 wk in the PG, the levels of NPY, MTL and GAS sharply increased, whereas

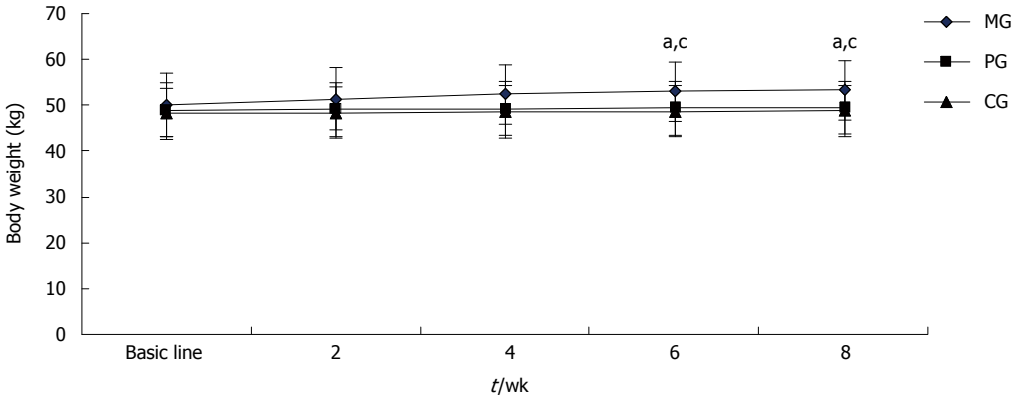

Figure 2 Change of body weight. [a]*P* < 0.05, MG *vs* PG; [c]*P* < 0.05, MG *vs* CG. MG: Mirtazapine group; PG: Paroxetine group; CG: Conventional group.

Table 2 Change of body weight and its composition in mirtazapine group

Variable	Baseline	2 wk	4 wk	6 wk	8 wk
Body weight (kg)	49.77 ± 6.79	51.35 ± 6.80	52.36 ± 6.60[a]	53.07 ± 6.46[a]	53.35 ± 6.52[a]
BMI (kg/m^2)	18.73 ± 5.62	18.97 ± 5.43	19.70 ± 4.52[a]	19.87 ± 4.62[a]	20.07 ± 5.23[a]
Body fat (kg)	8.36 ± 2.53	9.28 ± 2.33	11.05 ± 1.92[a]	11.09 ± 1.87[a]	11.13 ± 2.86[a]
Body fat ratio (%)	0.17 ± 0.02	0.18 ± 0.04	0.20 ± 0.07[a]	0.20 ± 0.09[a]	0.21 ± 0.08[a]
Visceral fat area (cm^2)	35.27 ± 8.12	36.12 ± 8.05	41.68 ± 9.23[a]	42.02 ± 9.14[a]	42.83 ± 10.64[a]
Muscle volume (kg)	38.25 ± 7.53	38.26 ± 7.49	38.46 ± 6.84	38.52 ± 6.64	38.62 ± 6.77

[a]*P* < 0.05 *vs* baseline. BMI: Body mass index.

Table 3 Changes of expression level of serum hormones

Group		Ghrelin (ng/mL)	Leptin (ng/mL)	NPY (pg/mL)	5-HT (ng/mL)	CCK (pg/mL)	MTL (pg/mL)	GAS (pg/mL)
MG	Baseline	4.62 ± 1.53	9.87 ± 4.65	107.52 ± 26.21	237.83 ± 56.94	392.36 ± 27.21	32.53 ± 6.28	56.37 ± 21.15
	4 wk	6.02 ± 3.43[a,c,e]	7.25 ± 3.47[a,c,e]	114.56 ± 25.10[a]	208.47 ± 52.48[a]	221.69 ± 23.77[a]	49.46 ± 6.10[a,c,e]	48.37 ± 11.93[a,c,e]
	8 wk	8.97 ± 3.64[a,c,e]	4.03 ± 2.77[a,c,e]	149.27 ± 39.53[a,c,e]	176.92 ± 53.38[a,c,e]	183.85 ± 27.65[a,c,e]	66.28 ± 3.97[a,c,e]	41.61 ± 10.52[a,c,e]
PG	Baseline	4.68 ± 2.12	9.07 ± 4.65	105.12 ± 29.52	232.83 ± 50.94	390.82 ± 27.54	31.98 ± 9.34	54.98 ± 15.24
	4 wk	5.12 ± 2.23	8.75 ± 3.05	112.31 ± 15.10[a]	215.32 ± 24.91[a]	291.57 ± 31.76[a]	40.37 ± 8.23[a]	52.37 ± 12.97
	8 wk	6.01 ± 3.27[a]	8.25 ± 2.13	114.27 ± 28.53[a]	210.17 ± 49.17[a]	283.85 ± 47.15[a]	53.28 ± 6.84[a]	48.61 ± 11.17[a]
CG	Baseline	4.89 ± 2.47	8.87 ± 2.65	104.93 ± 17.95	235.81 ± 61.82	391.75 ± 24.96	36.26 ± 6.22	54.23 ± 26.83
	4 wk	5.48 ± 2.15	8.32 ± 3.57	108.92 ± 29.64	211.26 ± 46.28	224.67 ± 23.45	43.92 ± 7.24	49.21 ± 12.15
	8 wk	6.93 ± 2.35	8.02 ± 1.45	121.43 ± 13.92	208.95 ± 38.29	303.12 ± 26.76	55.53 ± 5.98	43.34 ± 13.72

[a]*P* < 0.05 *vs* baseline in the same group; [c]*P* < 0.05, MG *vs* PG; [e]*P* < 0.05, MG *vs* CG. MG: Mirtazapine group; PG: Paroxetine group; CG: Conventional group; NPY: Neuropeptide Y; 5-HT: 5-hydroxytryptamine; CCK: Cholecystokinin; MTL: Motilin; GAS: Gastrin.

the levels of 5-HT and CCK decreased. There was no obvious difference in CG hormone expression levels over the treatment period (Table 3).

Adverse effects and treatment response

The adverse effects associated with the different protocols were recorded for the 20 patients assigned to each treatment group**:** in the MG, these were dizziness (10%), lethargy (15%), and fatigue (15%); in the PG, they were dizziness (15%), lethargy (20%), nausea (5%) and fatigue (20%). As these adverse effects were mild, they dissipated without treatment within 1 wk. No obvious adverse effects were reported in the CG.

After 8 wk, 85% of patients in the MG, and 80% in the PG responded positively to treatment, which were significantly higher than that (55%) found in the CG; however, there was no significant difference in the results between the MG and PG.

DISCUSSION

FD is a common psychosomatic disease associated with a variety of mental disorders including anxiety, depression, panic attacks, and post-traumatic stress disorder, of which anxiety and depression are the most common. Negative spiritual, psychological and social factors can accelerate the onset of FD symptoms and exacerbate them and thereby ultimately affect treatment efficacy. However, at present, the impact of such factors on the incidence and progression of FD is not very clear; one intriguing possibility is that

they may work to change gastrointestinal motor or sensory function through the brain-gut axis. Weight loss is a common symptom of digestive diseases, and may indicate an organic disease[6], but recently, certain studies have found that patients with functional gastrointestinal diseases often showed weight loss[11,20].

Currently, there is no very effective treatment for depressive FD patients with weight loss, because of the chronic and recurrent characteristics of the disease. Antidepressants are often used to treat patients with depression. One of these, mirtazapine, a serotonin-norepinephrine reuptake inhibitor that is clinically used for the treatment of depression, acts rapidly with positive effects on sleep disorders, appetite loss, depressive symptoms, *etc*.

Herein, we analyzed the effects of mirtazapine on depressive FD patients with weight loss**.** Mirtazapine showed higher efficacy in relieving dyspeptic symptoms and lowering NDSI scores when compared to paroxetine and conventional treatment, and was equal to paroxetine in mitigating depressive symptoms. After 8 wk of treatment, 85% of MG patients were classified as treatment responsive, a proportion higher than 80% as observed in the PG, and significantly higher than 55% as seen in the CG. This may be related to specific aspects of mirtazapine action that not only may alleviate depression and improve the function of the nervous system, but also regulate gastrointestinal motor or sensory function.

In this study, we show that mirtazapine treatment of depressive FD patients with weight loss not only effectively treated symptoms of dyspepsia and depression, but also induced significant weight gain, an effect not observed with either paroxetine or conventional treatment. Specifically, 80% of the patients experienced weight gain after 4 wk of treatment with mirtazapine; furthermore, 95% of these patients continued to gain weight until the end of the treatment. The average weight gain was 3.58 ± 1.57 kg, resulting in significantly higher weight than the baseline weight recorded before treatment. In humans, weight is mainly composed of muscle volume, body fat, and inorganic salts, and muscle volume and body fat are the most affected. Through dynamic observation of the weight distribution of the various body components, we found that muscle volume stayed relatively constant throughout treatment, whereas body fat significantly changed. Body fat, which includes subcutaneous fat, visceral fat, muscle clearance fat, proved to be the main contributor to body weight gain. Further analysis of body fat distribution revealed that visceral fat showed a marked increase with mirtazapine treatment at 4 wk and 8 wk, which indicated that visceral fat was the key element responsible for the observed body weight gain.

Generally, significant imbalances of visceral fat are known to increase the incidence of cardiovascular disease, digestive disease, urinary disease, *etc*. In our study, although visceral fat did indeed increase after 8 wk of mirtazapine treatment, body fat ratios remained at normal levels. We speculate that most of the patients were at a low level of body weight and BMI before treatment, and even underweight according to BMI, whereas muscle volume remained at normal levels throughout; thus, the amount of body fat must have been seriously deficient before treatment. Moreover, through appetite growth and symptom relief, muscle volume may have gradually increased with treatment, whereas overall body fat may have grown at a slower rate.

In recent years, functional gastrointestinal disease has been closely associated with dysregulation of the brain-gut axis. The brain-gut axis, which is regulated by neuroendocrine and immune factors, is a bipolar system between the gastrointestinal tract and brain that is affected by psychosocial factors. The coordination between the central nervous system and gastrointestinal contractility is regulated through a variety of brain-gut peptides and gastrointestinal hormones. In this study, the levels of ghrelin, NPY, MTL, and GAS which may increase appetite, food intake or gastrointestinal dynamic promotion were significantly upregulated, whereas the levels of leptin, 5-HT and CCK which may decrease food intake, block gastrointestinal motility or increase gastrointestinal sensitivity were significantly downregulated.

In conclusion, antidepressant mirtazapine not only improved patients' conditions concerning indigestive and depressive symptoms, but also increased appetite and body weight (mainly the visceral fat in body fat), much more effectively than either paroxetine or conventional therapy. The clinical efficacy of mirtazapine may be mediated in part through the regulation of brain-gut or gastrointestinal hormones. To clarify these effects and the underlying mechanisms of mirtazapine action in FD patients with weight loss will require bigger sample sizes, and multi-center, randomized controlled trials in future studies.

COMMENTS

Background

Functional dyspepsia (FD) is a common psychosomatic disease associated with a variety of mental disorders, and weight loss was often found in FD patients. Such patients had lower body mass index, more frequent physician visits, higher psychological disorders, poorer appetite and lower quality of life. In recent years, many clinical trials indicated that antidepressant mirtazapine are effective in treating FD patients. Whereas some side effects may limit the general application of mirtazapine in antidepressant therapy, these may prove to be beneficial in treating FD patients with weight loss.

Research frontiers

This study comprehensively explored the effect of mirtazapine on depressive FD patients with weight loss by dynamic observation not only of the changes in dyspepsia and depressive symptoms but also the modifications of body weight and fat distribution and the level of serum hormones.

Innovations and breakthroughs

This study showed that antidepressant mirtazapine not only improved patients' conditions concerning indigestive and depressive symptoms, but also increased appetite and body weight, mainly the visceral fat in body fat, much more effectively than either paroxetine or conventional therapy. The clinical efficacy of mirtazapine may be mediated in part through the regulation of brain-gut or gastrointestinal hormones.

Applications

The findings can supply the evidence for the clinical application of mirtazapine in FD patients with weight loss.

Terminology

The Nepean Dyspepsia Index-symptom is a scale that evaluates the frequency, intensity, and practical impediments of 15 gastrointestinal symptoms.

Peer-review

This is a good and practical study in which the authors found that the beneficial effects and mechanism of action of antidepressant mirtazapine in FD patients with weight loss. It is believed that the findings can provide a new angle and evidence for the clinical application of mirtazapine in FD patients with weight loss.

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ORIGINAL ARTICLE

Observational Study

Inflammatory bowel disease: A descriptive study of 716 local Chilean patients

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Abstract

AIM: To demographically and clinically characterize inflammatory bowel disease (IBD) from the local registry and update data previously published by our group.

METHODS: A descriptive study of a cohort based on a registry of patients aged 15 years or older who were diagnosed with IBD and attended the IBD program at Clínica Las Condes in Santiago, Chile. The registry was created in April 2012 and includes patients registered up to October 2015. The information was anonymously downloaded in a monthly report, and the information on patients with more than one visit was updated. The registry includes demographic, clinical and disease characteristics, including the Montreal Classification, medical treatment, surgeries and hospitalizations for crisis. Data regarding infection with *Clostridium difficile*

(*C. difficile*) were incorporated in the registry in 2014. Data for patients who received consultations as second opinions and continued treatment at this institution were also analyzed.

RESULTS: The study included 716 patients with IBD: 508 patients (71%) were diagnosed with ulcerative colitis (UC), 196 patients (27%) were diagnosed with Crohn's disease (CD) and 12 patients (2%) were diagnosed with unclassifiable IBD. The UC/CD ratio was 2.6/1. The median age was 36 years (range 16-88), and 58% of the patients were female, with a median age at diagnosis of 29 years (range 5-76). In the past 15 years, a sustained increase in the number of patients diagnosed with IBD was observed, where 87% of the patients were diagnosed between the years 2001 and 2015. In the cohort examined in the present study, extensive colitis (50%) and colonic involvement (44%) predominated in the patients with UC and CD, respectively. In CD patients, non-stricturing/non-penetrating behavior was more frequent (80%), and perianal disease was observed in 28% of the patients. There were significant differences in treatment between UC and CD, with a higher use of corticosteroids, and immunosuppressive and biological therapies was observed in the patients with CD ($P < 0.05$ and $P < 0.01$). Significant surgical differences were also observed: 5% of the UC patients underwent surgery, whereas 38% of the CD patients required at least one surgery ($P < 0.01$). The patients with CD were hospitalized more often during their disease course than the patients with UC (55% and 35% of the patients, respectively; $P < 0.01$). *C. difficile* infection was acquired by 5% of the patients in each group at some point during the disease course. Nearly half of the patients consulted at the institution for a second opinion, and 32% of these individuals continued treatment at the institution.

CONCLUSION: IBD has continued to increase in the study cohort, slowly approaching the level reported in developed countries.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; South America; Latin America; Chile; Epidemiology

Core tip: Several studies have found that the incidence of inflammatory bowel disease (IBD) has increased over the past several decades, even in countries where the frequency was extremely low. Industrialization, increased physician awareness, advancements in diagnostic methods and better access to medical services are factors that might explain this increase. Although few epidemiological studies have been conducted in Latin America, these analyses have described an increased incidence of IBD. In the present study, we analyzed single-center data of 716 patients with IBD. We collected data from a considerable number of patients diagnosed with IBD, enabling the demographic and clinical characterization of these individuals.

Simian D, Fluxá D, Flores L, Lubascher J, Ibáñez P, Figueroa C, Kronberg U, Acuña R, Moreno M, Quera R. Inflammatory bowel disease: A descriptive study of 716 local Chilean patients. *World J Gastroenterol* 2016; 22(22): 5267-5275 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5267.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5267

INTRODUCTION

Inflammatory bowel disease (IBD) includes a spectrum of typically progressive chronic diseases, including Crohn's disease (CD), ulcerative colitis (UC) and unclassified colitis. Unclassified colitis occurs in patients who have clinical and endoscopic evidence of chronic IBD affecting the colon without small bowel involvement and no definitive histological or other evidence suggesting either CD or UC[1]. Although IBD mortality is low, the onset of this disease during early adulthood and its chronicity as a lifelong disease result in a significant decline in the quality of life of the patients and a heavy burden on the healthcare system due to high treatment costs[2]. Natural history studies have helped identify subsets of patients whose disease prognosis can be stratified according to clinical features. These data might improve the management of patients with IBD by defining changes in disease phenotype and risks of relapse, hospitalization and surgery[3].

Several studies have reported that the incidence of IBD has markedly increased over the latter part of the 20th century, whereas other studies have suggested a plateau or even a decline in IBD incidence in certain geographical areas[4,5]. However, an increase in these diseases has been described in countries where their frequency was very low[6-9]. IBD has been associated with the industrialization of nations[10,11], and thus, the increasing incidence of these diseases in developing countries might reflect this phenomenon. However, other factors, such as increased physician awareness, advancements in diagnostic methods and better access to medical services, such as colonoscopies, should be considered[12]. Although few epidemiological studies have been conducted in developing Latin American countries, these analyses have also described an increased incidence of IBD[13-19]. As we previously published, the incidence and prevalence of IBD in Chile are unknown; however, consistently with two other studies, our data suggest increases in the numbers of local cases of CD and UC[16,18,20]. The objective of this study was to demographically and clinically characterize IBD from a local registry and thereby update previously published data.

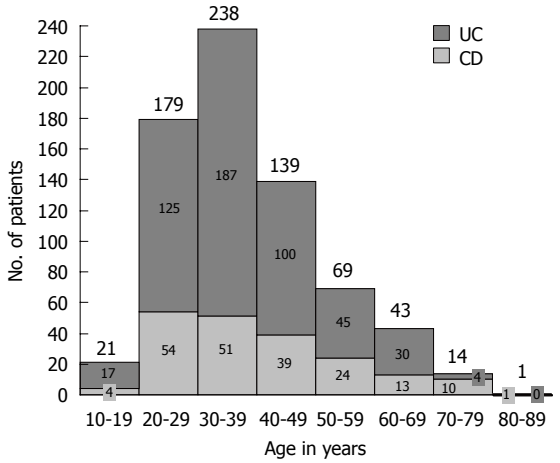

Figure 1 Frequency of patients with ulcerative colitis and Crohn´s disease at age of diagnosis. UC: Ulcerative colitis; CD: Crohn´s disease.

MATERIALS AND METHODS

This was a descriptive study of a cohort based on a registry of patients aged 15 years and older, diagnosed with IBD according to clinical, endoscopic, histological and radiologic findings and attending the IBD program at Clínica Las Condes in Santiago, Chile. The registry was created in April 2012 and includes patients who attended the program until October 2015. Retrospective data were obtained from those patients diagnosed prior to the indicated date. The registry is composed of online forms available in the electronic medical record of each patient that were completed by gastroenterologists and colorectal surgeons during clinic visits. On each subsequent visit, the information was prospectively updated as deemed necessary. The information was anonymously downloaded in a monthly report, and the information for patients with more than one total visit was updated. The registry includes demographic, clinical and disease characteristics, such as extension, location and behavior, changes in diagnosis from UC to CD, phenotype changes in CD, medical treatment, surgeries and hospitalizations for crisis. Data concerning *Clostridium difficile* (*C. difficile*) infection were incorporated into the registry in 2014. A polymerase-chain reaction assay for *C. difficile* detection was requested for patients presenting with moderate-to-severe activity. In CD, the Montreal Classification was used to define the phenotype as follows: B1, non-stricturing/non-penetrating; B2, stricturing; and B3, penetrating. A "p" was added to any of these classifications in case of perianal disease. The same classification was used to define the location of the disease: L1, ileum; L2, colon; L3, ileocolonic; and L4, concomitant upper gastrointestinal involvement. The extension of UC was defined according to the Montreal Classification: E1, ulcerative proctitis; E2, left-sided UC (distal UC); and E3, extensive UC (pancolitis)[1]. However, because Clínica Las Condes is a tertiary center that receives patients from locations throughout the country, the data for patients who received consultations as a second opinion and continued treatment at this institution were also analyzed. Patients with two or more visits over the next year were considered patients who were continuing treatment with the IBD program at this institution. This study was approved through the Institutional Ethics Committee.

Statistical analysis

The data were analyzed using the R Commander program. Continuous variables did not have a normal distribution and were described based on medians and ranges and compared using the Mann Whitney rank test for independent groups. Qualitative categorical variables were described with absolute frequency and percentage, and we used the χ^2 test for comparative statistical analysis. When the sample was less than 20, Fisher's exact test was used. Differences with a *P* value less than 0.05 were considered to be statistically significant. A biomedical statistician conducted a statistical review of the present study.

RESULTS

The study included 716 patients with IBD: 508 patients (71%) were diagnosed with UC, 196 patients (27%) were diagnosed with CD, and 12 patients (2%) were diagnosed with unclassifiable IBD. The UC/CD ratio was 2.6/1. The median age was 36 years (range 16-88), and 58% of the patients were female, with a median age at diagnosis of 29 years (range 5-76). Most patients with UC and CD were diagnosed between the ages of 20 and 29 years (Figure 1), without differences in gender. However, 22 patients (3%) were diagnosed when over 60 years of age. In the past 15 years, a sustained increase in the number of patients diagnosed with IBD has been observed, and significant increases were obtained from the comparison of the periods 1971-1985, 1986-2000 and 2001-2015, with 87% of patients diagnosed in the last period. The frequency of patients with IBD distributed according to the year of diagnosis is shown in Figure 2, illustrating an increase in the diagnosis of new cases of UC and CD over time. The demographic and disease characteristics of patients are shown in Table 1. In both UC and CD patients, articular symptoms were the most frequent extraintestinal manifestations. Primary sclerosing cholangitis (PSC) was diagnosed in eight patients (2%) with UC and two patients (1%) with CD.

Extent, location and behavior of IBD

Regarding the extent of UC, 50% of patients had extensive colitis. In CD, 44% of patients had colonic involvement, and only 3% of patients presented with concomitant upper disease. One patient in the registry presented with isolated perianal disease, and CD was confirmed through biopsies of the fistula,

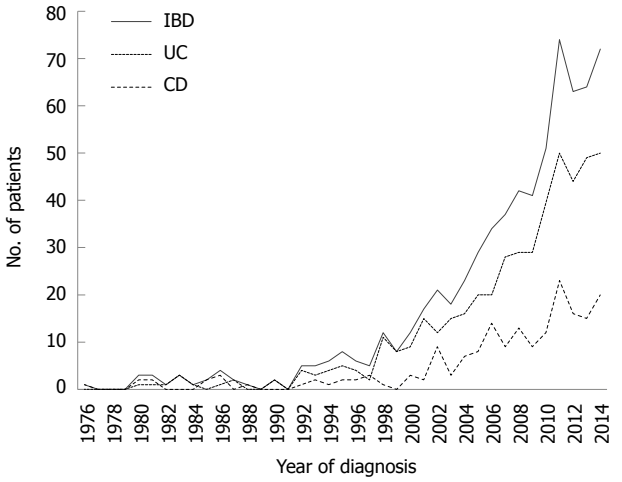

Figure 2 Frequency of patients with inflammatory bowel disease distributed by year of diagnosis. IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn´s disease.

Table 1 Demographic and disease characteristics *n* (%)

	UC (*n* = 508)	CD (*n* = 196)
Smoking habit		
Active	47 (9)	31 (16)
Discontinued	75 (15)	34 (17)
Family history of IBD	59 (12)	19 (10)
Extraintestinal manifestations		
Articular	156 (31)	87 (44)
Dermatological	11 (2)	10 (5)
Ocular	8 (2)	3 (2)
Other	31 (6)	15 (8)

UC: Ulcerative colitis; CD: Crohn´s disease.

which demonstrated granulomas. Non-stricturing/non-penetrating behavior was predominant in CD (80%). Stricturing and penetrating behavior was observed in 10% and 9% of patients, respectively. Perianal disease was observed in 28% of the patients with CD (Table 2). During the course of IBD, the diagnosis of 19 patients changed: one patient with unclassifiable IBD was newly diagnosed with CD, and 18 UC patients were newly diagnosed with CD. In six patients, CD was posteriorly diagnosed because these individuals developed perianal fistulas. In addition, 16 patients with CD showed modified behavior, nine of these patients showed changes from non-stricturing/non-penetrating to stricturing disease and seven of them showed changes from non-stricturing/non-penetrating to penetrating disease. In addition, two patients who are included in the 16 patients mentioned above developed perianal disease. Changes in disease extension were observed in 36 UC patients. Specifically, 12 of these 36 patients presented disease extension from proctitis to left colitis, and the remaining 24 patients exhibited disease extension from proctitis or left colitis to extensive colitis.

Medical treatment for IBD

Significant differences in treatment were found between

Table 2 Extent, location and behavior of inflammatory bowel disease according to the montreal classification *n* (%)

	UC (*n* = 508)	CD (*n* = 196)
UC Extent		
E1: Ulcerative proctitis	142 (28)	
E2: Left sided UC	112 (22)	
E3: Extensive UC (pancolitis)	254 (50)	
CD Location		
L1: Ileal		53 (27)
L2: Colonic		87 (44)
L3: Ileocolonic		55 (28)
L4: Upper gastrointestinal		5 (3)
CD Behavior		
B1: Non-stricturing/non-penetrating		157 (80)
B2: Stricturing		20 (10)
B3: Penetrating		18 (9)
p: Perianal disease		55 (28)

UC: Ulcerative colitis; CD: Crohn´s disease.

Table 3 Medical treatment, hospitalizations and surgery in patients with inflammatory bowel disease *n* (%)

	UC (*n* = 508)	CD (*n* = 196)	*P* value
Corticosteroids	297 (58)	133 (68)	< 0.05
Mesalazine (oral, local or both)	497 (98)	133 (68)	< 0.01
Immunosupressive agents	166 (33)	132 (67)	< 0.01
Ciclosporine	7 (1)	2 (1)	
Biologic therapy	34 (7)	67 (34)	< 0.01
Surgery	27 (5)	75 (38)	< 0.01
Intestinal resection	27 (5)	50 (25)	< 0.01
Hospitalizations	176 (35)	108 (55)	< 0.01
1	113 (64)	50 (46.3)	< 0.01
2-3	45 (26)	35 (32.3)	
⩾ 4	18 (10)	23 (21.3)	< 0.01[1]

[1]Fisher´s Test. UC: Ulcerative colitis; CD: Crohn´s disease.

UC patients and CD patients (Table 3). Mesalamine was used to treat 98% of UC patients and 68% of CD patients. Patients with CD received corticosteroids, mesalamine and immunosuppressive agents at equal frequency. A comparison of both groups revealed that the use of corticosteroids and immunosuppressive and biological therapies was significantly higher in patients with CD. A total of 102 patients (14%) were treated with biological therapy; specifically, 83 patients received infliximab, 13 patients received adalimumab, one patient received certolizumab pegol, one patient received golimumab and four patients received natalizumab. Biological therapy was initiated one year (median) after diagnosis for patients diagnosed since 2010 (39 patients) because biological therapy has become more accessible since then.

Surgery in IBD

A comparison revealed that more CD than UC patients required surgery. Specifically, only 27 UC patients (5%) underwent surgery, whereas 75 CD patients (38%) underwent surgery ($P < 0.01$) (Table 3). Fifty of the patients diagnosed with CD (25%) underwent intestinal resection, and six of these patients (3%)

required surgery for posteriorly based perianal disease. In addition, 24 CD patients (12%) underwent surgery due only to perianal disease, and one patient underwent a loop ileostomy.

Hospitalization and C. difficile infection

CD patients were hospitalized more often during the disease course than UC patients (55% and 35% of patients, respectively; $P < 0.01$). In addition, 21.3% of the CD patients had more than four hospitalizations compared with 10% of the UC patients ($P < 0.01$). The median number of hospitalizations for both was 2, with ranges from 1 to 24 for UC and 1 to 40 for CD (Table 3).

As previously described, data concerning *C. difficile* infection were incorporated into the registry in 2014, and a total of 490 patients (344 UC patients and 141 CD patients) were analyzed. No differences in the prevalence of infection was found between the groups, and 5% of the patients in each group acquired *C. difficile* infection at some point during the disease course.

Second opinion

The data obtained from the registry showed that 328 patients (46%) received consultations at the institution for a second opinion. Most of these patients (72%) were diagnosed with UC, and 107 of the 328 patients (32%) continued treatment at this center. However, 130 patients were not analyzed because these individuals received consultations late in 2015 and had therefore not completed more than one year of treatment since the first consultation at this institution. In addition, many patients live in other cities and receive consultations only for complex situations.

DISCUSSION

The number of patients in the registry more than doubled compared with the number detailed in our previous publication[20]. This increase not only allowed us to better characterize these patients but also facilitated a comparison with studies conducted worldwide. This population increase reflects not only the recognition of this institute as a referral center but also the increased rate of IBD diagnoses in recent years.

The cohort UC/CD ratio in 2014 was 2.9/1, and the current ratio has increased 2.6/1, showing a slow approaching to the ratio reported in developed countries (1/1)[6,13,21,22]. However, genetic, environmental and geographic factors may influence this difference. A gender-based analysis showed that the percentages of UC and CD were slightly higher in women, which is consistent with the results obtained by other series[13,14,17]. The median age at diagnosis was 29 years, and 64% of patients were diagnosed between 20 and 39 years of age. No second peak was observed at an older age, which is consistent with the results from recent studies[6,7,23-25].

An active smoking habit was almost twice as frequent in patients with CD (16%) compared with patients with UC (9%), regardless of whether the patients with CD received counseling and were told of the particular deleterious effect of smoking. This finding demonstrates that smoking education is important and that smoking cessation should be emphasized on every visit. Nonetheless, the frequencies of patients with active smoking habits observed in the present study were low compared with the frequency of active smokers in the general population. The last National Health Survey has demonstrated that 40.6% of the adult population smokes regularly, indicating that cigarette smoking is an important health problem in Chile and that other factors, such as environmental factors, may be influencing the increase in the diagnosis of CD[26].

A family history was obtained for 12% and 10% of the patients diagnosed with UC and CD, respectively, similarly to the results reported by Moller *et al*[27], who published a much larger study. The analysis of the clinical characteristics revealed that the most frequent extra-intestinal manifestation was articular symptoms, with frequencies of 31% and 44% in UC and CD patients, respectively. In previous studies, musculoskeletal manifestations were described as the most common extra-intestinal manifestation, and UC patients are more affected than CD patients. However, the percentage of affected patients was lower than that observed in the present study (20%-30%)[28]. It has been suggested that the risk of developing peripheral arthritis increases with an increase in the extent of IBD activity[29,30], and 70% of patients diagnosed with CD who showed musculoskeletal manifestations had either colonic or ileocolonic involvement, whereas 55% of the patients diagnosed with UC who showed musculoskeletal manifestations had pancolitis. This observation reflects the recognition of this institution as a tertiary referral center that receives complex patients. In addition, the etiology of peripheral arthritis in IBD might reflect a combination of genetic predisposition and exposition to the luminal bacterial bowel contents[30].

The analysis of the extent of UC showed that half of the patients had extensive colitis, different from the frequency described in previous studies, which reported that distal location predominates[9] and that extensive colitis varies between 20 and 40%[7,9,19,22,24,31]. Even a previous study published in Chile, which involved two different institutions, reported 38% and 15% extensive colitis; notably, the frequency of 15% was previously reported at our institution 10 years ago[18], before this institution was recognized as a referral center and before its association with an IBD program. The higher percentage of extensive colitis observed in the population examined in the present study might reflect the fact that it was conducted at a tertiary referral

center and included refractory cases that were difficult to treat.

Colonic involvement was more frequent in the CD patients examined in the present cohort, with a frequency of 44%. This finding is consistent with the results of a study conducted in Brazil, which found that colonic involvement predominates, although at a lower frequency (36%)[19]. Non-structuring/non-penetrating behavior was more frequently observed in the cohort examined in the present study, with a frequency of 80%. This value is consistent with the frequency described in previous studies, which showed that inflammatory phenotypes predominate during the first years of the disease[24]. During the disease course, approximately 10% of patients with non-stricturing/non-penetrating behavior exhibited a modification of this characteristic to a more aggressive behavior. However, other studies have reported that 31% to 60% of patients exhibit a disease progression to a more severe behavior[32-34]. Indeed, after 40 years, most patients experience complications and are classified as having a penetrating, or less often, a stricturing disease[16]. These differences might reflect the short follow-up period used in the present study. Additionally, it has been reported that colonic disease remains uncomplicated or inflammatory for many years[24], so predominant colonic involvement found in our study might play a role in this progression. Another factor potentially explaining this difference is that the patients at this institution were aggressively treated upon diagnosis with an "accelerated step-up approach", involving the initiation of biological therapy a median of one year after diagnosis for patients diagnosed since 2010; thus, patients might have a lower probability to exhibit a change in behavior[35,36].

During the IBD course, 12 patients (2.3%) with an initial diagnosis of UC developed perianal fistulas or showed ileal involvement, changing their diagnosis to CD, as confirmed through histological and image analyses. Previous studies have described a 5%-10% change in diagnosis after 25 years of the disease course[37]. This finding might reflect the short follow-up period in the present study.

The analysis of IBD treatment revealed that mesalamine was the most used drug in UC treatment (98%), whereas corticosteroids, mesalamine and immunosuppressive agents were used at equal frequencies (67%-68%) in CD treatment. In the present study, despite the frequent use of mesalamine for patients with CD, the use of this agent in CD is controversial. Indeed, the European Crohn's and Colitis Organization Consensus recently stated that oral aminosalicylates are not recommended for the treatment of mild to moderate CD[38]. However, both the American and British National Gastroenterology Associations recommend the use of high-dose 5-aminosalicylic acid as the first-line treatment of mild ileal, ileocolonic or colonic CD[38]. Because we had a high percentage of patients with colonic involvement, treatment with mesalamine could have had some implications because the action of mesalamine is predominantly topical at the site of inflammation, particularly within the colon[38]. However, many of these patients received mesalamine, regardless of the severity of the disease, prior to evaluation at this institution, possibly resulting from misinformation regarding the role of mesalamine in CD. On the other hand, according to the latest clinical guidelines, the use of immunosuppressive and biological therapies is significantly higher in CD patients compared with UC patients, as observed in the present study. In the series examined in the present study, the use of infliximab in UC treatment (7%) was similar to that reported in other countries[31,39,40]; however, the use of this drug in CD treatment (34%) was considerably higher than that detailed in Saudi Arabia, Israel and some European countries, which report frequencies between 2% and 10%[31,40]. Similarly, the use of immunomodulators was considerably higher in both groups compared with that observed in some European countries[31]. This discrepancy reflects the type of center where the present study was conducted, *i.e.*, a tertiary center that treats patients with more complex diseases. Unfortunately, the use of adalimumab and certolizumab pegol was extremely low in the cohort examined in the present study, reflecting the low coverage of these therapies by insurance companies. In addition, vedolizumab is still not available for use in Chile.

The investigation of the use of surgery for IBD treatment revealed that 38% of the CD patients required surgery for either intestinal resection or perianal disease. The frequency of intestinal resection was significantly higher in CD patients (25%) compared with that of colectomy in UC patients (5%), a result consistent with the findings reported by Niewiadomski *et al*[41], who showed that the risk of intestinal resection in CD was 13% after one year and 26% after five years. However, the colectomy rates in UC obtained in this study were 2% and 13% after one and five years, respectively. Notably, the early use of immunomodulators and biological therapies during the disease course could reduce the risk of surgery[24,42], particularly for those patients who achieve mucosal healing[43].

Relatively few data are available regarding the hospitalization rates in population-based cohorts[44,45]. The CD patients in the cohort examined in the present study had significantly more hospitalizations than the UC patients; however, higher percentages of patients belonging to both groups in this cohort were affected compared with the frequencies obtained in previous studies[41,46]. Nevertheless, it has previously been reported that more than one-third of UC patients require hospitalization within one year after diagnosis in the biological era[44]. Among the UC patients examined

in the present study, 35% required hospitalization at some point during the disease course. The disease extent at diagnosis and the need for steroids and anti-TNF therapy were associated with the risk of UC-related hospitalization[44]. For CD, a 52.7% cumulative risk of hospitalization within ten years of diagnosis has previously been described[45]. Among the CD patients in the present study, 55% required hospitalization at some point during the disease course.

The analysis of our data concerning IBD and *C. difficile* infection demonstrated that this bacterium was equally prevalent in patients with UC and patients with CD (5%). Notably, it is difficult to clinically distinguish between *C. difficile* infections and IBD flare-ups because both pathologies have similar presentations, *i.e.*, diarrhea and abdominal pain. Indeed, C. *difficile* might mimic or even trigger an IBD flare-up, and screening is therefore recommended at every flare-up experienced by these patients[47]. Because IBD patients with concomitant *C. difficile* infections have been associated with longer hospital stays, colectomy and even higher mortality, the diagnosis of this bacterial infection is important[48]. The data obtained in the present study differ from those detailed in previous publications, which reported that UC patients exhibit increased susceptibility to *C. difficile* compared with CD patients[48-50]; however, previous studies have reported that one of the major risk factors for *C. difficile* infection in patients with IBD is colonic IBD[51], and 44% of the patients with CD in the present study showed colonic involvement. Importantly, only patients with moderate-to-severe activity were examined for *C. difficile* infections, and hence, these data might be underestimated because patients with mild activity were not examined.

In conclusion, IBD has continued to increase in the present cohort, slowly approaching the levels reported in developed countries. The association of this institution with a multidisciplinary IBD program has improved the characterization of these patients and had therefore improved management options.

Limitations

The present study was conducted in a private single tertiary center, which may have resulted in bias because many of the patients received consultations for second opinions. Some of these individuals were inadequately treated, whereas others are refractory to therapy; therefore, these patients could represent more complex cases. In addition, more drugs are available for treatment in our center compared with those available at the public hospitals in Chile. In addition, the presented findings were obtained retrospectively, implying a selection bias. Nevertheless, we collected data from a considerable number of patients diagnosed with IBD, enabling a demographic and clinical characterization of these individuals. Unfortunately, in the present study, we were unable to determine incidence or prevalence rates because we did not receive patients from a determinate geographic area.

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COMMENTS

Background

Several studies have reported that the incidence of inflammatory bowel disease (IBD) has increased over the past several decades, even in countries where the frequency of this disease is low. Industrialization, increased physician awareness, advancements in diagnostic methods and greater access to medical services are factors that might explain this rise.

Research frontiers

Although few epidemiological studies have been conducted in Latin America, these studies have also described an increased incidence of IBD. The incidence and prevalence of IBD in Chile are unknown; however, increases in the numbers of Crohn's disease (CD) and ulcerative colitis (UC) cases have been suggested. The research goal of this study was to actualize previously published data to better demographically and clinically characterize IBD in patients from Chile.

Innovations and breakthroughs

The present study represents the largest series of IBD patients reported in Chile and even in South America. These data demonstrated an increase in the number of IBD cases.

Applications

The data used in this study not only enable the characterization of patients locally but also facilitate the comparison of these individuals with those included in other studies conducted worldwide. The characterization of these patients enabled treatment optimization, thereby improving patient quality of life.

Terminology

IBD includes a spectrum of typically progressive chronic diseases, including CD, UC and unclassified colitis. Although IBD mortality is low, the onset of this disease during early adulthood and its chronicity as a lifelong disease lead to a significant decline in the quality of life of IBD patients and an increase in the burden on the healthcare system due to high treatment costs.

Peer-review

The paper is good written, an intersting paper regarding IBD in developing country with different results about disease distrubution, severity and treatment.

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ORIGINAL ARTICLE

Observational Study

Primary biliary cirrhosis degree assessment by acoustic radiation force impulse imaging and hepatic fibrosis indicators

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Author contributions: Zhang FC and Hu RF designed the research; Zhu T, Tong L, Zhang QQ, and Zhang HC performed the research; Hu RF contributed new reagents/analytic tools; Zhang HC and Zhu T analyzed data; and Tong L and Zhang QQ wrote the paper.

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Abstract

AIM: To evaluate the assessment of primary biliary cirrhosis degree by acoustic radiation force impulse imaging (ARFI) and hepatic fibrosis indicators.

METHODS: One hundred and twenty patients who developed liver cirrhosis secondary to primary biliary cirrhosis were selected as the observation group, with the degree of patient liver cirrhosis graded by Child-Pugh (CP) score. Sixty healthy individuals were selected as the control group. The four indicators of hepatic fibrosis were detected in all research objects, including hyaluronic acid (HA), laminin (LN), type Ⅲ collagen (PC Ⅲ), and type Ⅳ collagen (Ⅳ-C). The liver parenchyma hardness value (LS) was then measured by ARFI technique. LS and the four indicators of liver fibrosis (HA, LN, PC Ⅲ, and Ⅳ-C) were observed in different grade CP scores. The diagnostic value of LS and the four indicators of liver fibrosis in determining liver cirrhosis degree with PBC, whether used alone or in combination, were analyzed by receiver operating characteristic (ROC) curve.

RESULTS: LS and the four indicators of liver fibrosis within the three classes (A, B, and C) of CP scores in the observation group were higher than in the control

group, with C class > B class > A class; the differences were statistically significant (P < 0.01). Although AUC values of LS within the three classes of CP scores were higher than in the four indicators of liver fibrosis, sensitivity and specificity were unstable. The ROC curves of LS combined with the four indicators of liver fibrosis revealed that: AUC and sensitivity in all indicators combined in the A class of CP score were higher than in LS alone, albeit with slightly decreased specificity; AUC and specificity in all indicators combined in the B class of CP score were higher than in LS alone, with unchanged sensitivity; AUC values (0.967), sensitivity (97.4%), and specificity (90%) of all indicators combined in the C class of CP score were higher than in LS alone (0.936, 92.1%, 83.3%).

CONCLUSION: The diagnostic value of PBC cirrhosis degree in liver cirrhosis degree assessment by ARFI combined with the four indicators of serum liver fibrosis is of satisfactory effectiveness and has important clinical application value.

Key words: Acoustic radiation force imaging technology; Hepatic fibrosis index; Primary biliary cirrhosis; Diagnostic value

Core tip: One hundred and twenty patients who had developed liver cirrhosis from primary biliary cirrhosis were assessed by ARFI imaging and hepatic fibrosis index alongside sixty healthy individuals. The ROC curves of LS combined with four liver fibrosis indexes showed that the AUC values (0.967), sensitivity (97.4%), and specificity (90%) of all indexes combined in the C grade of CP score were higher than in those of LS alone (0.936, 92.1%, and 83.3%). The diagnostic value of PBC cirrhosis degree in liver cirrhosis degree assessment by ARFI combined with the four indicators of serum liver fibrosis is of satisfactory effectiveness and has important clinical application value.

Zhang HC, Hu RF, Zhu T, Tong L, Zhang QQ. Primary biliary cirrhosis degree assessment by acoustic radiation force impulse imaging and hepatic fibrosis indicators. *World J Gastroenterol* 2016; 22(22): 5276-5284 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5276.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5276

INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic disease[1] that can develop into liver fibrosis, cirrhosis[2-4], and even lead to liver failure[5]. When a patient is already in the liver cirrhosis stage, accurate diagnosis, and assessment of the extent of liver cirrhosis is vital to the diagnosis, treatment, and prognosis of the disease[6]. Therefore, exploring a high value examination method to diagnose liver cirrhosis is very significant[7-9]. It has been reported that liver cirrhosis can be divided into three classes according to the Child-Pugh (CP) scoring criteria, and the accuracy of their assessment methods have been demonstrated[10-13]. Although liver biopsy is still currently the preferred diagnostic method for cirrhosis, the resulting trauma to the patient's body leads to low acceptance[14-17]. Serum fibrosis indicators are a non-invasive examination method of cirrhosis diagnosis with a wide range of applications[18], however its accuracy in the assessment of cirrhosis degree remains to be studied[19]. Acoustic radiation force impulse imaging (ARFI) is a new ultrasound elastography technique[20] that can detect the hardness of the liver parenchyma for liver disease accurate assessment, and is non-invasive, simple, repeatable[21-23], and it can effectively compensate for the lack of liver biopsy and serum liver fibrosis markers. ARFI technology in China remains at the clinical development phase[24-26]. However, comparative studies of ARFI technology and other methods to assess the degree of liver cirrhosis and joint applications are few[27-30]. This study intends to use the CP score as a grading standard, as well as to observe the comparison of ARFI technology measured serum fibrosis markers alone and in combination with diagnostic accuracy to find a more satisfactory diagnostic method for PBC, with the aim of providing a theoretical basis for the clinical diagnosis and treatment of liver cirrhosis.

MATERIALS AND METHODS

General information

From January 2014 to September 2015, 120 patients with primary cholestatic cirrhosis that had developed to the stage of cirrhosis and were admitted to Huashan Hospital (Baoshan Branch Affiliated to Fudan University, Shanghai, China) were selected as the observation group. The patients consisted of 35 males and 85 females, with an average age of 56.33 ± 7.42 years. Patients were divided into different groups according to Child-Pugh score as follows: grade A, 39 cases; grade B, 43 cases; and grade C, 38 cases. Meanwhile, 60 healthy subjects were chosen as the control group, and consisted of 24 males and 36 females, with an average age of 54.27 ± 8.31 years. General information on the differences between these two groups was not statistically significant (P > 0.05). This study was approved by the ethics committee.

Diagnostic criteria

The degree of liver cirrhosis in patients was diagnosed based on symptoms, signs, CT, MRI, biochemical examination, and liver biopsy results.

Table 1 Child-Pugh scoring criteria

Indicator	Score		
	1 point	2 point	3 point
Hepatic encephalopathy (grade)	None	Slight	Occasional drowsiness
Ascites	None	Small amount of diuretics can be controlled	Numerous
Total bilirubin (μmol/L)	< 34	34-51	> 51
Albumin (g/L)	> 35	28-35	< 28
Prolonged prothrombin time(s)	< 4	4-6	> 6

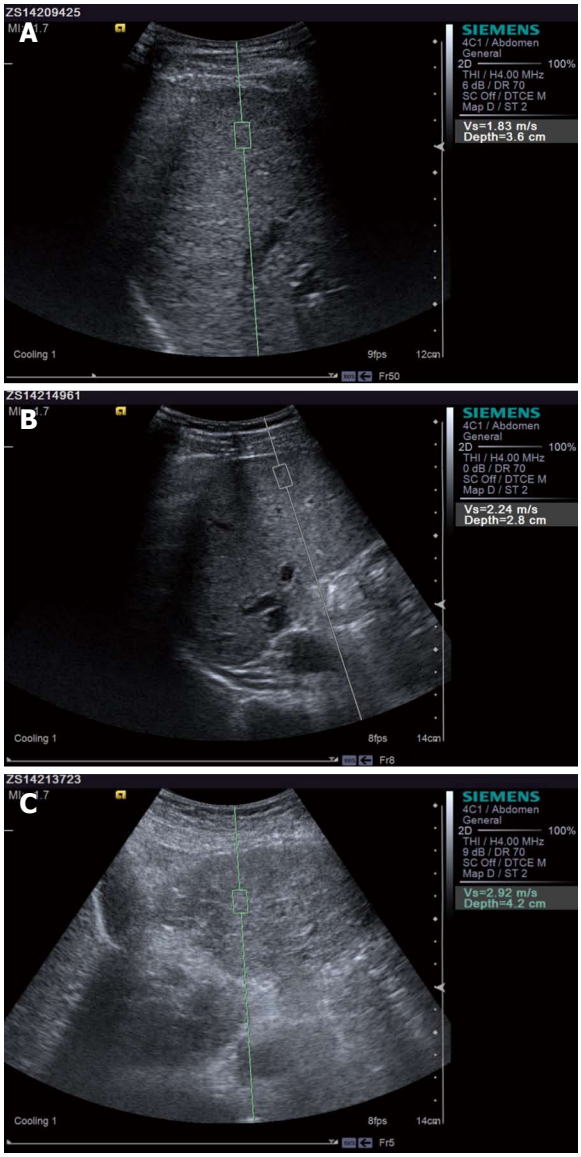

Figure 1 Observation group CP scores in the three classes. A: CP score patients with cirrhosis (Vs = 1.83 m/s); B: CP score patients with cirrhosis (Vs = 2.24 m/s); C: CP score patients with cirrhosis (Vs = 2.92 m/s).

Inclusion criteria

(1) Diagnosed with PBC that has developed to liver cirrhosis; (2) healthy subjects with no hepatobiliary diseases; (3) independent and able to cooperate with the test; and (4) provided written informed consent.

Exclusion criteria

(1) Patients with liver cancer or heart, lung, or other vital organs diseases; (2) disturbance of consciousness or mental illness; and (3) patients who provided written informed consent, but failed to cooperate with the test.

Child-Pugh scoring criteria

Patients were scored according to hepatic encephalopathy, peritoneal effusion, total bilirubin and albumin content, prolonged prothrombin time, and other conditions. Child-Pugh classification criteria (Table 1): class A, 5-6 points; class B, 7-9 points; and class C, ≥ 10 points.

Research methods

Liver fibrosis index detection: (1) After fasting, 5 ml of morning blood samples were collected from patients and kept at room temperature for approximately 30 min; (2) serum was separated and stored at -70 ℃; and (3) four indexes of liver fibrosis were determined using fluorescence immunoassay: hyaluronic acid (HA), laminin (LN), procollagen Ⅲ (PC Ⅲ), and collagen Ⅳ (Ⅳ-C).

ARFI detection: Siemens ACUSON S2000 color ultrasound diagnostic apparatus was used to conduct ARFI detection. (1) After fasting, the patient was placed on the left lateral position with the right hand on the head, and the right lobe of the liver tissue was detected; (2) elastic sampling frame was perpendicular to the surface of the liver, with a depth of approximately 2-5 cm while avoiding the surrounding blood vessels, and the patient was asked to hold their breath; and (3) the update button was pressed, a high-strength low-frequency pulse was launched, and the transverse shear wave velocity (Vs) was received. Units were in m/s and the value was recorded. Measurements were repeated 10 times and Vs were averaged to determine liver parenchyma hardness LS value.

Statistical analysis

SPSS 17.0 statistical software was used for all data results. LS value and the four indicators of liver fibrosis were measurement data presented as mean ± SD, with groups compared using two independent samples *t*-test. To evaluate the diagnostic value of LS value and the four serum indicators for liver fibrosis detected by ARFI (HA, LN, PCⅢ, and Ⅳ-C) for PBC, receiver operating characteristic (ROC) curve analysis with the area under the ROC curve (AUC), sensitivity and specificity representations were used. $P < 0.05$ was

Table 2 Test results of two groups of indicators (mean ± SD)

Item	Control group (n = 60)	Observation group		
		A class (n = 39)	B class (n = 43)	C class (n = 38)
LS value (m/s)	1.03 ± 0.03	1.90 ± 0.07[a]	2.31 ± 0.02[a]	2.92 ± 0.17[a]
HA (ng/mL)	54.96 ± 21.13	431.01 ± 118.04[a]	619.03 ± 164.28[a]	857.13 ± 192.05[a]
LN (ng/mL)	79.11 ± 15.37	116.14 ± 18.77[a]	153.42 ± 36.25[a]	211.09 ± 30.18[a]
PCⅢ (ng/mL)	89.91 ± 18.76	142.51 ± 30.07[a]	227.93 ± 69.11[a]	367.39 ± 99.21[a]
Ⅳ-C (ng/mL)	51.32 ± 9.27	104.58 ± 42.17[a]	168.99 ± 32.14[a]	193.36 ± 30.22[a]

[a]P < 0.01 *vs* the control group.

Table 3 Receiver operating characteristic curves results of different CP score classifications of liver cirrhosis with different indicators of diagnosis

Item	A Class			B Class			C Class		
	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity
LS value	0.852	57.9%	93.3%	0.911	97.4%	75.0%	0.936	92.1%	83.3%
HA	0.694	97.4%	55.0%	0.852	97.4%	65.0%	0.888	63.2%	96.7%
LN	0.707	97.4%	43.3%	0.746	46.2%	96.7%	0.828	97.4%	58.3%
PCIII	0.741	57.9%	86.7%	0.823	53.8%	96.7%	0.871	86.8%	75.0%
IV-C	0.688	78.9%	56.7%	0.785	97.4%	51.7%	0.889	94.7%	73.3%

considered statistically significant.

RESULTS

Test result indicators in the two groups

CP scores, LS values, and the four serum indicators for liver fibrosis in the three classes (A, B, and C) of patients in the observation group were significantly higher than controls; the difference was statistically significant (P < 0.01). In the observation group, CP scores in the three classes of patients, LS values (Figure 1), and the four serum indicators for liver fibrosis revealed that class C > class B > class A; differences were statistically significant (P < 0.01), as shown in Table 2.

ROC curve analysis of LS value and the four indicators of serum liver fibrosis in the observation group

ROC curve analysis of LS values and the four diagnostic indicators of liver fibrosis of CP rates in different cirrhosis grades and each index of the AUC showed: grade C > grade B > grade A, as well as that the sensitivity and specificity were different (Table 3). Comparison of results of CP levels of LS values and the four indicators of liver fibrosis in the ROC curve are as follows:

In CP score grade A, LS values in the AUC and the specificity were high compared with serum liver fibrosis, albeit with lower sensitivity (Figure 2A).

In grade B, the AUC value of LS and specificity were high compared with HA and Ⅳ-C, but with lower sensitivity; AUC and sensitivity were high compared with LN and PCⅢ, but with lower specificity (Figure 2B).

In grade C, AUC values of LS, sensitivity, and specificity were high compared with PCⅢ; AUC and sensitivity were high compared with HA, but with lower specificity; AUC and specificity were high compared with LN and Ⅳ-C, but with lower sensitivity (Figure 2C).

ROC curve analysis of LS value in the observation group combined with the four indicators of serum liver fibrosis

LS values detected by ARFI in the observation group combined with the four indicators of serum liver fibrosis in the ROC curve show the following (Table 4): in each indicator of CP score grade A, the AUC and sensitivity were higher than the LS value detected by ARFI alone, although its specificity decreased slightly (Figure 3A); in CP score grade B, the AUC and sensitivity were higher than LS detected by ARFI alone, with sensitivity being constant (Figure 3B); in CP score grade C, the AUC, sensitivity, and specificity were higher than the LS values detected by ARFI alone (Figure 3C).

DISCUSSION

Cholestatic liver cirrhosis is a chronic liver disease with a long and gradual progression to liver cirrhosis[31-34]. An accurate assessment of early liver cirrhosis can effectively prevent further liver damage that can result in liver failure[35-37]; this has great significance for the diagnosis, treatment, and prognosis of chronic liver disease[38-40]. In this study, by comparing the diagnostic values of AFRI detected LS values and the four indicators (HA, LN, PCⅢ, and Ⅳ-C) of serum liver fibrosis alone or in combination, we aimed to accurately and effectively explore this examination

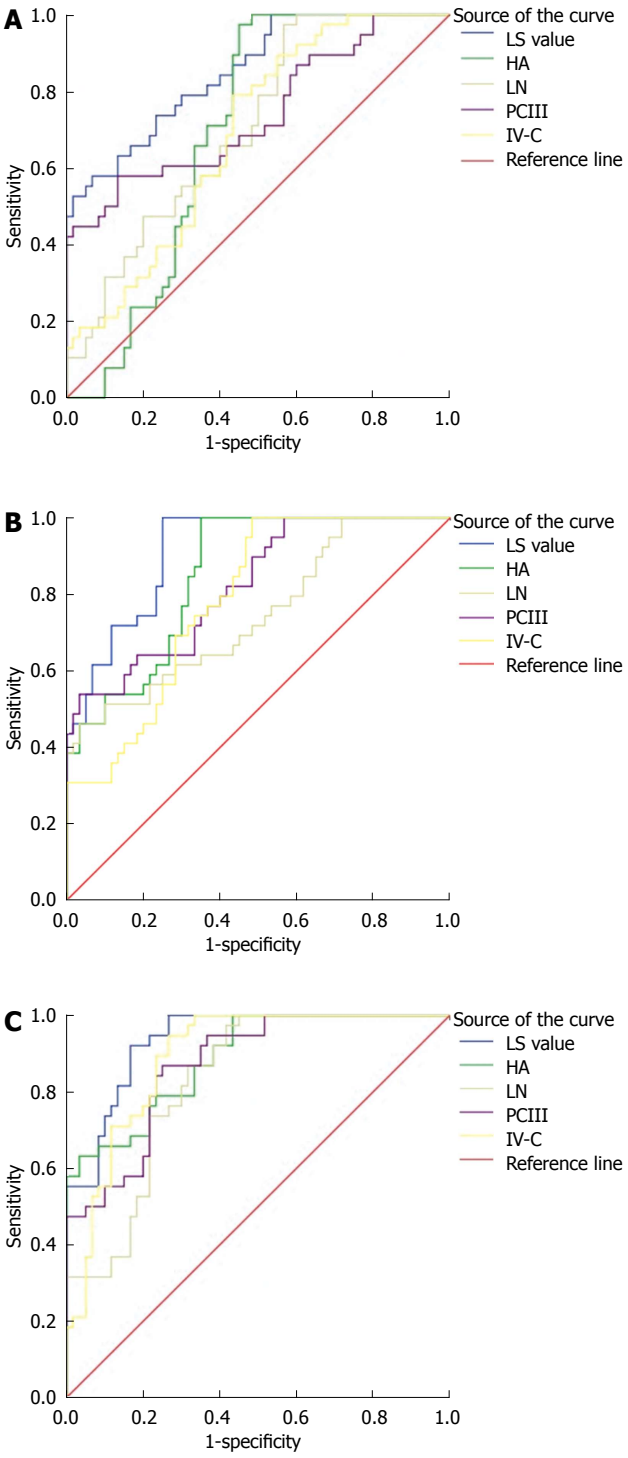

Figure 2 Comparison of the results of CP levels of LS values and the four indicators of liver fibrosis in the ROC curve. A: CP score for each indicator; B: CP score of the various indicators; C: CP score of the various indicators.

method for the assessment of cirrhosis degree.

LS values and the four indicators of serum liver fibrosis in observation and control groups

Liver stiffness increases as chronic liver disease develops to liver fibrosis and cirrhosis. In this study, the LS value results and four indicators of serum liver fibrosis in the observation group showed class C > class B > class A trends; the level of indicators were significantly higher. LS values and the four indicators of serum liver fibrosis of cirrhotic patients were higher than in the control group; this increased as liver cirrhosis degree increased. This also proves that ARFI-detected LS values and the four indicators of serum liver fibrosis can reflect changes in the degree of cirrhosis. Studies have reported[41] that AFRI-detected LS values increased as the degree of hepatic fibrosis increased; this can be widely used in patients with chronic liver disease. In recent years, this research has garnered more attention. The four serum fibrosis indicators for liver damage can be assessed via changes in each indicator, and thus can effectively diagnose cirrhosis. However, its detection accuracy for liver cirrhosis degree remains as yet unconfirmed[42].

Table 4 Receiver operating characteristic curve analysis for LS values combined with the four indicators of serum liver fibrosis

	LS value			Combination		
	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity
A Class	0.852	57.9%	93.3%	0.881	68.4%	91.7%
B Class	0.911	97.4%	75.0%	0.973	97.4%	85.0%
C Class	0.936	92.1%	83.3%	0.967	97.4%	90.0%

ROC curve analysis of LS values and the four indicators of liver fibrosis

In the ROC curve analysis of LS value and the four indicators of liver fibrosis, we found the following: LS value and the four indicators of liver fibrosis in the AUC are present in class C > class B > class A trends, and that the diagnostic accuracy of each indicator can increase with increased liver cirrhosis degree (*i.e.*, each indicator can assess the degree of cirrhosis). While each indicator for the diagnostic value of different grades of liver cirrhosis are different, a comparison of results from the ROC curves show that the CP score of the three classes in the AUC were higher than in the four indicators of liver fibrosis, but that its sensitivity and specificity were unstable. CP score class A: LS values were higher than that of serum-specific liver fibrosis, but with lower sensitivity; CP score class B: LS values and specificity were higher than HA and Ⅳ-C, but with lower sensitivity (sensitivity was higher than LN and PCⅢ, but with lower specificity); CP score class C: LS values and sensitivity were higher than HA, but with lower specificity (specifically was higher than LN and Ⅳ-C, but with relatively lower sensitivity). The results show that the diagnostic value of LS values is high compared to the four indicators of liver fibrosis and that it has high diagnostic accuracy, although its diagnostic sensitivity and specificity is unstable. The sensitivity of the four indicators of liver fibrosis for the diagnosis of cirrhosis degree is strong, but its

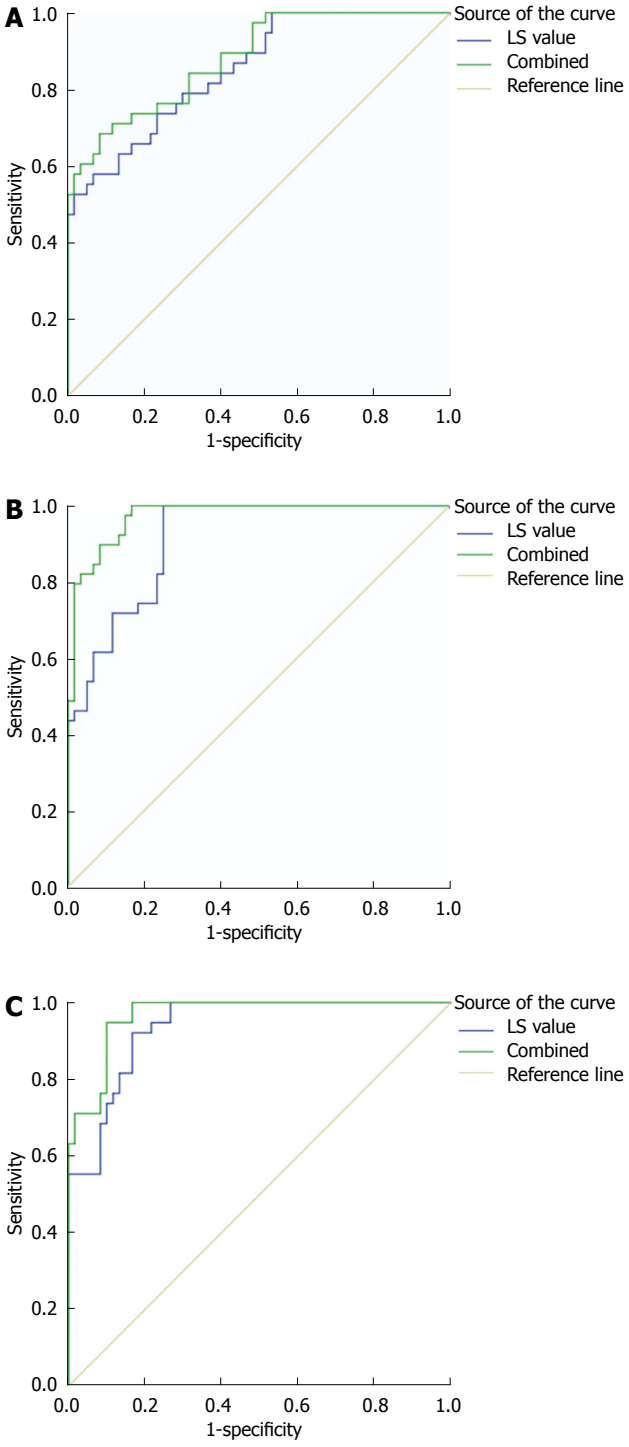

Figure 3 Receiver operating characteristic curve analysis for LS values combined with the four indicators of serum liver fibrosis. A: CP score of all indicators combined; B: CP score of all indicators combined; C: CP score of all indicators combined.

specificity and overall diagnostic value are insufficient. Detection of the four indicators of serum liver fibrosis can effectively diagnose cirrhosis, but lacks specificity in the accurate assessment of cirrhosis degree; thus, its technical support requires improvement[43]. The most commonly used method for the clinical diagnosis of cirrhosis is liver biopsy. However, due to its invasiveness, it has low acceptance limitations and causes more distress in clinical diagnosis and treatment to a certain extent[44-46]. On the other hand, ARFI ultrasound is a non-invasive detection technology. The degree of liver fibrosis can be determined by detecting LS value, which can compensate for the weakness of liver biopsy in detecting liver fibrosis[47].

ROC curve analysis of LS value combined with the four indicators of liver fibrosis

In the observation group, the results of the ROC curve analysis of LS value combined with the four indicators of serum liver fibrosis revealed that the CP score of the three classes combined with the diagnosis of AUC values were higher than ARFI-detected LS values alone, with sensitivity and specificity also improving. CP score class A: combined diagnosis sensitivity was higher than the LS value, albeit with slightly decreased specificity; CP score class B: the combined diagnostic specificity value was higher than the LS value, but sensitivity remained unchanged and there was no reduction; CP score class C: combined diagnostic sensitivity and specificity values were higher than the LS value. These results show that combined diagnosis improves the diagnostic accuracy of single-use LS values, and that diagnostic sensitivity and specificity can be guaranteed. The combined diagnostic value of LS values is high compared to the four indicators of liver fibrosis. It also proves that the LS value combined with the four indicators of serum liver fibrosis in the diagnosis of cirrhosis degree is higher than the diagnostic value of each indicator alone.

Limitation and prospects

Requirements for AFRI examination in patients were stringent. This may be due to insufficient coordination between doctors and patients, which affects the accuracy of the examination[48-50]. The detection operation for the four indicators of serum liver fibrosis is relatively simple, but also has its own shortcomings. Combined diagnosis can therefore play a complementary role and help improve diagnostic accuracy. Furthermore, LS values and indicators of liver fibrosis by way of motion detection can assist doctors in understanding the condition of a patient's liver disease, which is of great significance in the diagnosis and prognosis of liver cirrhosis.

In summary, the clinical diagnostic value of AFRI-detected LS value for determining liver cirrhosis degree is high compared to the four indicators of serum liver fibrosis. The diagnostic value of two combined diagnostics was more satisfactory compared to the indicators alone. Thus, detection by AFRI technology combined with the four indicators of serum liver fibrosis may serve as a powerful tool for determining liver cirrhosis degree, which has important clinical value and is worthy of wide promotion.

COMMENTS

Background

Primary biliary cirrhosis (PBC) is a chronic cholestatic disease that may develop into liver fibrosis, cirrhosis, and even lead to liver failure. When the patient is already in the liver cirrhosis stage, the accurate diagnosis and assessment of the extent of liver cirrhosis is vital in the diagnosis, treatment, and prognosis of the disease. Therefore, exploring a high value examination method to diagnose liver cirrhosis is very significant.

Research frontiers

It has been reported that liver cirrhosis can be divided into three classes according to the Child-Pugh (CP) scoring criteria, and the accuracy of their assessment methods have been demonstrated. Although liver biopsy is still currently the preferred diagnostic method for cirrhosis, the resulting trauma to the patient's body leads to low acceptance. Serum fibrosis indicators are a non-invasive examination method of cirrhosis diagnosis with a wide range of applications; however its accuracy in the assessment of cirrhosis degree remains to be studied. Acoustic radiation force impulse imaging (ARFI) is a new ultrasound elastography technique that can detect the hardness of the liver parenchyma for liver disease accurate assessment, and is non-invasive, simple, repeatable, and can effectively compensate for the lack of liver biopsy and serum liver fibrosis markers.

Innovations and breakthroughs

The clinical diagnostic value of AFRI-detected LS value for determining the degree of liver cirrhosis is high compared to the four indicators of serum liver fibrosis (HA, LN, PCIII, and IV-C). The diagnostic value of two combined diagnostics was more satisfactory compared to the indicators alone. Thus, detection by AFRI technology combined with the four indicators of serum liver fibrosis may serve as a powerful tool for determining liver cirrhosis degree, which has important clinical value and is worthy of wide promotion.

Applications

The diagnostic value of cirrhosis degree with PBC through liver cirrhosis degree assessment by ARFI combined with the four indicators of serum liver fibrosis is more satisfactory compared to the indicators alone and has important clinical application value. Results have shown that the higher the LS value, the higher the degree of liver fibrosis. This also confirmed that the diagnostic value of LS value was higher than that of the four indicators of liver fibrosis and, despite high diagnostic accuracy, that the diagnostic sensitivity and specificity were not stable. Further, the diagnosis value of liver cirrhosis degree for LS value combined with the four serum liver fibrosis was higher than each index alone.

Peer-review

The diagnostic value of cirrhosis degree with PBC through liver cirrhosis degree assessment by ARFI combined with the four indicators of serum liver fibrosis is more satisfactory compared to the indicators alone and has important clinical application value. The combination of AFRI and serum liver fibrosis four indicators can be used as a powerful tool to evaluate the degree of cirrhosis. It has important clinical application value and is worthy of clinical application.

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META-ANALYSIS

Hepatitis C virus genotype 3: Meta-analysis on sustained virologic response rates with currently available treatment options

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Abstract

AIM: To address the therapeutic efficacy of various treatment regimens in genotype 3 selecting randomized clinical trials and prospective National Cohort Studies.

METHODS: (1) PEG-INF-based therapy including sofosbuvir (SOF) + RBV for 12 wk *vs* SOF + RBV 24 wk; (2) SOF + RBV therapy 12 wk/16 wk *vs* 24 wk; and (3) the role of RBV in SOF + daclatasvir (DCV) and SOF + ledipasvir (LDV) combinations. This meta-analysis provides robust information with the intention of addressing treatment strategy for hepatitis C virus genotype 3.

RESULTS: A combination treatment including SOF + RBV + PEG-IFN for 12 wk notes better SVR than with only SOF + RBV for 12 wk, although its association with more frequent adverse effects may be a limiting factor. Longer duration therapy with SOF + RBV (24 wk) has achieved higher SVR rates than shorter durations (12 or 16 wk). SOF + LDV are not an ideal treatment for genotype 3.

CONCLUSION: Lastly, SOF + DCV combination is probably the best oral therapy option and the addition of RBV does not appear to be needed to increase SVR rates substantially.

Key words: Hepatitis C; Genotype 3; Sofosbuvir; Daclatasvir; Ledipasvir

Core tip: The landscape of therapy for hepatitis C virus infection is changing rapidly. In genotype 3, the improvement in SVR rates has not been hugely spectacular, being considered the most difficult genotype to treat and representing a major challenge. The advent of direct acting antivirals has not solved all questions about the treatment, while challenges remain such as the use of RBV, the duration of PEG-IFN-free treatment and whether PEG-IFN still plays an important role. These questions are difficult to elucidate with the current data because of the small number of patients included in clinical trials (particularly, those with cirrhosis) and their different designs.

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INTRODUCTION

The landscape of therapy for hepatitis C virus (HCV) infection is changing rapidly[1]. Ideally, new drugs should be all-oral regimen (once-daily, single pill) with pangenotypic activity, and have short treatment course (no more than 12 wk), and with high sustained virological response (at least 90%-95%). A multitude of direct acting antivirals (DAAs) have been developed with or without pegylated interferon (PEG-IFN) and ribavirin (RBV)[2], and others are being tested in promising clinical trials[3]. In genotype 3, the improvement in SVR rates has not relatively suboptimal and is being considered the most difficult genotype to treat and thus representing a major challenge[4]. Unique clinical features of genotype 3 and possible reasons for suboptimal response are: (1) a close relationship with insulin resistance and disturbances in lipid metabolism[5]; and (2) fibrosis progression[6] and higher incidence of hepatocellular carcinoma[7].

The advent of DAAs has not solved all questions regarding the treatment in genotype 3, and with emerging new challenges such as RBV use[8], duration of PEG-IFN-free treatment and whether PEG-IFN still plays an important role[9]. These questions are difficult to elucidate with the current data because of the small number of patients included in clinical trials (particularly, those with cirrhosis) and their different designs. In fact, more valuable data have been derived from prospective observational studies (clinical practice), and beyond randomized clinical trials. In this study, we aimed to address key questions on treatment outcomes through a meta-analysis.

MATERIALS AND METHODS

Data sources and search

The search strategy was in accordance with the recommendations of meta-analysis of clinical trials and observational studies. We searched in MEDLINE, EMBASE and Cochrane Library databases (to November 2015), as well as abstracts published and presented at EASL and AASLD (to November 2015) to identify potentially relevant publications in English language. We included FDA-approved DAA therapies that included SVR as a primary end point. Search terms were: "hepatitis C", "genotype 3", "HCV treatment", "sofosbuvir", "ledipasvir", "daclatasvir", "ribavirin", "interferon". The preceding terms were combined with appropriate Boolean logic. Manual search of cited bibliographies was also performed. Duplicated publications were deleted. Two researchers independently performed the literature search and data abstraction with regard to the inclusion and exclusion criteria by reading titles and abstracts. When reading titles and abstracts did not allow identification of eligible studies, articles were read in full. Relevant reviews and letters to the editor were excluded from the analysis, but read in full to identify potential relevant original studies. Disagreements between two observers were resolved by discussion.

Study selection criteria and data extraction

We selected randomized clinical trials (preferably) and prospective National Cohort Studies in which therapies were administrated in different arms. Therefore, studies including only a combination testing different doses or being administrated to different subset of patients were excluded. Inclusion and exclusion criteria (studies involving genotypes other than 3) were defined prior to initiation of the literature search. Twelve studies were included and classified according to the aims (Figure 1). The following data were extracted: (1) Study: year of publication, number of patients, location, design; (2) Patients: stage of liver disease (cirrhosis or chronic hepatitis), previous HCV treatment (naïve or treatment-experienced); (3) HCV treatment regimen and duration; and (4) SVR rates.

Objectives

We aimed to address the therapeutic efficacy of various treatment regimens in genotype 3. Firstly, we compared a PEG-INF-based therapy including sofosbuvir (SOF) + RBV during 12 wk with SOF + RBV 24 wk. Secondly, we assessed the importance of extending the course of SOF + RBV therapy (12 wk/16 wk *vs* 24 wk). Thirdly, we analyzed the role of RBV in SOF + daclatasvir (DCV) and SOF + ledipasvir (LDV) combinations.

Statistical analysis

Statistical analysis was performed using the Meta-Disc

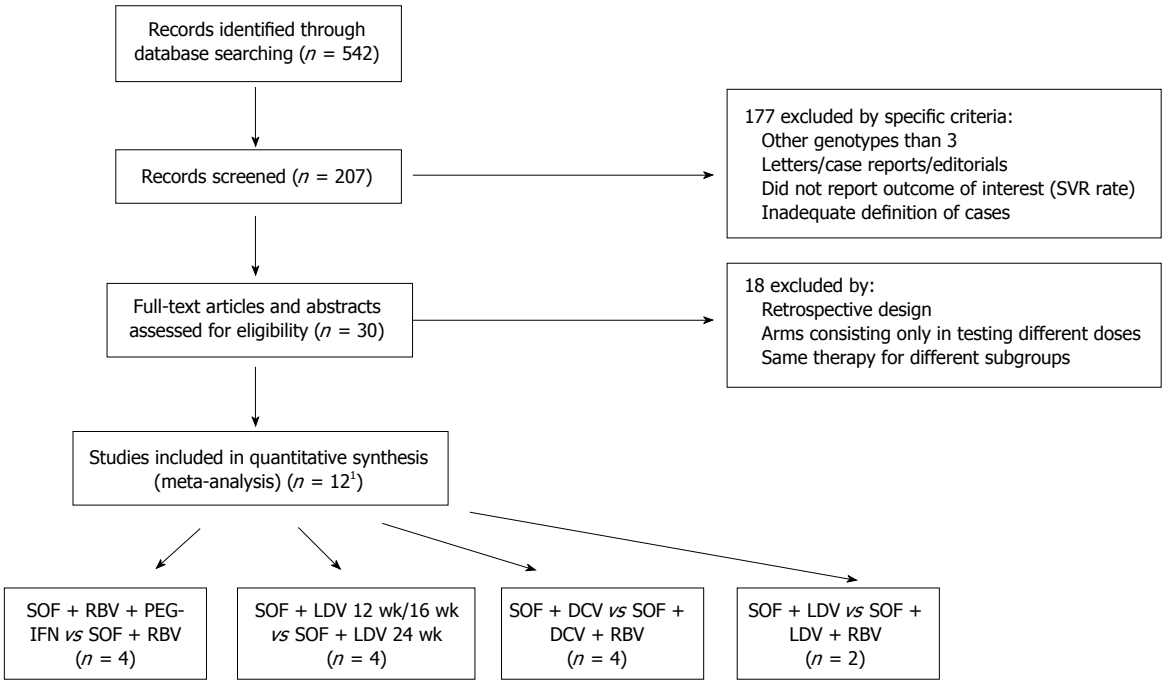

Figure 1 Flow chart of studies screened and included in meta-analysis. [1]Two studies included in two different sub-meta-analysis. SOF: Sofosbuvir; DCV: Daclatasvir; LDV: Ledipasvir; PEG-IFN: Polyethylene glycol interferon.

software 1.4[10], considering: (1) a summary of data from individual studies; (2) an investigation of the studies homogeneity, graphically and statistically; (3) calculation of clustered indexes; and (4) exploration of heterogeneity. Our assumption of heterogeneity was tested for each planned analysis using the Cochran-*Q* heterogeneity and I^2 statistics (low, moderate, and high heterogeneity according to I^2 values of 25%, 50%, and 75%, respectively)[11]. Random effects model using Der Simonian and Laird method and fixed effects model were used according to the presence of heterogeneity. To check for publication bias, we used the Begg and Egger tests. Only two-sided tests with a significance level of 0.05 were used. Confidence intervals (CIs) of individual studies were determined or approximated from the available data. Further, we assessed the quality of the studies using the "Quality Assessment of Diagnostic Accuracy Studies" (QUADAS) tool for observational studies ($\geqslant$ 10 were considered as high-quality studies[12]) and Jadad scale for randomized clinical trials ($\geqslant$ 3 were considered as high-quality ones[13]).

RESULTS

Comparison between INF-based and IFN-free regimens

We evaluated four studies that met the selection criteria and that were identified using the search strategy described. Studies characteristics are shown in Table 1. Pooled data included 807 patients. The meta-analysis demonstrated that triple therapy including SOF + RBV + PEG-IFN was able to achieve higher SVR rates (92.5%; 236/255) than SOF + RBV (75.2%; 415/552), using fixed effects model [OR = 3.51 (95%CI: 2.08-5.92)] (Figure 2A). We found neither heterogeneity between these studies [(Cochran-*Q* = 0.94; *df* = 3, *P* = 0.8157); inconsistency I^2 = 0%, and τ^2 = 0.0000)] nor publication bias [(Begg test: Kendall' s tau 1.70, *P* = 0.1); (Egger test: -1.14, *P* = 0.37)].

Course of SOF + RBV treatment

We included four studies involving 850 patients. The meta-analysis demonstrated that a 24 wk-course of SOF + RBV (85.5%; 501/586) combination was better than 12 wk-16 wk (70%; 185/264) in terms of SVR rates, using random effects model [OR = 3.51 (95%CI: 1.59-7.70)] (Figure 2B). We found a moderate heterogeneity between these studies [(Cochran-Q = 7.77, *df* = 3, *P* = 0.0511); inconsistency I^2 = 61%, and τ^2 = 0.3718], but no publication bias [(Begg test: Kendall's tau 0.34, *P* = 0.73); (Egger test: 0.81, *P* = 0.50)]. Three of these studies evaluated SVR rates according to the presence of cirrhosis. In non-cirrhotic patients, longer therapy of SOF + RBV (89.7%; 218/243) achieved higher SVR rates than shorter one (78.2%; 144/184) using random effects model (OR 2.44 (95%CI: 1.41-4.23). We did find a moderate heterogeneity between these studies [(Cochran-*Q* = 4.42; *df* = 2, *P* = 0.11); inconsistency I^2 = 55%, and τ^2 = 0.3987], with no publication bias. Similarly, this effect was observed in cirrhotic population (78.5%; 73/93 *vs* 55%; 38/69) using the random effects model [OR = 2.79 (95%CI: 1.34-5.78)].

Role of RBV in SOF + DCV and SOF + LDV combinations

Additionally, we assessed the role of adding RBV in

Table 1 Overall characteristics of studies included in meta-analysis

Ref.	Year	Patients characteristics	Study design	Outcome (SVR %)
Alqahtani *et al*[31]	2015	HCV mono-infected patients TARGET cohort Randomized by cirrhosis and previous treatment 50% Treatment naïve 51% Cirrhosis	a) SOF + RBV + PEG-IFN (n = 18) b) SOF + RBV (n = 133)	a) 89% b) 65%
Chulanov *et al*[32]	2014	HCV mono-infected patients Russian multicenter cohort Randomized by cirrhosis 100% Treatment naïve 18% Cirrhosis	a) SOF + RBV 16 wk (n = 30) b) SOF + RBV 24 wk (n = 31)	a) 87% b) 90%
Dalgard *et al*[33]	2015	HCV mono-infected patients Scandinavian cohort study 51% Treatment naïve 82% Cirrhosis	a) SOF + RBV + PEG-IFN 12 wk (n = 25) b) SOF + RBV 24 wk (n = 33)	a) 92% b) 79%
Foster *et al*[17](BOSON)	2015	HCV mono-infected patients Randomized study 51% Treatment naïve 31% Cirrhosis	a) SOF + RBV + PEG-IFN 12 wk (n = 181) b) SOF + RBV 16 wk (n = 181) c) SOF + RBV 24 wk (n = 182)	a) 93% b) 71% c) 84%
Foster *et al*[27]	2015	HCV mono-infected patients NHS England Early Access Program 100% Decompensated Cirrhosis	a) SOF + DCV 12 wk (n = 7) b) SOF + DCV + RBV 12 wk (n = 113) c) SOF + LDV 12 wk (n = 7) d) SOF + LDV + RBV 12 wk (n = 61)	a) 71% b) 81% c) 57% d) 72%
Gane *et al*[29] (ELECTRON-2)	2015	HCV mono-infected patients Randomized study 50% Treatment naïve 32% Cirrhosis	a) SOF + LDV 12 wk (n = 25) b) SOF + LDV + RBV 12 wk (n = 26) c) SOF + LDV + RBV 12 wk (n = 50)	a) 64% b) 100% c) 82%
Hezode *et al*[34]	2015	HCV mono-infected patients French Compassionate Use Program 27% Treatment naïve 94% Cirrhosis	a) SOF + DCV 12 wk (n = 26) b) SOF + DCV + RBV 12 wk (n = 4) c) SOF + DCV 24 wk (n = 35) d) SOF + DCV + RBV 24 wk (n = 13)	a) 85% b) 100% c) 91% d) 92%
Ingiliz *et al*[35]	2015	HCV-HIV co-infected patients German multicenter cohort study 50% Treatment naïve38% Cirrhosis	a) SOF + RBV + PEG-IFN 12 wk (n = 31) b) SOF + RBV 24 wk (n = 23)	a) 94% b) 91%
Sulkowski *et al*[22](PHOTON)	2014	HCV-HIV co-infected patients International multicenter cohort 25% Treatment naïve	a) SOF + RBV 12 wk (n = 42) b) SOF + RBV 24 wk (n = 123)	a) 67% b) 89%
Sulkowski *et al*[36]	2014	HCV mono-infected patients Randomized study 100% Treatment naïve 14% Cirrhosis	a) SOF + DCV 24 wk (n = 13) b) SOF + DCV + RBV 24 wk (n = 5)	a) 92% b) 80%
Welzel *et al*[28]	2015	HCV mono-infected patients European Compassionate Use Program 72% Cirrhosis	a) SOF + DCV 24 wk (n = 11) b) SOF + DCV + RBV 24 wk (n = 13)	a) 100% b) 85%
Zeuzem *et al*[37] (VALENCE)	2014	HCV mono-infected patients Randomized study 41% Treatment naïve 24% Cirrhosis	a) SOF + RBV 12 wk (n = 11) b) SOF + RBV 24 wk n = 250)	a) 27% b) 84%

HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; SOF: Sofosbuvir; DCV: Daclatasvir; LDV: Ledipasvir; PEG-IFN: Polyethylene glycol interferon.

IFN-free regimens. Four studies have evaluated this point regarding the combination treatment of SOF + DCV. Pooled data included 502 patients. The meta-analysis demonstrated that adding RBV was not essential to achieve optimal SVR rates (83%; 173/209 *vs* 86.3%; 253/293), using fixed effects model [OR = 1.09 (95%CI: 0.35-3.40)] (Figure 2C). We did not find heterogeneity between these studies [(Cochran-Q = 2.38; df = 3, P = 0.4981); inconsistency I^2 = 0%, and τ^2 = 0.0000], and did not seem to have publication bias. On the other hand, two studies have evaluated the role of adding RBV in SOF + LDV combination. Pooled data included 169 patients. The meta-analysis demonstrated that adding RBV was important to achieve better SVR rates (81%; 111/137 *vs* 62.5%; 20/32), using fixed effects model [OR = 3.30 (95%CI: 1.35-8.04)] (Figure 2D). We did not find heterogeneity between these studies [(Cochran-Q = 0.61, df = 1, P = 0.4335); inconsistency I^2 = 0%, and τ^2 = 0.0000], and no publication bias was found [(Begg test: Kendall's tau 0.01, P = 0.99)].

DISCUSSION

New challenges have emerged in the evolving era of HCV therapy, particularly with genotype 3, and these

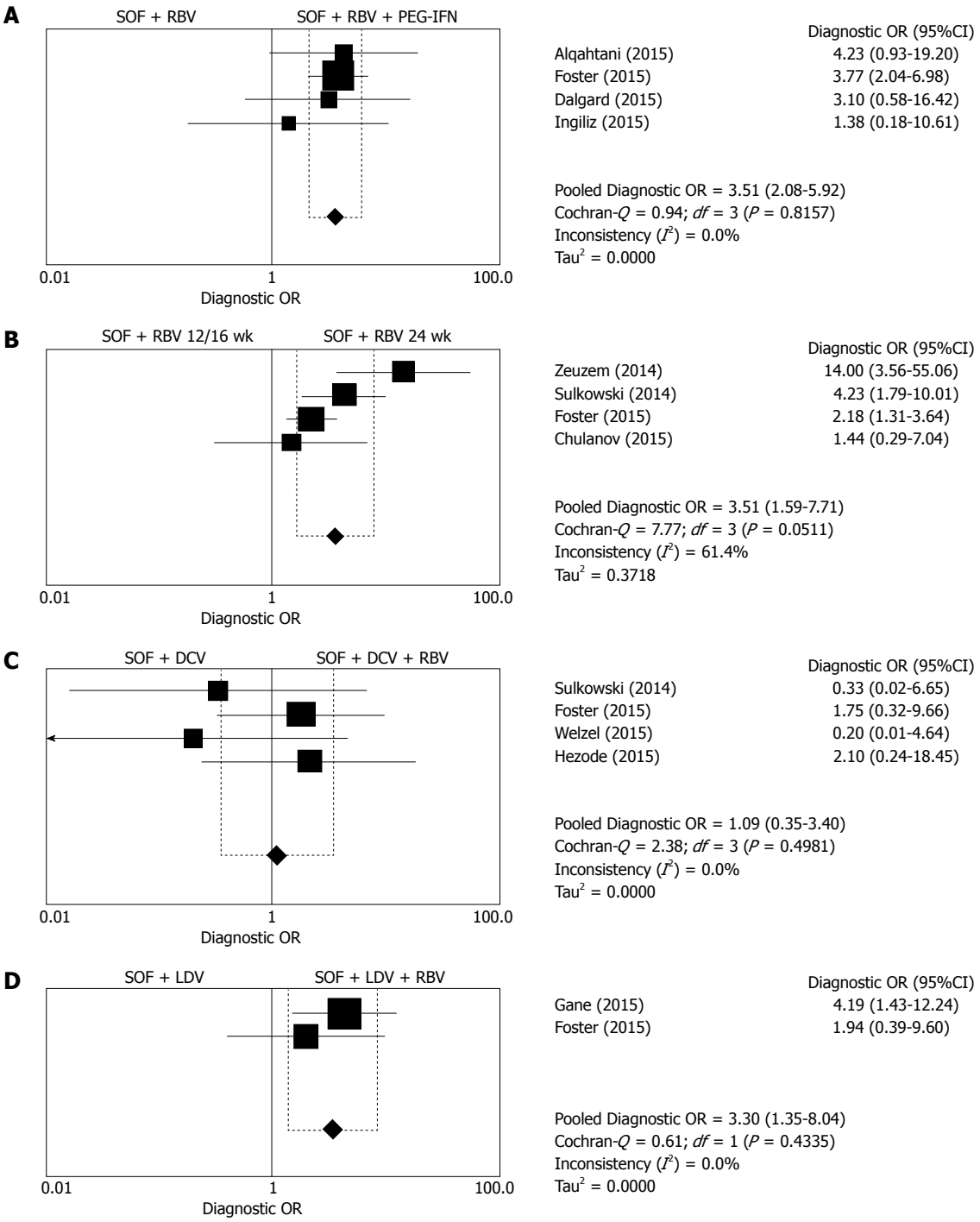

Figure 2 Odds ratio (95%CI) and Forest plot for SVR rates. A: SOF + RBV + PEG-IFN *vs* SOF + RBV combinations; B: SOF + RBV 12 wk/16 wk *vs* SOF + RBV 24 wk combinations; C: SOF + RBV 12 wk/16 wk *vs* SOF + RBV 24 wk combinations; D: SOF + LDV *vs* SOF + LDV + RBV combinations.

include the ongoing role of PEG-IFN, the addition of RBV and the adequate duration of the therapy[13]. The rapid development and use of DAAs in several heterogeneous studies including small number of patients has made robust guideline development and recommendation rather challenging. Thus, a meta-analysis is needed pooling all patients to address these questions.

In this new era, PEG-IFN is being abandoned as part of standard HCV therapy because of the association with serious adverse effects (and the parenteral administration)[14]. From now on, PEG-IFN will not be used for genotypes 1, 2 or 4 anymore. For genotype 3, there are only two DAAs (SOF and DCV) with a significant inhibitory activity *in vitro*[15]. In this context, PEG-IFN could potentially play a role in HCV treatment and could be the last such indication for its use. We demonstrated that the addition of PEG-IFN to SOF + RBV 12 wk was superior to only SOF + RBV combination (92% *vs* 75%, OR = 3.51). BOSON study

represents the main study evaluating this comparison, and it included nearly two hundred patients per arm[16]. Additionally, DCV has been evaluated in combination with PEG-IFN + RBV, although SVR rates were not higher than those patients treated with dual standard therapy (65% *vs* 59%)[17]. Both EASL and AASLD recommend SOF + RBV + PEG-IFN as a good alternative in non-cirrhotic and compensated-cirrhotic patients[18]. On the other hand, no data is available evaluating SOF + RBV + PEG-IFN *vs* SOF + DCV.

We analyzed the combination of SOF + RBV, in terms of duration of therapy. To date, this combination has been evaluated for 12, 16 and 24 wk duration. We compared SOF + RBV 12 wk/16 wk *vs* SOF + RBV 24 wk, and the latter achieved higher SVR rates (89% *vs* 70%, OR = 3.51). Furthermore, SOF + RBV 12 wk (56%) was associated with poorer SVR rates than dual standard therapy with PEG-IFN + RBV 24 wk (63%) in FISSION study[19], and showing similar results than POSITRON study (61%)[20]. Both studies demonstrated that SOF + RBV combination 12 wk was suboptimal, especially in the cirrhotic population. In FISSION study, a longer course of therapy (16 wk) with SOF + RBV showed better results than a shorter one (62% *vs* 30%)[21]. Overall SVR rates with SOF + RBV 12/16 wk were about 60%, which is considered suboptimal in the evolving era of hepatitis C therapy where response rates far below 90% are considered suboptimal. We included four studies that evaluated the course of 24 wk of SOF + RBV and noted an overall SVR rate around 90%. In addition, PHOTON study confirmed the extrapolation of these results in HIV-co-infected patients[22]. Taking into account all of these results, EASL and AASLD guidelines recommend extending SOF + RBV treatment to 24 wk (especially indicated in non-cirrhotic population).

In this meta-analysis, we demonstrated that SOF + LDV combination needs the addition of RBV to achieve optimal SVR rates in patients with genotype 3 (81% *vs* 62%, OR = 3.30). In contrast, RBV did not play any role in the combination of SOF + DCV because it did not improve SVR rates. DCV and LDV are HCV NS5A inhibitors[23], although DCV shows a pangenotypic activity[24] while LDV has a low activity in genotypes 2 and 3[25]. Currently, SOF + DCV combination is the first option to treat patients with genotype 3 in EASL guidelines, 12 wk in non-cirrhotic and 24 wk (with RBV) in cirrhotic patients. This recommendation is mainly based on ALLY-3 study in which SOF + DCV 12 wk achieved 97% and only 58% SVR in non-cirrhotic and cirrhotic population respectively[26]. The UK Early Access Program did not show any impact of adding RBV to SOF + DCV 24 wk in cirrhotic patients (70% *vs* 71%)[27], as well as the European Compassionate Use Program in patients at high risk of hepatic decompensation or death within 12 mo (100% *vs* 85%, *P* = NS)[28]. In a relatively small study, ELECTRON-2 trial, SOF + LDV for 12 wk achieved suboptimal SVR rates while the addition of RBV substantially increased it (100% in non-cirrhotic naïve patients, and 89% in non-cirrhotic and 73% in cirrhotic treatment-experienced patients)[29]. However, this trial should be interpreted with caution because it has very limited data from a phase Ⅱ single-center study and comprising a homogenous population which could limit the generalizability of the results. This, together with the high EC50 of LDV for genotype 3[30], has lead EASL and AASLD to not recommend SOF + LDV±RBV combination for genotype 3.

Recommendations made by EASL and AASLD guidelines were based on few data derived from randomized clinical trials and, due to the rapid and wide use in clinical practice, modified by prospective national cohorts. This meta-analysis provides solid and robust information to address several important questions, regarding the treatment of HCV genotype 3. First, combination including SOF + RBV + PEG-IFN shows better results than only SOF + RBV, although its association with adverse effects may limit the use (*i.e.,* cirrhotic population). Second, longer therapies including SOF + RBV (24 wk) have higher SVR rates than shorter ones (12 or 16 wk). Therefore, SOF + RBV for 24 wk are ideal. Third, SOF + LDV should not be used in genotype 3 and, if so, necessarily with RBV. Lastly, SOF + DCV combination is probably the best option and the addition of RBV does not appear to be needed to increase substantially the SVR rates.

COMMENTS

Background

The advent of direct acting antivirals has not solved all questions of successfully and effectively treating all hepatitis C virus (HCV) genotypes. Genotype 3, a common genotype globally, remains the last challenge.

Research frontiers

Nowadays, it remains unclear if Peg-IFN and RBV are still required to treat HCV genotype 3 effectively. The worldwide research is directed towards a more suitable combination of DAA.

Innovations and breakthroughs

In the present study, the authors investigated the SVR rates of different DAA combinations. This is the first report of a meta-analysis including sofosbuvir, daclatasvir, ledipasvir, peginterferon and ribavirin showing the eradication of the HCV infection.

Applications

The present report allows understanding the role of DAAs in the treatment of HCV genotype 3.

Peer-review

This systematic review and meta-analysis adds useful information for clinical practice and research.

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CASE REPORT

Laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis for Peutz-Jeghers syndrome with synchronous rectal cancer

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Author contributions: Wu B and Zhong ME designed the report; Zhong ME, Niu BZ and Ji WY collected the patient's clinical data and wrote the paper.

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Abstract

We report on a patient diagnosed with Peutz-Jeghers syndrome (PJS) with synchronous rectal cancer who was treated with laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA). PJS is an autosomal dominant syndrome characterized by multiple hamartomatous polyps in the gastrointestinal tract, mucocutaneous pigmentation, and increased risks of gastrointestinal and nongastrointestinal cancer. This report presents a patient with a 20-year history of intermittent bloody stool, mucocutaneous pigmentation and a family history of PJS, which together led to a diagnosis of PJS. Moreover, colonoscopy and biopsy revealed the presence of multiple serried giant pedunculated polyps and rectal adenocarcinoma. Currently, few options exist for the therapeutic management of PJS with synchronous rectal cancer. For this case, we adopted an unconventional surgical strategy and ultimately performed laparoscopic restorative proctocolectomy with IPAA. This procedure is widely considered to be the first-line treatment option for patients with ulcerative colitis or familial adenomatous polyposis. However, there are no previous reports of treating PJS patients with laparoscopic IPAA. Since the operation, the patient has experienced no further episodes of gastrointestinal bleeding and has demonstrated satisfactory bowel control. Laparoscopic restorative proctocolectomy with IPAA may be a safe and effective treatment for patients with PJS with synchronous rectal cancer.

Key words: Peutz-Jeghers syndrome; Laparoscopy; Ileal pouch-anal anastomosis; Restorative proctocolectomy; Multiple polyps in gastrointestinal tract

Core tip: Few options currently exist for the therapeutic management of Peutz-Jeghers syndrome with synchronous rectal cancer. Here, we present a patient diagnosed with Peutz-Jeghers syndrome with synchronous rectal cancer treated with laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA). The patient has experienced no further episodes of gastrointestinal bleeding and has demonstrated satisfactory bowel control. To our knowledge, this is the first report on laparoscopic restorative proctocolectomy with IPAA performed for the treatment of Peutz-Jeghers syndrome with synchronous rectal cancer.

Zhong ME, Niu BZ, Ji WY, Wu B. Laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis for Peutz-Jeghers syndrome with synchronous rectal cancer. *World J Gastroenterol* 2016; 22(22): 5293-5296 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5293.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5293

INTRODUCTION

Peutz-Jeghers syndrome (PJS) is a rare, hereditary, autosomal dominant disorder characterized by multiple hamartomatous polyps in the gastrointestinal tract (mainly in the jejunoileum but also in the stomach and colon) and mucocutaneous pigmentation. It has also been reported to be associated with a high risk of malignancy, with a lifetime cancer risk of up to 93%, and it is caused by a germline mutation in the STK11 gene[1,2].

Few options currently exist for the therapeutic management of PJS with synchronous rectal cancer. Surgical strategies are commonly used to treat the sequelae of PJS, such as small bowel intussusception or neoplastic lesions, which may require enterectomy. To date, no studies have focused on PJS patients treated with laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA).

Here, we present a patient diagnosed with PJS with synchronous rectal cancer that was treated with laparoscopic restorative proctocolectomy with IPAA.

CASE REPORT

A 42-year-old female patient presented with a 20-year history of intermittent bloody stool, poor appetite, abdominal pain and body-weight loss. Twenty years prior, she had experienced occasional abdominal pain, which subsided without treatment. Her family history confirmed that her father and children had similar symptoms. She had presented to our emergency department one month prior with acute gastrointestinal hemorrhaging.

Physical examination showed scattered, punctate, dark-blue areas of pigmentation on her lips (Figure 1) and the distal parts of her fingers. The patient was pale and extremely emaciated. Her body mass index (BMI) was 16. Rectal examination (in the knee-chest position) revealed the presence of a soft mass in the rectal lumen that was 6 cm from the anal verge, approximately 3 cm in diameter, located at nine o' clock, and difficult to move.

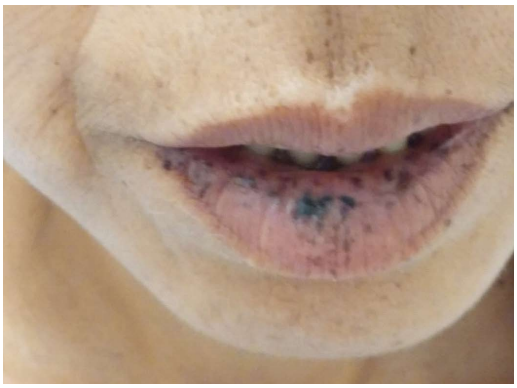

Figure 1 Scattered, punctate, dark blue macules on the mucosa of the lips.

Laboratory tests revealed that she had iron deficiency anemia (69 g/L hemoglobin). Her serum CA125 and serum CEA levels were within the normal limits. A computed tomography scan of the abdomen and pelvis revealed the presence of multiple polyps in the ileum and colorectum. Colonoscopy revealed the presence of multiple serried giant pedunculated polyps, with involvement of the rectum, sigmoid colon, descending colon, transverse colon and part of the ascending colon. In addition, a large cauliflower-like mass was identified at 7 cm from the anal verge (Figure 2). Biopsy of the tumor was performed, which revealed that the cauliflower-like mass was rectal adenocarcinoma (Figure 3A).

Finally, PJS with synchronous rectal adenocarcinoma was diagnosed. Surgical intervention consisting of laparoscopic restorative proctocolectomy and IPAA with a covering ileostomy was performed. During the operation, the patient was noted to have areas of intussusception of the small bowel secondary to the giant polyps. Several adenomatoid polyps were found at locations 30, 50 and 70 cm from the Treitz ligament, and consequently, enterotomy and polypectomy were performed.

The final pathological examination confirmed the diagnosis of moderately differentiated rectal adenocarcinoma (Figure 3B). Of the 37 lymph nodes examined, metastatic adenocarcinoma was detected in 3, and hamartomas with atypical hyperplasia were identified in the polyps, thereby confirming the diagnosis of PJS.

The patient underwent ileostomy closure 6 mo later. After 14 mo of follow-up, no further episodes of gastrointestinal bleeding occurred. This patient has demonstrated satisfactory bowel control to date. Her Wexner incontinence score is zero, indicating that she has no fecal incontinence. Her defecation frequency

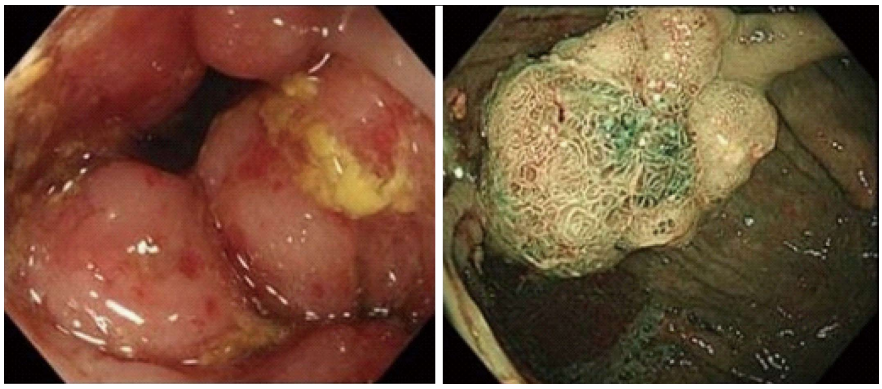

Figure 2 Colonoscopy revealed the presence of multiple polyps and a rectal cauliflower-like mass.

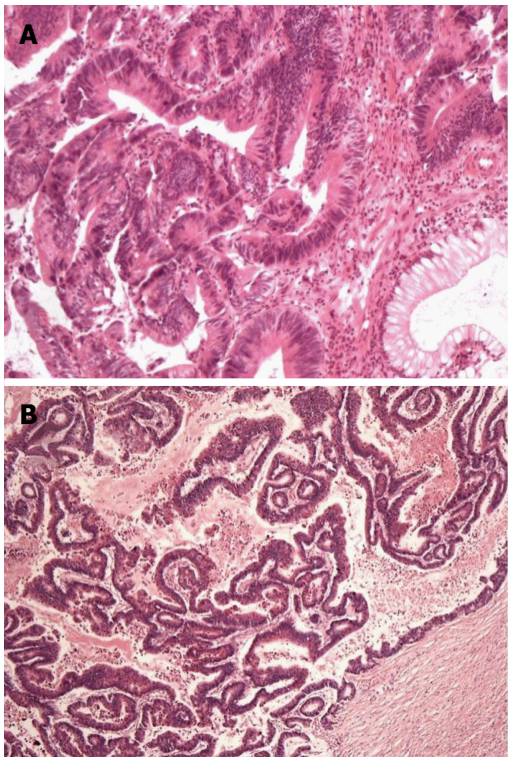

Figure 3 Biopsy revealed that the rectal mass was rectal adenocarcinoma (A) and postoperative pathological examination showed moderately differentiated rectal adenocarcinoma (B).

is 1-2/d. In addition, she is very satisfied with the cosmetic results.

One month ago, this patient underwent gastroenterography, and several polyps were found in the small bowel. As a result, double balloon enteroscopy (DBE) was performed. The ileal polyps were removed using an endoscopic snare.

DISCUSSION

PJS is an inherited, autosomal dominant disorder with variable inheritance and is characterized by hamartomatous polyps in the gastrointestinal tract, mainly in the small bowel, as well as pigmented mucocutaneous lesions. It is also characterized by increased risks for gastrointestinal and nongastrointestinal cancer. The prevalence of PJS is estimated to be between 1 in 50000 and 1 in 200000 individuals[2].

In this case, the patient had typical manifestations and a family history of PJS. Thus, it was not difficult to diagnose her. In addition, colonoscopy and biopsy revealed the presence of synchronous midrectal cancer.

Few options currently exist for the therapeutic management of PJS with synchronous rectal cancer. Surgical strategies are commonly used to treat the sequelae of PJS, such as small bowel intussusception or neoplastic lesions. Enterotomy and polypectomy or limited resection is considered to be the procedures of choice. Polypectomy using DBE is now recommended for small bowel polyps[3]. DBE is well recognized as a new enteroscopic method that allows for the examination and treatment of the jejunum and ileum in almost all patients. However, it cannot completely replace the surgical treatment of malignancies.

In the present case, we adopted an unconventional surgical strategy and performed laparoscopic restorative proctocolectomy and IPAA with a covering ileostomy.

Laparoscopic restorative proctocolectomy with IPAA is generally considered to be the first-line treatment option for patients with ulcerative colitis or familial adenomatous polyposis (FAP)[4]. However, no previous reports have focused on treating PJS patients with laparoscopic IPAA.

This report describes a patient who presented with severe gastrointestinal hemorrhage. Multiple polyps were found in her gastrointestinal tract, most of which were located in the colorectum and not the small bowl. Giant pedunculated polyps covered the patient's rectum, sigmoid colon, descending colon, transverse colon and part of the ascending colon, and surgery was necessary. In addition, colonoscopy and biopsy indicated the presence of a midrectal malignancy. Due to the locations of the giant polyps and the midrectal malignancy, restorative proctocolectomy with IPAA was considered. This procedure effectively minimizes

the risk of recurrence through the maximal removal of the involved bowel tissue while maintaining bowel continence. A large, retrospective cohort study from the Cleveland Clinic has shown that IPAA is a relatively safe and effective procedure with a low perioperative mortality rate of 0.1%[5]. Compared to conventional laparotomy, laparoscopic restorative proctocolectomy with IPAA is associated with less blood loss, fewer respiratory complications, a faster return of bowel function and a shorter hospital stay[6,7]. In addition, patients are satisfied with laparoscopic surgery[5], which significantly improves cosmesis and quality of life. In this case, the patient has not suffered from further episodes of gastrointestinal bleeding or fecal incontinence since the operation, and she is satisfied with the cosmetic results and the therapeutic effects.

We suggest that laparoscopic restorative proctocolectomy with IPAA may be a safe and effective treatment for PJS with synchronous rectal cancer in patients similar to the one presented in this report. However, this procedure cannot be applied to all patients with PJS. Polyps in the small intestine still require endoscopic polypectomy or resection of the involved regions of the small intestine.

COMMENTS

Case characteristics

A 42-year-old female patient presented with a 20-year history of intermittent bloody stool. Physical examination showed areas of pigmentation on the lips and extremities.

Clinical diagnosis

Peutz-Jeghers syndrome (PJS) with synchronous rectal cancer.

Differential diagnosis

Familial adenomatous polyposis.

Laboratory diagnosis

The hemoglobin level was 69 g/L, CEA, CA19-9 levels and metabolic panel and liver function test results were within the normal limits.

Imaging diagnosis

CT scan revealed the presence of multiple polyps in the ileum and colorectum.

Pathological diagnosis

Colonoscopy and biopsy revealed that the rectal mass was an adenoma with high-grade intraepithelial neoplastic changes. The final pathological examination confirmed the diagnosis of moderately differentiated rectal adenocarcinoma.

Treatment

The patient was treated with laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA).

Experiences and lessons

The authors suggest that laparoscopic restorative proctocolectomy with IPAA may be a safe and effective treatment for treating patients with PJS with synchronous rectal cancer, with the advantage of minimal invasiveness.

Peer-review

In this manuscript, the authors reported a patient with PJS with synchronous rectal cancer who was treated with laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis. Overall, this case report is very interesting, and worthy to be published.

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CASE REPORT

Application of cystoscope in surgical treatment of hepatocellular carcinoma with portal vein tumor thrombus

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Author contributions: Cheng SQ designed the report and carried out the operation; Li N and Wei XB collected the clinic data and wrote the paper; Li N and Wei XB contributed equally to the article.

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Abstract

Development of portal vein tumor thrombus deteriorates the prognosis of hepatocellular carcinoma, while surgical treatment can offer a promising prognosis for selected patients. However, the possibility of residual lesions in portal vein after conventional thrombectomy is a main risk factor leading to postoperative recurrence. Therefore, ensuring the complete removal of tumor thrombus during operation is critical to improve prognosis. For the first time, we report here one case of hepatocellular carcinoma with portal vein tumor thrombus in which cystoscope was successfully applied as a substitute of intravascular endoscope to visualize the cavity of the portal vein. The patient was a 61-year-old man with a 7-cm tumor in the right lobe of the liver, with tumor thrombus invading the right branch and adjacent to the conjunction of the portal vein. After removal of the tumor, the Olympus CYF-VA2 cystoscope was used to check the portal vein from the opening stump of the right branch of the portal vein. In this case, residual thrombus tissue was found near the opening stump and the conjunction of the portal vein. The residual lesion was carefully retrieved from the stump after retraction of the cystoscope. The procedure was repeated until no residual lesion was found. The whole duration time of thrombectomy was 22.5 (15 + 7.5) min. The patient was free from recurrence at 8 months after the procedure. Our work indicated that the cystoscope is a suitable substitute, with a proper size and function to check the portal vein system and ensure the curability of thrombectomy. Although well-designed clinic trails are still needed, this procedure may further improve the postoperative prognosis of hepatocellular carcinoma with portal vein tumor thrombus.

Key words: Hepatocellular carcinoma; Portal vein tumor thrombus; Surgical treatment; Thrombectomy; Cystoscope

Core tip: Inability to ensure the curability of the thrombectomy has been a main obstacle to improving postoperative prognosis of hepatocellular carcinoma with portal vein tumor thrombus, especially for cases with invasion in the main trunk of the portal vein. In this report, we firstly applied the cystoscope as an intravascular endoscope to investigate the cavity of the portal vein after primary tumor removal. The cystoscope offered a clear view of the portal vein cavity from the main trunk to the secondary branch, indicating its suitability as a substitute with a proper size and function to check the portal vein system.

Li N, Wei XB, Cheng SQ. Application of cystoscope in surgical treatment of hepatocellular carcinoma with portal vein tumor thrombus. *World J Gastroenterol* 2016; 22(22): 5297-5300 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5297.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5297

INTRODUCTION

Hepatocellular carcinoma (HCC) has a propensity to invade the intrahepatic vasculature, especially the portal vein system, leading to the formation of portal vein tumor thrombus (PVTT). PVTT is the most important significant factor for a poor prognosis, with a median survival of only 2.7 mo if patients are untreated[1]. Although sorafenib was recommended by the Barcelona Clinic Liver Cancer (BCLC) guideline as the only therapy for these patients, recent studies have demonstrated that surgical resection may offer a more promising prognosis for selected HCC patients with PVTT[2,3].

In the surgical operation, when the PVTT and tumor could not be resected *en-bloc*, thrombectomy was carried out after the removal of tumor. Theoretically, when thrombectomy was performed, squeezing or fragmenting the tumor thrombus could not be avoided, which would increase the risk of scattering tumor tissue within the portal vein cavity. What's more, there will be a possibility of residual PVTT tissues adhering to the inner wall of the portal vein even after careful extraction[4-6]. Those factors may lead to the early intrahepatic recurrence of tumor or PVTT[6]. Therefore, ensuring the complete removal of PVTT during operation is critical to improving the prognosis. With the development of endoscopy, it is theoretically ideal to achieve this goal by direct visual observation under intravascular endoscope. However, to the best of our knowledge, there is currently no angioscope specially designed for the portal vein system. Here, we describe one case of an HCC patient with PVTT in which the cystoscope was successfully applied as a substitute to the intravascular endoscope to visualize the cavity of the portal vein.

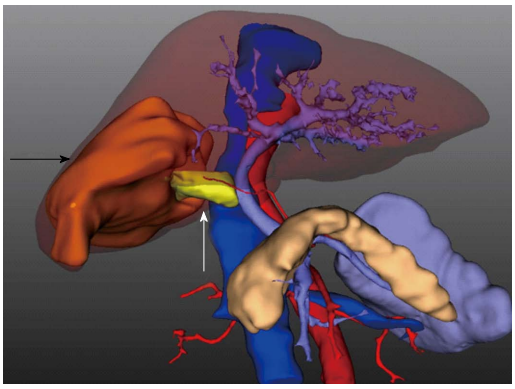

Figure 1 3D reconstruction of the tumor and portal vein tumor thrombus. A 7-cm hepatocellular carcinoma (white arrow) was located in segments Ⅴ, Ⅵ and Ⅶ. The tumor thrombus (black arrow) extended into the right branch and was adjacent to the conjunction of the portal vein.

CASE REPORT

This study was approved by our Institutional Review Board and written informed consent was obtained from the patient for this research. A 61-year-old man with hepatitis B virus infection presented to our department with a 7-cm HCC in the right lobe of the liver, with tumor thrombus that had invaded the right branch and was adjacent to the conjunction of the portal vein. Figure 1 shows a 3D reconstruction of the tumor and PVTT. The patient had Child-Pugh class A liver function and the other laboratory tests were normal. Intraoperative assessment confirmed the preoperative diagnosis. During operation, Pringle's maneuver was applied distal to the PVTT to occlude the blood inflow using a clamp/unclamp cycle of 15 min/5 min. According to characteristics of the tumor and the PVTT, a right semi-hepatectomy was carried out with a clamp crushing method.

After removal of the tumor, the Olympus CYF-VA2 cystoscope was used to check the portal vein. First, the streamlined tip was inserted into the opening stump on the right branch of the portal vein. The function of flush-and-suction was used to keep the field of view clear. In this case, scattered PVTT tissue was found near the opening stump. Further inspection revealed a residual lesion near the conjunction of the portal vein (Figure 2A and B). Then, the cystoscope was retracted from the stump and the residual PVTT was carefully retrieved using a clamp. After that, the portal vein cavity was reexamined meticulously from the main trunk to the left secondary branch by bending the flexible tip and drawing the insertion tube in and out. The procedure was repeated until no residual lesion was found (Figure 2C and D). Then, the stump was closed using a continuous suture. The whole duration time of the thrombectomy was 22.5 (15 + 7.5) min. The patient was discharged home without

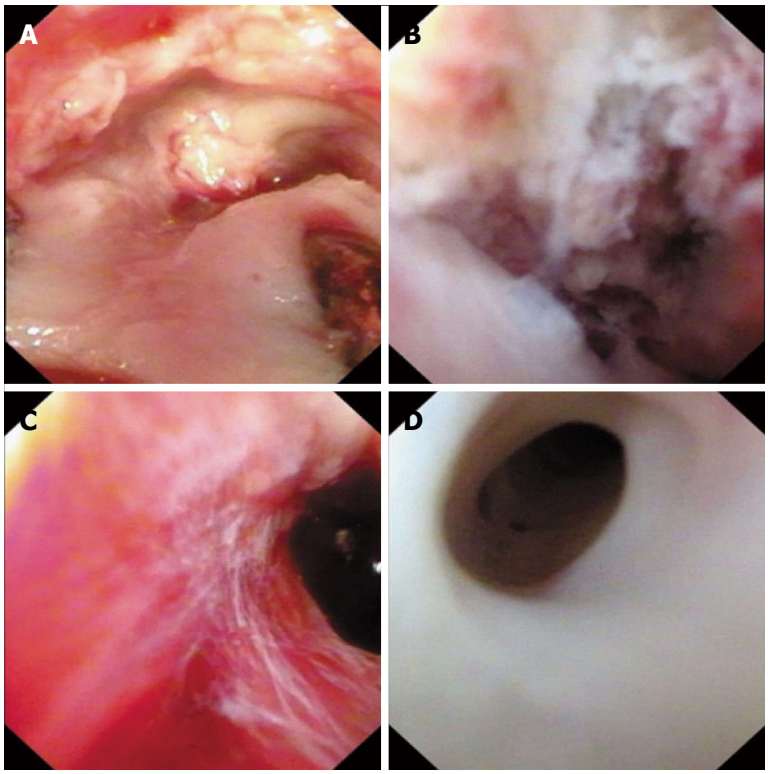

Figure 2 Endoscopic images of portal vein before and after thrombectomy. A: Before thrombectomy, endoscopy revealed scattered tissue of tumor thrombus near the opening stump; B: Residual tumor thrombus was adhered to the inner wall of the portal vein near the conjunction; C: After repeated retraction of the residual tumor thrombus, endoscopy revealed a clean inner wall of the portal vein with no macroscopic thrombus remaining; D: The left secondary branch of the portal vein was clean with no scattered thrombus.

complications on postoperative day 7 and was free from recurrence at 8 mo after the procedure when the last follow-up was attended.

DISCUSSION

Curative resection of tumor and complete removal of PVTT is essential to improve the oncological prognosis of HCC patients with PVTT. For PVTT confined to the ipsilateral branch of the portal vein, *en-bloc* resection of the ipsilateral portal vein branch containing the tumor thrombus has been recommended, whenever the liver remnant is sufficient[4,7]. However, for patients with PVTT extending to the main portal trunk, or patients with insufficient liver remnant after en-bloc resection, thrombectomy would be inevitably carried out after resection of the primary tumor. Patients who underwent thrombectomy have been reported to have a poor prognosis, with a 6-mo PVTT recurrence rate of 63.8% and the 1-year intrahepatic recurrence rate of 78.8%[4]. For these patients, residual or disseminated tumor thrombus in portal vein may be a significant risk factor leading to the high recurrence rate[6]. Therefore, it is crucial to eliminate the risk of residual thrombus while performing thrombectomy. Fortunately, the portal vein has no blood flow inside during the application of Pringle's maneuver, allowing the possibility of endoscopic inspection. In this case, the cystoscope we used could view the portal vein cavity clearly from the main trunk to the secondary branch, indicating it is a suitable substitute with a proper size and function to check the portal vein system. Despite the possibility that a microscopic lesion may still exist, this procedure theoretically eliminated the possibility of a residual and scattered macroscopic tumor thrombus in the portal vein and further ensured curability of the thrombectomy. This procedure may further improve the postoperative prognosis of HCC with PVTT**.** It will also be worthwhile to carry out a well-designed clinical trail to measure the significance of intravascular endoscopy to prove the postoperative prognosis of HCC with PVTT.

COMMENTS

Case characteristics

A 61-year-old man presented to our department with a 7-cm hepatocellular carcinoma (HCC) in the right lobe of the liver, with tumor thrombus invading the right branch and adjacent to the conjunction of the portal vein.

Treatment

Using a cystoscope to check the portal vein cavity after removal of the tumor in surgical treatment of HCC with portal vein tumor thrombus (PVTT).

Term explanation

PVTT means "portal vein tumor thrombus".

Experiences and lessons

The cystoscope we used could view the portal vein cavity clearly from the main trunk to the secondary branch, indicating it is a suitable substitute with a proper size and function to check the portal vein system in the surgical treatment of HCC with PVTT.

Peer-review

This is a novel idea to ensure the curability of hepatectomy for HCC with PVTT. The effectiveness of the treatment will require a well-designed clinical trail to further confirm.

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