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2016 Hepatitis B virus: Global view

Natural regression of fibrosis in chronic hepatitis B

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Abstract

The fibrosis of liver cirrhosis was considered to be irreversible before the anti-viral drugs showed that it is reversible when they lead to continuous suppression of viral replication and inflammation. However, several reports previously showed that fibrosis of type B liver cirrhosis was almost completely absorbed after the natural remission of chronic inflammation. This phenomenon might not be limited to exceptional patients, but rather occur commonly, considering the dynamic clinical features of chronic hepatitis B (CHB), where inactive carrier stage normally follows aggravation of hepatitis and progression of fibrosis at the time of HBeAg seroconversion. Thus, fibrosis levels of CHB as a hepatocellular carcinoma (HCC)-surveillance marker, particularly those of the inactive stage, could be underestimated, because some of them might have been (pre)cirrhotic in the past and recovered with the natural regression of fibrosis. We argue that cirrhosis-induced HCC mechanisms, rather than direct action of viral genome, may be more common than generally considered in CHB patients. This may have some impact on reconsidering the surveillance rationale for HCC in CHB, from where advanced HCCs tended to be missed. In addition, a molecular marker to assess the cancer-prone characteristics of the liver will definitely be needed to resolve the issue.

Key words: Chronic hepatitis B; Cirrhosis; Spontaneous remission; Regression of fibrosis; Occult hepatitis B infection; Hepatocellular carcinoma surveillance of hepatitis B virus

Core tip: The fibrosis of liver cirrhosis may be reversible. Regression of fibrosis in hepatitis B virus (HBV) patients with (pre)cirrhosis might be a more common phenomenon than generally considered. This might cause the underestimation of fibrosis levels in chronic hepatitis B, suggesting a difficulty with the surveillance system of HBV-hepatocellular carcinoma (HCC). That is, some HCC patients with non-cirrhotic liver might have been cirrhotic in the past, after which spontaneous regression of fibrosis occurred. Cirrhosis-HCC mechanisms, compared to the direct action of the viral genome, might be more prevalent than generally considered in HBV patients.

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INTRODUCTION

Continuous inflammation of the liver induces deposition of extracellular matrix (ECM) and results in liver cirrhosis. Although several reports showed regression of fibrosis in animal models with liver cirrhosis[1,2], and in human case reports[3-5], established fibrosis of cirrhosis was generally considered to be irreversible[6,7]. However, reversibility of fibrosis in the liver became commonly-known recently because elimination of viruses with anti-hepatitis B virus (HBV) or hepatitis C virus (HCV) drugs caused regression of fibrosis even in cirrhotic cases[8,9].

The natural history of chronic HBV infection pursues a dynamic process caused by the conflict between virus and immune system, which results in the seroconversion of HBeAg to anti-HBe, followed by that of HBsAg to anti-HBs[10]. Long-standing liver injury in the process of HBeAg-seroconversion induces particularly rapid progression of fibrosis that frequently results in cirrhosis. However, due to the dynamic course of the disease, stable clinical remission with low HBV DNA and transaminase levels follows, and the majority of patients become inactive carriers. Natural regression of fibrosis induced by this clinical remission has been reported[3,11].

In general, hepatocellular carcinoma (HCC) with HBV arises more frequently in non-cirrhotic liver than in HCV infection[12,13]. This is considered mainly due to the direct mutagenesis effect of the HBV genome that integrates into the host genome or the direct hepatocarcinogenic action of viral genes such as hepatitis B virus X (HBx)[14]. However, if HCCs have arisen in the liver in which fibrosis naturally regressed from advanced liver fibrosis, they might be misinterpreted as having arisen from a non-cirrhotic liver.

Consequently, it should always be kept in mind that fibrosis levels of chronic hepatitis B (CHB) always have the possibility of being underestimated in terms of HCC-risk, because they might have regressed from a more advanced stage of fibrosis[15]. We attempt to spotlight the natural regression of fibrosis in chronic HBV infection and re-evaluate the clinical background of HBV-induced HCC in this review.

CHRONIC HBV DISEASE AND PROGRESSION OF FIBROSIS

About 350 million people in the world have chronic HBV infection[16]. Chronic HBV infection is a significant cause of liver cirrhosis, which bears a high risk of HCC. The annual rate of development of HCC has been reported to be 10% to 17% in HBV-induced liver cirrhosis[17]. In general, fibrosis levels correlate well with the risk of HCC, as in the case with HCV infection.

The immunological attack against hepatocytes infected with HBV induces a dynamic clinical course which is represented by seroconversion of HBeAg to anti-HBe. Almost no or very mild liver injury is observed in the HBeAg-positive immunotolerant phase[18]. The severity of liver injury and the progression of fibrosis depend on how long and severe the immunological attacks continue in the phase of HBeAg seroconversion. A rapid transition to anti-HBe normally results in a silent clinical course, referred to as the inactive carrier state, while a difficult transition that is accompanied by aggravation of chronic hepatitis result in liver cirrhosis or HBeAb-positive chronic active hepatitis, which frequently bears core-promoter mutations. Importantly, the majority of patients become inactive carriers afterwards, irrespective of their clinical course[10,19].

MECHANISM OF HBV-INDUCED HCC AND CLINICAL BACKGROUND CHARACTERISTICS

Chronic inflammation induces a progression of fibrosis resulting in liver cirrhosis in which HCC frequently develops. This cirrhosis-HCC mechanism is common to both HBV and HCV infections and is considered to play the main role in hepatocarcinogenesis, to which a process of accumulation of genetic mutations and epigenetic events contributes. In addition to this common carcinogenetic mechanism, each virus infection has its own carcinogenic mechanism.

On the other hand, HBV, which is related to retroviruses, integrates into the host genome during the process of replication and acts as a mutagen. In addition, the *HBx* gene, which is a pleiotropic transactivator of many genes, has been known to be directly involved in hepatocarcinogenesis[20]. Because "cirrhosis to HCC" (indirect) and these

Table 1 A comparison of clinic-pathological data between type-B and type-C hepatocellular carcinoma

	Type B (*n* = 97)	Type C (*n* = 81)	*P* value
Age (yr)	56.1 ± 11.9	66.7 ± 9.8	< 0.0001
Sex (M:F)	71.1%:28.9%	60.5%:39.5%	0.18
AST (IU/L)	82.2 ± 98.6	81.8 ± 44.1	0.18
ALT (IU/L)	67.7 ± 70.9	83.2 ± 56.9	0.11
T.Bil (mg/dL)	1.2 ± 1.1	1.0 ± 0.6	0.36
Alb (g/dL)	4.0 ± 0.6	3.9 ± 0.5	0.22
Plt (/mm^3)	13.9 ± 8.1	10.8 ± 5.5	< 0.005
Histology			
F1:2:3:4	2:20:3:32	1:6:8:21	< 0.005
A1:2:3	17:25:12	1:14:17	< 0.005
Clinical stage (Ⅰ:Ⅱ:Ⅲ:Ⅳ)	12:29:25:31	15:32:29:5	< 0.0005

direct mechanisms are not mutually exclusive, it is unknown how much a role each mechanism plays in real hepatocarcinogenesis, especially in individual HCC patients with variable clinical backgrounds.

DIFFICULTY OF HCC SURVEILLANCE IN CHRONIC HBV PATIENTS

Because the carcinogenic processes of HBV and HCV are different, the rationale to perform effective cancer surveillance should be confirmed based on the difference in the specific clinical courses of these infections. Age, sex, fibrosis levels, the presence of HBeAg, HBV viral load, HBsAg levels accounts for the risks of HBV-HCC[21]. In reports from Japan, HCC patients with HBV tended to be younger and have advanced tumor when diagnosed, as well as lower fibrosis levels with good liver function when compared to those with HCV[22,23]. Our result also showed that patients with HBV were younger, with lower fibrosis levels and higher platelet counts than those with HCV (Table 1). Notably, the clinical stages of HBV-HCC were more advanced. These clinical features suggest that cancer surveillance of HBV is much more difficult than that of HCV infection, where continuous elevation of transaminase values induces gradually increasing fibrosis levels, and a low platelet count may be associated with advanced fibrosis levels[24]. In general, a direct hepatocarcinogenic mechanism of HBV, such as the integration of the genome or a direct role of an HBV gene such as HBx, may be considered to be involved in the occurrence of HCC in these "unexpected" patients. However, our question is "Is this the full explanation?"

It seems very paradoxical that HBeAg-positive carriers in the immunotolerant phase with high viral loads and little inflammation, who are supposed have abundant viral gene expression and integration event, seldom suffer from HCC[18]. On the other hand, most HCC patients who were younger than 20 years old were already seroconverted to anti-HBe, and the majority of them were cirrhotic[25-27]. Thus, the cirrhosis to HCC mechanism might play a central role even in these young HCC patients. These facts render the HCC surveillance system for HBV more complex.

REVERSIBILITY OF FIBROSIS WITH LIVER CIRRHOSIS WITH ANTI-HBV TREATMENT AND NATURAL COURSE

The generally accepted idea was that the fibrosis of liver cirrhosis is an irreversible process, and established fibrosis could not regress. However, the development of innovative anti-viral drugs for HBV and HCV changed this long-lasting concept[8]. Fibrosis is reversed when inflammation is suppressed by effective anti-viral agents, and even cirrhosis can be cured. Dienstag *et al*[28] reported that the fibrosis of cirrhosis was reversed during 3.5 years lamivudine treatment. Several reports also confirmed the improvement of fibrosis levels with anti-HBV drugs[29,30].

However, the regression of fibrosis occurs even in the natural course of CHB, where remission of inflammation usually follows the aggravation of hepatitis and progression of fibrosis that occurred during the HBeAg-positive stage[31]. In the past, several reports showed the improvement of fibrosis during the natural course of CHB. Fong *et al*[3] showed the improvement of histology comparing before and after the seroclearance of HBsAg. Waneless *et al*[32] reported that most cirrhotic livers showed some findings indicating the regression of fibrosis, irrespective of etiology. Natural regression of fibrosis consists of three steps, that is, thinning of fibrous septa, regeneration of hepatocytes and recovery of acinal structure. The regression of fibrosis occurs at a particularly early stage of improvement[6].

Recently, Jang *et al*[33] reported improvement of the long-term prognosis with anti-HBV drugs for patients with HBV-cirrhosis. However, suppression of HCC development was not observed in these cirrhotic patients, while it has been confirmed in patients with chronic hepatitis[8]. These results strongly suggest that liver cirrhosis still provides a strong risk for HCC, even after the eradication of virus and suppression of

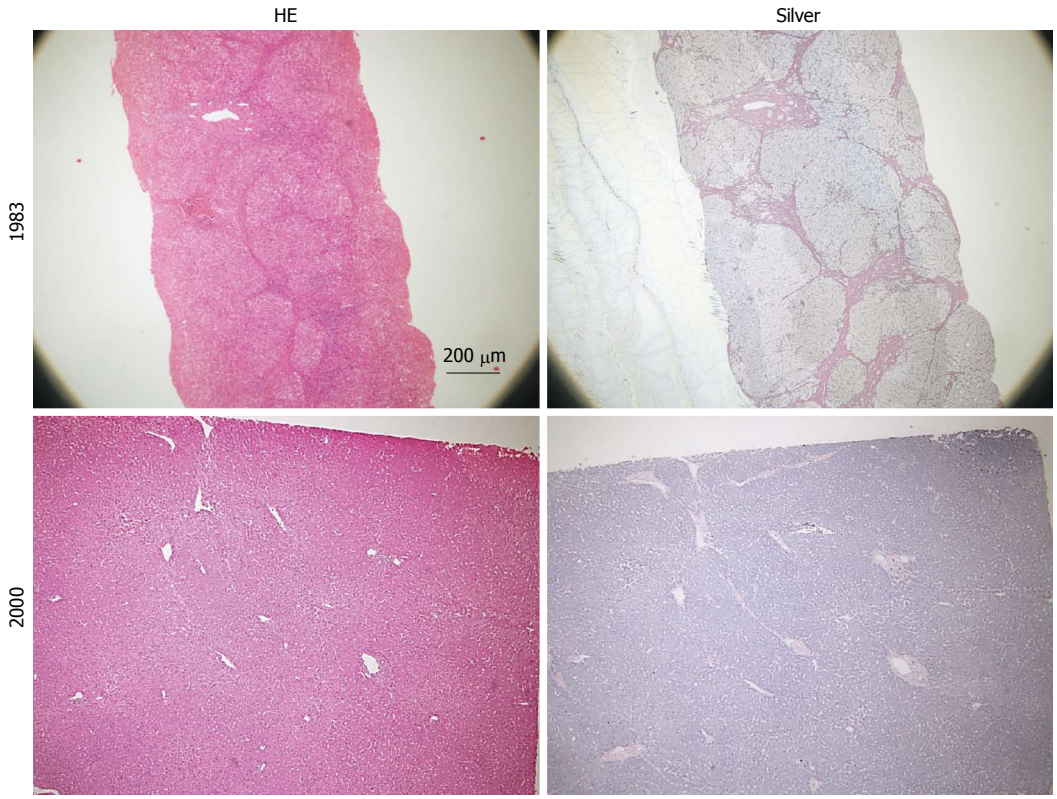

Figure 1 Liver histology of a patient obtained in 1983 (58 year old, needle biopsy) and 2000 (surgical resection of hepatocellular carcinoma). Slides stained with hematoxylin-eosin are shown on the left and those stained with silver are on the right. Marked reduction of fibrosis with improvement of inflammation is observed (This patient was patient 1 in our previous report[4]).

inflammation.

THE LESSONS FROM THE HCC CASES THAT OCCURRED AFTER HBSAG SEROCLEARANCE

We previously reported several cases with HCC that developed after seroconvesion from HBsAg to anti-HBs[4,34]. They progressed to liver cirrhosis or precirrhosis when they had HBeAg-positive chronic hepatitis several decades earlier and became inactive carriers with low viral load and long-standing normal transaminase levels after the seroconversion to anti-HBe. HCC developed further after the seroconversion to anti-HBs. Hepatic fibrosis was dramatically reversed and found to become only thin septa. Changes in liver histology of one patient[4] are shown in Figure 1. Similarly, Bortolotti *et al*[5] reported 2 pediatric patients with HBV-induced liver cirrhosis, whose fibrosis was almost completely resolved after normalization of transaminase levels. Similar to our cases, these results suggested that fibrosis of cirrhosis could be dramatically resolved when viral replication and inflammation were suppressed[31,35]. These cases, considering the dynamic natural course of HBV infection, give us some important suggestions about the clinical background characteristics of HBV-induced hepatocarcinogenesis.

First, as with anti-HBV treatment, these cases are typical examples showing that advanced fibrosis could be absorbed even in the natural course of CHB. After HBeAg-positive chronic hepatitis progresses to liver cirrhosis, the majority of patients follow the clinical course of long-standing remission with a low viral load. Considering this typical clinical course of CHB, natural regression of fibrosis might not be an exceptional phenomenon limited to these case reports, but it might occur commonly. This also suggests that some population of HBV-HCC patients with non-cirrhotic liver might have regressed fibrosis that was once cirrhotic or pre-cirrhotic. Consequently, our hypothesis is that the incidence and role of cirrhosis-derived HCC might be underestimated in HBV-HCC, and the roles of direct carcinogenesis due to HBV integration or viral gene function with a slight involvement of necroinflammation in non-cirrhotic HCC are overestimated.

HBV-cirrhosis is "macronodular" and has thinner septa than HCV[32]. Micronodular cirrhosis changes to macronodular with the suppression of inflammation[36]. This morphological observation might reflect the clinical course of these viruses, where HCV takes a continuous progressive course, and HBV is characterized by the alternating increases and normalization of transaminase levels.

Another important suggestion relates to the assess-

ment of fibrosis in CHB patients for the establishment of an effective HCC surveillance system. Recently, not only liver biopsy which is a gold standard for the evaluation of fibrosis, but a handful of methods to assess fibrosis levels have been used clinically. However, these methods may not discriminate these cases of "regressed fibrosis from cirrhosis", which might have more risk of HCC than "ongoing fibrosis". Thus, it might be possible to underestimate the risk of HCC in these patients with regressed fibrosis levels using these current methods. Indeed, needle biopsy might give the diagnosis of an almost normal liver with completely regressed fibrosis[32].

Cirrhosis-HCC mechanisms can be glimpsed in studies that examined the incidence of HCC after the seroclearance of HBsAg, which normally has a good prognosis[10,37,38]. Yuen *et al*[39] reported that the risk factors of HCC at this stage were the presence of liver cirrhosis and old age. Liver cirrhosis was also counted as a significant factor for the seroclearance of HBsAg[40]. This indicates that the strong immune reaction that caused liver cirrhosis eventually contributed to the seroclearance of HBsAg, providing some risk of HCC.

Similar suggestions could be applied to HCC cases with occult HBV infection (OHB). Some patients might have overt chronic HBV infection before entering the stage of OHB[41]. These patients might have "regressed fibrosis from advanced fibrosis" during the process of becoming seronegative for HBsAg. They can easily be missed during clinical surveillance of HCC, unless they were recognized as HBV carriers, possibly in early life[15]. Integration of HBV DNA or the direct action of an HBV gene such as HBx from long-standing viral replication is considered to play a role in the occurrence of HCC with OHB[42]. However, cirrhosis-HCC mechanism might play a central role in more number of patients with OHB-HCC than generally considered.

Liver biopsy has been a gold standard of evaluation for liver fibrosis[43]. Non-invasive methods for evaluation of fibrosis are widely used in clinical settings. They have been primarily used for the assessment of HCV-related fibrosis, and their application to HBV-induced fibrosis has always been under constant debate[44]. Transient elastography (TE) is one of the most reliable recent modalities to assess the fibrosis level without biopsy[45]. Several reports evaluated the efficacy of TE in both CHB and CHC patients and reported that diagnostic accuracy was almost equal between them[46,47]. However, a lower liver stiffness (LS) cut-off in CHB than in CHC in the advanced fibrosis stage was reported[48]. The interpretation of this was that fibrous septa were thinner in CHB than in CHC. Moreover, because CHB tended to have macronodular cirrhosis, TE waves go through liver parenchyma and produce a low stiffness value[49]. Likewise, Castéra *et al*[50] reported that the cut off values of TE in cirrhosis were higher in HCV-LC than that of HBV-LC and this difference came from the high prevalence of macronodular LC in HBV. Thus, although fibrosis markers are considered to be useful in the evaluation of fibrosis levels of CHB in general[49], there have been no studies attempting to assess the fibrosis levels following CHB patients from aggravation of hepatitis to the inactive stage.

APPLICATION OF MOLECULAR ANALYSIS TO THE "HCC FROM REGRESSED FIBROSIS"

Innovative advances of molecular analysis recently allow comprehensive cancer genome analysis in a short time, resulting in the accumulation of an overwhelming body of information about the cancer genome[51]. Schulze *et al*[52] analyzed the whole exon of 243 HCC tissues and reported that genomic pathways centered on three signaling abnormalities, β-catenin (CTNNB1), TP53, and AXIN1-related. They also suggested that they can distinguish HCC that arose from cirrhosis from those derived from non-cirrhosis using the difference in incidence of CTNNB1 TP53, a mutation of telomerase reverse transcriptase (TERT). In addition, mutations of TERT promoter have been noted as a characteristic genomic change of HBV-HCC, in which integration of the HBV genome frequently occurs[53,54]. It is interesting to know whether HCC that arose from "liver with regressed fibrosis from cirrhosis" is close to a cirrhotic or a non-cirrhotic pattern.

Telomere shortening is a general genetic marker of liver cirrhosis causing senescence of hepatocytes that is associated with the development of HCC[55-57]. Recently, TERT promotor mutations were observed already in premalignant nodules in cirrhotic liver[58]. In addition, Hartmann *et al*[59] reported that TERT gene mutations were frequently detected in liver cirrhosis irrespective of etiology, suggesting that they may be used to evaluate cancer-prone characteristics of liver. This result might be applicable in the evaluation of whether the liver with regressed fibrosis has oncogenic potential close to that of real cirrhosis, helping the understandings of the carcinogenic potential of that state. Such molecular markers that can assess cancer-prone characteristics of cirrhotic or non-cirrhotic liver are much needed to prove that a cirrhosis-HCC mechanism might be involved in the development of HCC than currently considered, even in low-fibrosis cases.

CONCLUSION

The clinical course of chronic HBV infection is dynamic and complex, with changes of serological markers, which is distinct from HCV infection that takes a more continuous clinical course. Because of this clinical profile, long-standing remission after established cirrhosis, result in the regression of fibrosis, where only thin septa are recognized. On the other hand, the integration of

the HBV genome or a direct gene function, such as HBx, may play an important role in so-called direct hepatocarcinogenesis. HCCs from regressed fibrosis and those *via* direct hepatocarcinogenesis might be categorized together into "HCC from non-cirrhotic liver". Thus, these mechanisms render the assessment of fibrosis as a risk factor for HCC very complex. Therefore, there is an urgent need to establish effective cancer surveillance system that addresses these complex issues. In addition, molecular markers to evaluate the risk of HCC in non-cirrhotic as well as cirrhotic liver should be clarified in a future analysis.

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

X region mutations of hepatitis B virus related to clinical severity

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Abstract

Chronic hepatitis B virus (HBV) infection remains a major health problem, with more than 240 million people chronically infected worldwide and potentially 650000 deaths per year due to advanced liver diseases including liver cirrhosis and hepatocellular carcinoma (HCC). HBV-X protein (HBx) contributes to the biology and pathogenesis of HBV *via* stimulating virus replication or altering host gene expression related to HCC. The HBV X region contains only 465 bp encoding the 16.5 kDa HBx protein, which also contains several critical cis-elements such as enhancer Ⅱ, the core promoter and the microRNA-binding region. Thus, mutations in this region may affect not only the HBx open reading frame but also the overlapped cis-elements. Recently, several types of HBx mutations significantly associated with clinical severity have been described, although the functional mechanism in most of these cases remains unsolved. This review article will mainly focus on the HBx mutations proven to be significantly related to clinical severity *via* epidemiological studies.

Key words: Hepatitis B virus infection; Hepatitis B virus-X protein mutation; Hepatocellular carcinoma; Clinical severity

Core tip: Of hepatitis B virus (HBV)-X protein (HBx) mutations related to clinical severity, the A1762T/G1764A BCP mutation is one of the most frequently

encountered HBx mutations and plays a significant role in liver disease progression in chronic patients with HBV infections. It also further contributes to disease progression by inducing mutations of other HBx mutations related to clinical severity, such as G1386A/C (V5M/L), C1653T (H94Y), T1753V (I127V) and HBx C-terminal deletion or insertion. Moreover, T1753V (I127L,T,N,S) and C1653T (H94Y) mutations in the enhancer Ⅱ/BCP region and A1383C, G1386A/C (V5M/L) and C1485T (P38S) in the negative regulation domain are significantly related to severe types of liver diseases, including hepatocellular carcinoma. Furthermore, deletions or insertions affecting the C-terminal region of HBx may also contribute to the severity of the clinical outcome in chronic patients. In addition, our recent study indicated that a novel mutation type (X8Del) composed of an 8-bp deletion in the C-terminal region of the HBx could contribute to occult HBV infection in vaccinated Korean individuals *via* a reduced secretion of HBsAg and virions. Therefore, several distinct types of HBx mutations may contribute to HBV pathogenesis by regulating HBV replication or host genes related to cell homeostasis.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection remains a major health problem with more than 240 million people chronically infected worldwide, which potentially causes 650000 deaths per year due to advanced liver diseases including liver cirrhosis and hepatocellular carcinoma (HCC), particularly in endemic areas such as China and South Korea[1,2]. It is generally accepted that HBV infection accounts for approximately 50% of the HCC cases worldwide and even 80%-90% in highly endemic areas[1].

HBV is an enveloped Hepadnavirus belonging to the *Hepadnaviridae* family, with an incomplete double-stranded DNA genome of approximately 3.2 kb in length with four overlapping open reading frames (ORFs) encoding the polymerase (P), core (C), surface antigen (S), and X protein[3]. The S gene encodes a family of surface antigen polypeptides embedded within the viral envelope, which is a major target for diagnosis and protective vaccines. The C gene encodes the core antigen, which forms the nucleocapsid, within which reverse transcription of pre-genomic RNA occurs. The P gene encodes the virus reverse transcriptase, which also has RNase H and DNA polymerase activities[4-6]. Transcription of HBV proteins is controlled under four promoters (preS1, preS2, core and X) and two enhancers (EnhI and EnhII) in the viral genome, which overlap with those ORFs. Because it contains a polymerase without proofreading activity and uses an RNA intermediate (pgRNA) during its replication, the HBV genome has a higher mutation ratio than other DNA viruses[7-11]. Moreover, host immune pressures and interventions such as antiviral drugs and vaccines make the viral mutations more complicated[12-18].

Based on an intergroup divergence of > 8% in its complete genome sequence, the HBV strains are classified into eight genotypes, which are designated A-H, with a distinct ethnic and geographical distribution[1,19-21]. Different genotypes have distinct geographical distributions and usually induce various clinical outcomes. For instance, genotype C, the most prevalent genotype in Asia, is more prone to mutations and is associated with more severe liver diseases and lower antiviral responses compared with genotype B[3,22,23]. In particular, genotype C2 is reportedly responsible for the most chronic infections in South Korea. Indeed, several types of HBV mutations that are never or rarely encountered in other areas have been found in South Korea and have been proven through molecular epidemiologic or functional studies to be related to disease progression in chronic patients[24-44].

HBV X PROTEIN STRUCTURE AND FUNCTION

The HBV X protein (HBx) is a multifunctional non-structural protein that contributes to HBV biology and pathogenesis by stimulating virus replication or altering host gene expression related to HCC. HBx contains only 465 bp encoding the 16.5 kDa protein, which also contains several critical cis-elements such as EnhII, the core promoter and the microRNA-binding region[45-47] (Figure 1).

HBx plays a significant role in sustained HBV replication, which is a major risk factor for HCC development *via* proteasome inhibition[48,49], transactivation of HBV enhancer or promoters[50], autophagy induction[51,52], or polymerase activation by Ca^{2+}-dependent signaling[53-55]. HBx can also regulate HBV replication through epigenetic modifications, by being recruited onto the viral minichromosome in the nuclei of infected hepatocytes along with cellular histone acetyltransferases such as CREB-binding protein (CBP)/p300[56,57] and histone deacetylases such as HDAC1 and hSirt1[58]. HBx can help establish and maintain chronic infection by altering the patterns of host innate immunity, which causes the development and progression of chronic liver diseases in the absence of virus elimination[59,60]. HBx blocks apoptotic signaling and activates signaling pathways (such as NF-κB and PI3K) that override apoptotic signals from extrinsic ligands such as Fas or TNF-alpha[61,62]. HBx also plays an important role in hepatocarcinogenesis by inactivating the tumor suppressor p53[63], promoting Rb inactivation by

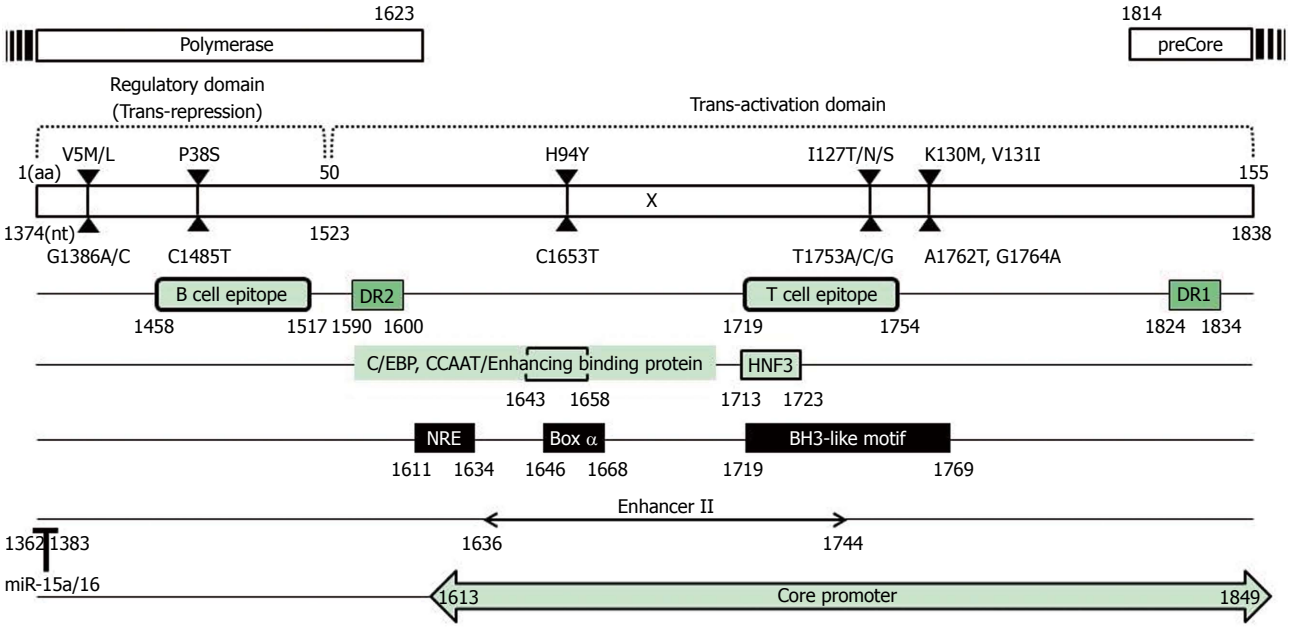

Figure 1 Hepatitis B virus-X protein genome structure. The HBV X region contains 465 bp (nt 1374 to 1838) encoding the 16.5 kDa HBx protein composed of 154 aa, which also contains several critical cis-elements such as EnhII (nt 1636 to 1744), the core promoter (nt 1613 to 1849) and the microRNA-binding region (nt 1362 to 1383). Thus, HBx mutations affected not only the HBx open reading frame but also the overlapped cis-elements. HBx: Hepatitis B virus-X protein.

phosphorylation[64], and compromising DNA repair mechanisms[65]. Consequently, mutations in the HBx ORF region may affect not only the HBx ORF and the overlapped cis-elements but also its binding capacity for host proteins. Recently, several types of HBx mutations significantly associated with clinical severity have been described mainly from chronic patients infected with genotype C[28,31,39,66-83], although the functional mechanism in most of these cases remains unsolved. This review article will mainly focus on the HBx mutations that have been proven to be significantly related to clinical severity *via* epidemiological studies (Table 1).

HBx MUTATIONS RELATED TO CLINICAL SEVERITY

Mutations in EnhII and (or) the core promoter region (BCP mutation, T1753V, and C1653T)

In general, 3 types of mutations in the EnhII/BCP region [one mutation in EnhII (H94Y: C→T of nt 1653) and two mutations in BCP (I127L,T,N,S: T→V of nt 1753, K130M and V131I: A→T of nt 1762 and G→A of nt 1764)] are mutational "hot spots", namely, the most frequently encountered among naturally occurring HBx mutations related to clinical severance from chronic hepatitis B patients, irrespective of genotype or geographical distributions (Table 1). The A1762T/G1764A BCP mutation leading to two overlapped HBx amino acid changes, K130M and V131I, is the most frequent HBV DNA mutation identified in many studies as being associated with HCC risk and outcomes[72,74,84-87]. The exact mechanism underlying the role of this mutation in hepatocarcinogenesis is still unknown. However, some underlying mechanisms have been recently elucidated. The mutation can cause a substantial decrease in HBeAg expression and enhancement of viral genome replication, which contribute to the liver disease progression *via* increased inflammation and viral invasion[88,89]. The mutation also leads to a truncated HBx protein, which not only promotes hepatocellular proliferation but also enhances HCC cell invasion and metastasis[90,91]. In particular, in chronic patients infected with genotype C2, this mutation is reported to be related to HBV genome deletion[26,31] or to be positively correlated with HBx M5V/L or H94Y mutations[28,37]. In addition, it may also contribute to hepatocarcinogenesis *via* reduced p21 expression, leading to rapid and uncontrolled cell proliferation[92]. A recent meta-analysis by Yang *et al*[82] revealed that the BCP mutation is present at significantly higher frequencies in HCC patients than in non-HCC controls, including patients with liver cirrhosis, chronic hepatitis and asymptomatic carriers. Our previous data using a Korean cohort with genotype C2-infected chronic patients also showed that the BCP mutation was the most frequently encountered mutation related to clinical severity (66.1%, 123/184 strains) and was significantly related to HCC [HCC (86.7%) *vs* chronic hepatitis (61%), $P = 0.017$; HCC (86.7%) *vs* asymptomatic carrier (24.4%), $P < 0.001$][28]. Our data also showed that during the natural course of HBV chronic infection, the most significant rise in the rate of the BCP mutation was found during the progression from asymptomatic carrier to chronic hepatitis (24.4%-61.0%), suggesting that the BCP mutation may play a major role in liver

Table 1 Mutations in the hepatitis B virus X region as related to clinical severity

Type of mutation	Mutations				Genotype	Clinical Significance			Region	Description	Ref.
	Amino acid		Nucleotide			HCC (%)	Non-HCC (%)	*P* value			
	aa	mutations	nt	mutations							
AS	4	NC	1383	A1383C	B/C	HCC (postoperative survival in patients with HBV-HCC) (P = 0.028)				Independent predictors of HCC survival	[65]
						52.8	25.8	0.003		miRNA binding site	[66]
AS	5	V5M/L	1386	G1386A/C	C	50.0	4.9	< 0.001			[27]
						47.8	0.0	< 0.001			[38]
						37.7	13.3	0.002			[67]
AS	30	NC	1461	G1461A/T/C	B/C	HCC (postoperative survival in patients with HBV-HCC) (P = 0.005)			B cell epitope	Independent predictors of HCC survival	[65]
AS	36	T36P/S/A	1479	A/G1479C/T/G	A/C/D	49.3	22.7	0.034	B cell epitope	B cell epitope	[66]
						15.3	7.8	< 0.001		B cell epitope	[66]
						80.0	17.1	< 0.010			[68]
AS	38	P38S	1485	C1485T	B/C	21.7	4.9	0.023	B cell epitope		[27]
						HCC (postoperative survival in patients with HBV-HCC) (P = 0.006)				Independent predictors of HCC survival	[65]
						48.7	13.9	0.001		Independent risk factor for the HCC	[69]
						29.9	16.8	0.001		B cell epitope	[66]
						30.4	15.0	0.038			[67]
AS	44	A44V	1504, 1505	C1504G, C1505G	A/D	35.9	6.9	0.012	B cell epitope		[70]
AS	50	G50R	1521	G1521A/C	A/D	60.0	4.3	< 0.010			[68]
AS	57	NC	1544	T1544A/C	B/C	HCC (postoperative survival in patients with HBV-HCC) (P = 0.039)				Independent predictors of HCC survival	[65]
AS	80	NC	1613	G1613A	B/C/C2	HCC (postoperative survival in patients with HBV-HCC) (P = 0.006)			Core promoter	Independent predictors of HCC survival	[65]
						54.7	28.3	0.001		Significance of association with HCC	[71]
						38.0	10.0	< 0.050			[72]
AS	81	NC	1613	G1613A	B/C	50.0	8.6	0.001	Core promoter		[73]
AS	86	NC	1631	C1631T	C	8.3	1.8	0.010	Core promoter	CP, NRE	[66]
AS	94	H94Y	1653	C1653T	B/C/C2	40.0	4.9	< 0.001	Box α, C/EBP,CCAAT/enhancing binding protein, Core promoter, EnhII		[27]
						HCC (postoperative survival in patients with HBV-HCC) (P = 0.015)				Independent predictors of HCC survival	[65]
						61.3	25.3	< 0.001		Significance of association with HCC	[71]
						56.0	30.0	0.0013			[74]
						45.0	19.0	< 0.050			[72]
						31.6	19.1	0.016		Box α	[75]
						35.4	18.6	< 0.001		Box α, CP, C/EBP, EnII	[66]
						41.2	13.3	< 0.001			[67]
						55.5	2.9	< 0.001			[73]
						8.9	2.2	0.017			[76]
AS	101	S101Stop	1675	C1675A	B/C	35.3	5.3	0.001	Core promoter, EnhII	EnhII	[77]
AS	106	S106T	1689	T1689A	C2	19.3	4.4	< 0.001	Core promoter, EnhII		[76]

AS	116	L/V116V/L	1719	T/G1719G/T	B/C	HCC (postoperative survival in patients with HBV-HCC) (P = 0.020)			BH3-like motif, Core promoter, EnhII, NRE	Independent predictors of HCC survival	[65]
						82.6	57.6	0.010		BH3-like motif, CP, EnhII, HNF3, T cell epitope	[66]
AS	117	NC	1724	T1724C	B/C	41.1	2.6	0.000	BH3-like motif, Core promoter, EnhII, NRE	EnhII	[77]
AS	118	NC	1727	A1727G	D1	35.0	5.0	0.001	BH3-like motif, Core promoter, EnhII, NRE		[78]
AS	123	L123S	1741	T1741C	D1	30.0	7.5	0.006	BH3-like motif, Core promoter, EnhII, NRE		[78]
AS, DM	127	I127L/T/N/S	1753	T1753C/A	C	36.7	12.2	0.007	BH3-like motif, Core promoter, NRE		[27]
			1753	T1753C	B/C	HCC (postoperative survival in patients with HBV-HCC) (P = 0.047)				Independent predictors of HCC survival	[65]
			1752, 1753	A1752C + T1753A/C/G	D	52.2	20.0	0.033			[79]
			1753	T1753A/C/G	C2	50.7	16.0	< 0.001		Significance of association with HCC	[71]
			1753	T1753A/C/G	C2	50.0	24.0	0.001			[74]
			1753	T1753C/G	A/D	43.6	17.2	0.041			[70]
			1753	T1753A/C/G	B/C	30.9	17.6	0.006		EnhII/BCP	[75]
			1753	T1753A/C/G	C	29.0	5.0	< 0.001			[67]
AS	130	K130M	1762	A1762T	C2	94.7	74.7	0.001	BH3-like motif, Core promoter	Significance of association with HCC	[71]
					B/C	80.0	25.7	< 0.001			[73]
AS	131	V131I	1764	G1764A	C2	98.7	78.7	< 0.001	BH3-like motif, Core promoter	Significance of association with HCC	[71]
					B/C	95.0	31.4	< 0.001			[73]
AS, DM	134	NC	1773	C1773T	D1	95.0	52.5	0.000	Core promoter		[78]
			1773, 1775	C1773T + A1775G	D1	17.5	0.0	0.010			[78]
AS	143	C143R	1800	T1800C	C	3.5	0.3	0.008	Core promoter	CP	[66]
AS, DM	100, 102	NC	1673, 1679	C1673T + A1679G	D1	17.5	0.0	0.010	Core promoter		[78]
AS, TM	128, 131	NC + V131L	1757, 1764, 1766	G1757A, G1764C + C1766G	D1	37.5	12.5	0.010	Core promoter		[78]
AS, DM	130, 131	K130M + V131I	1762, 1764	A1762T + G1764A	C	86.7	24.4	< 0.001	Core promoter		[27]
					D	HCC (HBV-DNA ⩾ 5 log copies/mL) *vs* CLD (HBV-DNA < 5 log copies/mL) (P < 0.05)					[79]
					C2	91.0	73.0	0.0035			[74]
					A/D	62.5	14.3	0.034			[80]
					A/D	64.1	20.7	0.000			[70]
					B/C	71.1	55.7	0.009		EnhII/BCP	[75]
					B/C	64.0	50.8	< 0.00001			[81]
					A/D	44.9	20.7	< 0.001			[82]
					C	91.5	53.3	< 0.001			[67]
					C2	60.7	22.2	< 0.001			[76]
Del, Ins	129-154, 120-148, 115-149, 135-154, 137-151	Deletion	93-94 (4aa), 79-80 (2aa), 93-94 (4aa), 151-152 (3aa)	Insertion	C2	HCC + LC (7.6%) *vs* CH + C (1.5%) (P = 0.017)					[30]

AA: Amino acid; AS: Amino acid substitution; BCP: Basal core promoter; C: Carrier; CH: Chronic hepatitis; CLD: Chronic liver disease; CP: Core protein; Del: Deletion; DM: Double mutation; EnhII: Enhancer II; HCC: Hepatocellular carcinoma; HNF3: Hepatocyte nuclear factor 3; Ins: Insertion; LC: Liver cirrhosis; miRNA: MicroRNA; NC: No change; NRE: Negative regulatory element; TM: Triple mutation.

disease progression, especially in the progression from asymptomatic carrier to chronic hepatitis in chronic patients infected with genotype C2[28]. This finding has also been confirmed by a recent meta-analysis[82]. Yang *et al*[82] also demonstrated that HBV-infected patients with genotype C, including HCC patients, have a significantly higher risk of BCP mutation compared with those with genotype B, suggesting that the BCP mutation can increase the risk of HBV-related hepatocellular carcinoma, particularly in an HBV genotype C population.

An HBV genome transfection-based experiment indicated that the BCP mutation can reduce the synthesis of HBeAg and enhance viral replication. However, a meta-analysis and our previous report also showed that there is no significant difference in BCP mutation prevalence between HBeAg-positive and HBeAg-negative chronic HBV-infected patients[28,82], suggesting that BCP mutation may occur in the HBeAg-positive immune tolerance phase earlier than in the HBeAg-negative immune clearance phase, at least in chronic patients infected with genotype C2.

The other HCC-associated T1753V mutation (I127L,T,N,S: T→V of nt 1753) was also shown to affect HCC survival[93,94]. The mutations in the HBx protein, which include an I127L,T,N,S amino acid substitution, can change the HBx binding affinity to BCL2, thereby affecting HBx-induced apoptosis[95]. Our previous data using Korean HBV-infected patients with genotype C2 showed that the prevalence of this mutation was also significantly higher in chronic patients with severe liver disease, HCC or liver cirrhosis than in patients who had milder types of diseases, were carriers or had chronic hepatitis [HCC and LC (34.3%) *vs* chronic hepatitis and carrier (13.4%), $P < 0.001$][28]. The other study using chronic patients from India who had genotype A or D revealed that this mutation is also usually associated with advanced forms of liver disease and had an increased risk of HCC[69], suggesting that the T1753V mutation may play a significant role in liver disease progression. Our previous report showed that the T1753V mutation is significantly related to the BCP double mutation [patients with the BCP mutation (31.7%) *vs* patients without the BCP mutation (11.5%), $P = 0.003$], but not to HBeAg serostatus[28]. A recent multivariate survival analysis by Xie *et al*[66] showed that the T1753V mutation is an independent predictor of HCC survival.

The C1653T mutation, leading to a simultaneous H94Y amino acid change in HBx, is located in box α, which is a strong activation element of the EnhII/core promoter, can enhance the box α binding affinity and EnhII/core promoter activity[96,97]. Because many trans-regulated nuclear factors bind HBV at the 1653 site, this mutation can alter the binding affinity of these nuclear factors. The C1653T mutation has been recently reported to be a predictive factor for HCC in Japan[75,98] and is associated with fulminant hepatitis and the acute exacerbation of HCC[99,100]. A recent multivariate survival analysis by Xie *et al*[66] showed that the C1653T mutation together with the T1753V mutation is also an independent predictor of HCC survival. Furthermore, our previous report showed that the C1653T mutation is significantly related to advanced liver diseases in Korean patients with genotype C2 infections [patients with HCC or LC (36.3%) *vs* patients who have chronic hepatitis or are carriers (12.2%), $P < 0.001$]. It has been reported that the C1653T mutation, together with 1762T/1764A mutations, can reduce the pre-C mRNA level (to approximately 55%) and HBeAg secretion in a transient transfection system using Huh7 cells[101]. Our previous study also demonstrated that this mutation tended to be related to an HBeAg-negative serostatus ($P = 0.087$) and was significantly related to the BCP mutation [patients with the BCP mutation (35.0%) *vs* patients without the BCP mutation (6.6%), $P < 0.001$].

Mutation in the negative regulation domain of HBx (aa 1-50) (A1383C, G1386A/C-V5M/L, C1485T-P38S)

The A1383T synonymous mutation, which does not cause an amino acid change in the HBx protein, is located in the negative regulation domain of HBx (aa 1-50), and this mutation was first found to be associated with HCC in a Korean cohort[28]. In one clinical study using Chinese cohort mostly infected with genotype B and C, this mutation was also associated with a worse prognosis in patients after liver transplantation[66]. Recently, a comprehensive analysis study based on global data by Li *et al*[67] showed that A1383T is one of the HBx mutations identified as independent risk factors for genotype C HBV-related HCC. It has also been reported that tumor suppressor microRNA 15a/16 (miR-15a/16) can directly target the wildtype HBx RNA sequence (nt1362-1383), inducing Bcl-2 expression by acting as a sponge to bind and sequester endogenous miR-15a/16. Consequently, this mutation can lead to a reduced binding capacity of miR-15a/16 to the HBx protein[47], which can prevent the infected cell from apoptosis by altering critical cell signal pathways and thereby contributing to hepatocarcinogenesis.

The G1386A/C mutation leading to a simultaneous V5M/L amino acid change at codon 5 of the HBx protein was first introduced by our previous study using a Korean cohort with genotype C2 infections[28]. Our data showed that this mutation was significantly more frequently found in HCC patients than in patients in other disease groups. Notably, the prevalence of this mutation was abruptly increased in HCC patients rather than in liver cirrhosis patients during disease progression (HCC *vs* liver cirrhosis; 49.2% *vs* 25.6%, $P = 0.024$), strongly suggesting that this mutation is a genuine HCC-specific mutation that possibly plays a pivotal role in the progression from liver cirrhosis to HCC[28]. Recently, the combination of both BCP double

mutations and both types of the V5M mutation, V5M and V5L, has also been reported to increase the HCC risk by 5.34 times compared with the wild type by inducing a higher NF-κB activity in transformed cells[86]. Our previous report showed that this mutation is significantly related to an HBeAg-negative serostatus [HBeAg-negative patients (40%) *vs* HBeAg-positive patients (19.1%), $P = 0.004$], suggesting that it may be generated from the immune clearance phase[28]. This mutation was also significantly related to the BCP mutation [patients with the BCP mutation (36.6%) *vs* patients without the BCP mutation (9.2%), $P < 0.001$]. To date, its clinical relevance has not been introduced except for a Korean cohort with genotype C2 infections. It is tempting to speculate that this mutation may play a pivotal role in hepatocarcinogenesis during the HBeAg-negative immune clearance phase during the natural course of genotype C2 HBV infection.

The C1485T mutation, leading to simultaneous P38S in the HBx protein, were first introduced as an independent risk factor for HCC development in a study by Muroyama *et al*[70] using a Japanese cohort with genotype C infections. Both studies using Korean cohorts with genotype C2 infections[28,68] and a recent investigation based on global data by Li *et al*[67] also revealed that this mutation is significantly related to HCC. A functional study supporting the relationship between the mutations with HCC still remains to be conducted. However, given that its mutation site is located at the B cell epitope region (Figure 1), this mutation may lead to persistent infection by providing a mechanism of evading the humoral immune response of the host.

Deletions or insertions in the C-terminal region of HBx

The C-terminal region of HBx plays a key role in controlling cell proliferation, viability, and transformation[102-105]. Therefore, C-terminally deleted or inserted HBx has reduced transactivation activity and inhibitory effects on cell proliferation and thus may contribute to HCC generation[106]. Moreover, its reduced transacting capacity might reduce HBV replication[107]. The C-terminal deletion or insertion is one of the most frequently reported mutations of HBx and has been frequently detected in tissues and serum samples from HCC patients, irrespective of genotype or geographical distribution[24,108,109]. Our previous report using a Korean cohort with genotype C2 infections showed that the prevalence of deletions or insertions was significantly higher in patients with severe liver disease, HCC, or cirrhosis of the liver (7.2%, 10/132), compared with patients who were carriers or had chronic hepatitis (1.5%, 2/135) ($P = 0.017$)[31]. All deletions in six strains were concentrated at the C-terminal end of HBx, encompassing the 113th to 154th codons. Four types of insertions (PKLL, GM, FFN, and tt) were observed in six patients. Notably, all insertions were accompanied by a BCP double mutation[31] (Figure 2). Furthermore, we first introduced a novel HBxAg vaccine escape mutation, X8Del with an 8-bp deletion in the C-terminal region of the HBx gene from 6 vaccinated Korean subjects[38]. Our *in vitro* and *in vivo* studies showed that this mutation causes a reduced secretion of HBsAg and HBV virions compared with the wild type, suggesting that the X8Del mutation may contribute to occult HBV infection in vaccinated individuals *via* the reduced secretion of HBsAg and virions, possibly by compromising the transacting capacity of HBxAg[38].

Other HBx mutations related to clinical severity

Recently, Xie *et al*[66] have reported 8 HBx mutational sites identified as significant independent risk predictors of HCC survival: 1383, 1461, 1485, 1544, 1613, 1653, 1719, and 1753 from a Chinese cohort mostly infected with genotype B and C. Despite the fact that the G1461V mutation is located at the B cell epitope, it (as a synonymous mutation) did not cause any simultaneous amino acid change in the HBx protein. Its regulatory modification in host cell or virion replication remains to be solved. The T1544V mutation also did not cause an amino acid change in the HBx protein. The G1613A mutation in the core promoter region is also a synonymous mutation, and its relationships with HCC have been reported in other previous studies.

Mutations in the BH-3-like motif of HBx can interfere with its interaction with two other Bcl-2 family members (Bcl-2 and Bcl-xL, which are critical for HBx to increase the intracellular calcium concentration), playing a significant role in viral replication and cell death[110]. Previous studies have reported that several types of mutations in the BH-3-like motif, T/G1719G/T, T1724C, and T1741C, were also significantly related to HCC[66,67,79].

The T1800C mutation leading to a simultaneous C143R amino acid change in the HBx protein is a novel genotype C HCC risk mutation identified by the Li *et al*[67] study, based on global data. To date, the function of this mutation in HCC remains unclear. However, of note, a recent study regarding HBV integration sites in 88 Chinese HCC patients showed that almost 40% of the integrated HBV genomes were cleaved at approximately nt1800, suggesting a potential role of this site in carcinogenesis, given that HBV genome integration has long been considered an important factor in HCC development.

CONCLUSION

In conclusion, HBx mutations may affect not only the HBx ORF but also the overlapped cis-elements. Considering all the HBx mutations related to clinical severity, the A1762T/G1764A BCP mutation is one of the most frequently encountered HBx mutations and plays a significant role in liver disease progression in

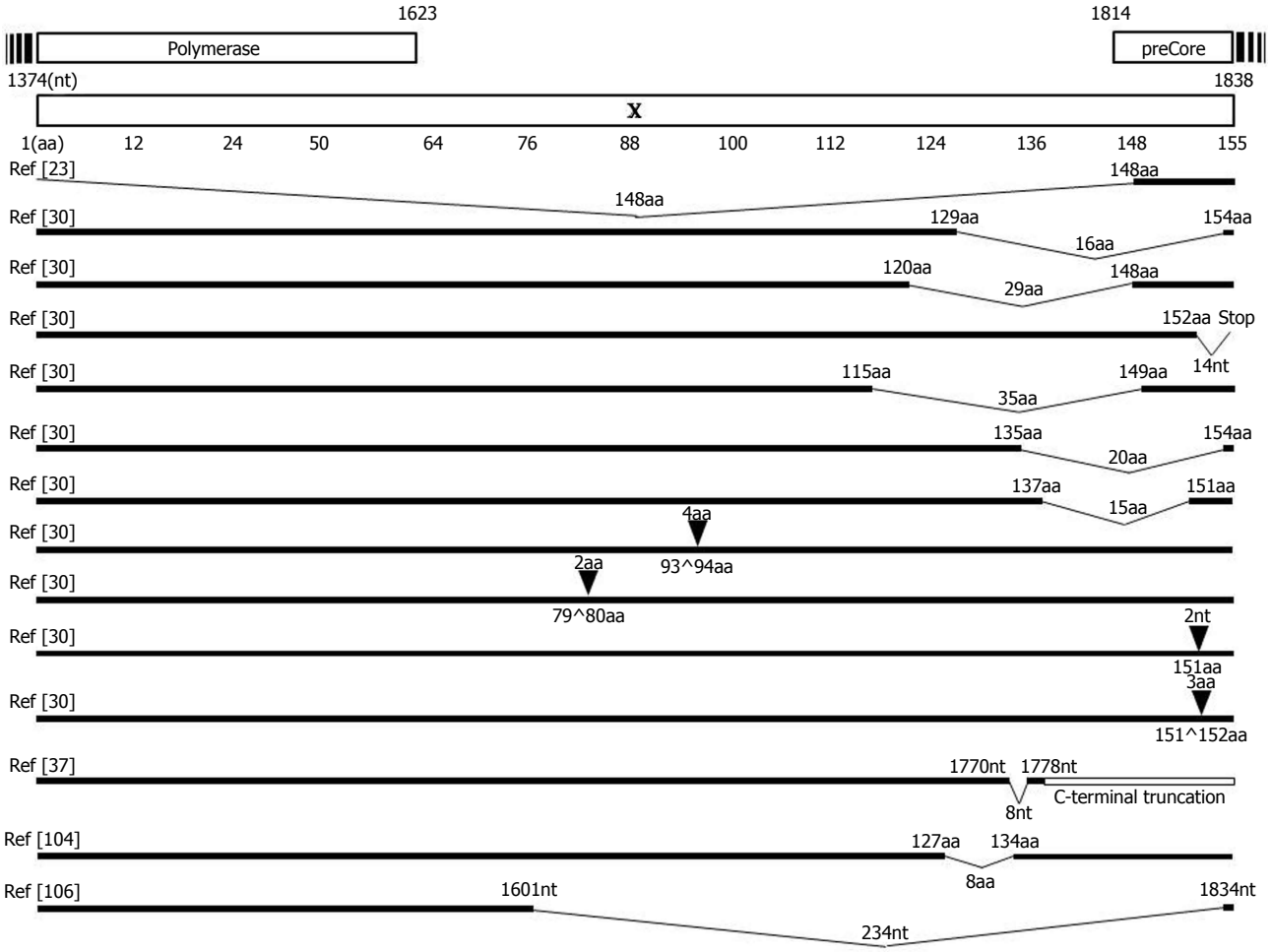

Figure 2 Mapping of deletions or insertions in the hepatitis B virus-X protein region. Deletions or insertions in the HBx region mainly occur in the C-terminal region of HBx, which may also contribute to the clinical outcome severity in chronic patients. HBx: Hepatitis B virus-X protein.

chronic patients with HBV infections. It also further contributes to disease progression by inducing mutations of other HBx mutations related to clinical severity, such as G1386A/C (V5M/L), C1653T (H94Y), T1753V (I127V) and HBx C terminal deletion or insertion. Moreover, T1753V (I127L,T,N,S) and C1653T (H94Y) mutations in the EnhII/BCP region and A1383C, G1386A/C (V5M/L) and C1485T (P38S) in the negative regulation domain were significantly related to severe types of liver diseases, including HCC. Furthermore, deletions or insertions affecting the C-terminal region of HBx can also contribute to the clinical outcome severity in chronic patients. In addition, our recent study indicated that a novel mutation type (X8Del) composed of an 8-bp deletion in the C-terminal region of the HBx contributes to occult HBV infection in vaccinated Korean individuals *via* a reduced secretion of HBsAg and virions. Thus, several distinct types of HBx mutations may contribute to HBV pathogenesis by regulating HBV replication or host genes related to cell homeostasis.

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REVIEW

Avoiding hepatic metastasis naturally: Lessons from the cotton top tamarin (*Saguinus oedipus*)

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Abstract

Much has been written about hepatic metastasis and animal models abound. In terms of the human experience, progress in treating this final common pathway, a terminal event of many human malignancies has been relatively slow. The current thinking is that primary prevention is best served by early detection of cancer and eradication of early stage cancers by screening. Some cancers spread early in their course and the role of screening may be limited. Until relatively recently there has not been a pathfinder model that makes the evasion of this unfortunate event a reality. This review discusses such an animal model and attempts to relate it to human disease in terms of intervention. Concrete proposals are also offered on how scientists may be able to intervene to prevent this deadly progression of the cancer process.

Key words: Cotton top tamarin; Hepatic metastasis; Carcinoembryonic antigen; Fibulin-5; Common marmoset

Core tip: Hepatic metastasis is a terminal event. Avoiding this complication would prolong life and current understanding of inflammatory mediators allows possible secondary intervention. The cotton top tamarin (CTT), like humans develops inflammatory bowel disease complicated by colorectal cancer but avoids liver metastasis. We suggest 5 mechanisms by which CTT avoid liver spread. They involve changes in ICAMs and their receptors, carcinoembryonic antigen (CEA) family mediators of angiogenesis, post translational modifications of molecules like CEA, and increased expression of anti-proliferative agents such as fibulins. This changes our perception from "monkey

see, monkey do" to "see what the monkeys do and do the same". Possible avenues of intervention are suggested.

Tobi M, Thomas P, Ezekwudo D. Avoiding hepatic metastasis naturally: Lessons from the cotton top tamarin (*Saguinus oedipus*). *World J Gastroenterol* 2016; 22(24): 5479-5494 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5479.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5479

INTRODUCTION

There are many animal models for human cancer and most of these do not occur naturally but are designed to mimic human disease by introduction of mutagen and may also employ promoters. Natural models lack this human "design" element but historically the differences may enhance understanding of disease causality and management for example, the Roux Sarcoma virus in the Plymouth Barred Rock fowl (chickens) and Bittner milk factor in murine mammary carcinoma among others. It is however unusual for a single animal to provide more than 1 natural model for disease[1].

Enter the cotton top tamarin (CTT), a pint-sized lower order, New World primate of the order Callitrichidae[2]. This remarkable monkey provides a model for human disease in inflammatory bowel disease and potential mechanisms[3,4], cancer of the lymphatic system[5], large bowel cancer[6], and immune-altered states[7], and recently liver metastases[8]. While a review of these models apart from the latter is beyond the scope of this review, it is significant that the example of the liver metastasis model could best be described as a "negative" or "avoidance model" in that the CTT with colon cancer avoids liver metastasis with an estimated frequency of 97.2%.

The hypotheses of how the CTT achieves this are open for discussion and debate has been ongoing since the first symposium, reported in 1985[9] where anatomical blood vessel variance was advanced as an explanation. This was refuted by veterinarians since the anatomy is similar to most mammals including humans (N. Clapp- personal communication). Our group has been exploring hypotheses based on adhesion molecules known to be important in human liver metastasis. While the article will focus on these molecular families in tamarins, it is important to first discuss our understanding of their importance in human "metastogenesis" as the findings in the CTT may enable intervention in the human where over 70% of GI cancer victims succumb to death caused by hepatic metastases.

Hypotheses of hepatic metastases in humans

Cancers of the colon and rectum comprise 14.5% of all cancers diagnosed in the United States (149880) and 11.1% of cancer deaths (51710) each year[10]. The primary treatment for large bowel cancer is surgery. Patients who recur do so largely in the liver, lung or peritoneum. While combination chemotherapy can increase survival even in advanced disease it is rarely long term. Metastatic colorectal cancer is therefore a major public health problem. Colorectal cancer is associated with chronic inflammation[11-13]. Inflammatory cytokines can be induced by interaction of terminally differentiated macrophages with the cancer associated glycoprotein carcinoembryonic antigen (CEA)[14]. CEA (CEACAM5) is a large heavily glycosylated glycoprotein of indeterminate function in the normal tissue in which it is expressed[15,16]. Discovered in 1965 by Gold and Freedman[17] it has become the most used tumor marker for colorectal cancers but failed to live up to the early expectations for use as an early detector of cancer[15,18]. It is a glycoprotein that is a member of a large gene family that consists of 29 genes divided into three subgroups that include the CEA-like glycoproteins and the pregnancy-specific glycoproteins (PSGs)[19]. These CEA like proteins are members of the much larger immunoglobulin supergene family[15]. The nomenclature for the entire CEA and PSG families is published in Beauchemin *et al*[20].

CEA

CEA is a GPI anchored glycoprotein with an average MW of 180 kDa this varies depending on the degree of glycosylation of the native protein. CEA contains 651 amino acids and these are found in 7 immunoglobulin like domains, an IgV domain at the N-terminus and six disulfide bridge linked IgC domains[15,16]. There are 29 possible sites for N-linked glycosylation and they tend to be of the complex tetra antennary type[15]. Recently a large number of functions in cancer cells have been attributed to CEA. These include a role in cell-cell adhesion[21,22], apoptosis (anoikis)[23], inflammatory responses leading to increased hepatic and possibly lung metastasis[14] and angiogenesis[24].

CEA AND ITS IMPACT ON LIVER METASTASIS: INDUCTION OF INFLAMMATORY RESPONSES IN THE LIVER

Liver metastasis is the major cause of death in patients with colorectal cancers. There is good evidence that cytokines play a major role in preparing the liver for implantation and subsequent growth of cancer cells[14]. It appears that a relationship exists between the colon cancer derived glycoprotein CEA and macrophage related cytokine production particularly associated with CEA Kupffer cell interactions in metastasis of colorectal

cancers to the liver. The discovery of CEA in 1965[17] marked a turning point in the study of cancer and led what might be called the age of tumor markers. CEA was the first commercially available tumor marker and may be considered the prototype[25]. Serum elevations of CEA (above 3 ng/mL) are seen in about 60% of colorectal cancer patients at presentation[26] and elevated levels are also seen in a number of other cancers including breast, gastric, lung and pancreas. Unfortunately, the early premise that CEA levels could be used as a screening test for colorectal cancer was not fulfilled. CEA was found not to be cancer specific and high false positive and false negative rates precluded its use in early detection of cancer. However, CEA is a useful marker in the determination of prognosis[27] and for detection of recurrence in the post-surgical follow up of colorectal cancer patients[28]. CEA has also been used for the detection of occult tumor by radioimmuno-detection[29] and as a target for treatment using radioimmuno-therapy[30]. In addition a number of studies have used CEA as the target antigen for immunotherapy[31]. Clinically CEA is very well understood, but its biological role is not clear and its function in the normal colonocyte still evades us. However over the years a number of functions mostly associated with tumor cell behavior have been attributed to CEA. We focused on the molecular interactions between CEA and its receptor CEAR in colorectal cancer cells and how disruption of these interactions and inhibition of cytokine production will affect the spread and growth of colorectal cancers. Designing an effective mimetic-based therapy requires the identification of the responsible molecules, mechanisms of action and regulation.

The most important functions of CEA are the effects associated with tumor cell survival whether it is at the primary site or at a distant metastasis. High levels of CEA are also associated with malignant ascites from colorectal cancer (CRC)[32]. CEA interacts with liver Kupffer cells through a cell surface receptor (CEAR) that we have identified as the heterogeneous RNA binding protein (hnRNP) M4[33]. The hnRNP group of proteins are multifunctional and are involved in many cellular events including alternative splicing, translational regulation and packaging of transcripts[34]. These proteins have also been implicated in the regulation of mRNA stability and translation in many cancers[35]. CEAR is a highly conserved protein that can bind both RNA and DNA and can transport mature RNA to the cytoplasm as well as acting as a splicing factor[36]. It can also be expressed both on the cell surface, in the cytoplasm and in the nucleus where it is most commonly found[37]. There are 4 isoforms of hnRNP M known[38]. Two of which we have shown bind CEA[33]. One form (hnRNP M4) which we originally identified as the CEA receptor has a 38 amino acid deletion between the first and second RNA binding domains. The longer form also binds CEA[33]. The two other forms have not been identified other than as proteins on 2D gels. CEAR will also bind to CEA in HT-29 colon cancer cells although the functional significance of this is not known. It has been suggested that it may be involved with the resistance afforded to anoikis[39,40]. Binding to CEAR occurs *via* a penta-peptide motif (PELPK) located at the hinge region between the CEA's N-terminal and first immunoglobulin loop domain. This interaction produces cytokines by activating a signaling cascade and these cytokines alter the liver microenvironment such that it becomes more hospitable to the implantation and growth of the cancer cells[41,42]. Production of both IL-6 and IL-10 by CEA stimulated Kupffer cells improves the survival of highly metastatic human colorectal cancer cells in the nude mouse intra-splenic injection model for liver metastasis[43] (see Figure 1).

CEA AS AN ADHESION MOLECULE

In 1989 Benchimol *et al*[22] demonstrated the first potential function for CEA. By transfecting tumor cells with the CEA gene they showed increased cell to cell adhesion and demonstrated that this could be inhibited using anti CEA antibodies. Adhesion was due to interactions between the N-terminal and the 5th and 6th (A3B3) immunoglobulin domains[44]. This suggested that these interactions may play a role in tumor cell behavior and in the development of metastases.

CEA AND ANOIKIS

Another important function that has been attributed to CEA expression is inhibition of apoptosis in particular the apoptosis caused by absence of attachment of cells to a substratum (anoikis)[23,40]. This would play an important role in metastases formation to a distant organ as unattached tumor cells in the circulation would be more susceptible to anoikis.

CEA AND ANGIOGENESIS

Here, we suggest a relationship between CEA, its receptor CEAR and angiogenesis. Others have also suggested a relationship between CEA and angiogenesis though they did not establish it as causal or identify potential mechanisms[45,46]. Recently, however they showed that secreted (soluble) CEA can directly activate endothelial cells *via* integrin β3 signaling[24,47]. We suggest that an alternate mechanism may also exist involving the relationship between CEA and its receptor CEAR. Interaction of CEAR with CEA in tumor associated stromal cells particularly macrophages is important. Disrupting that relationship may affect tumor growth and progression

It is significant that Low-Marchelli *et al*[48] have shown that CCL2 recruits macrophages to promote angiogenesis. We therefore suggest that colon cancer cells recruit macrophages by secreting MCP-1 and these macrophages are activated by tumor derived

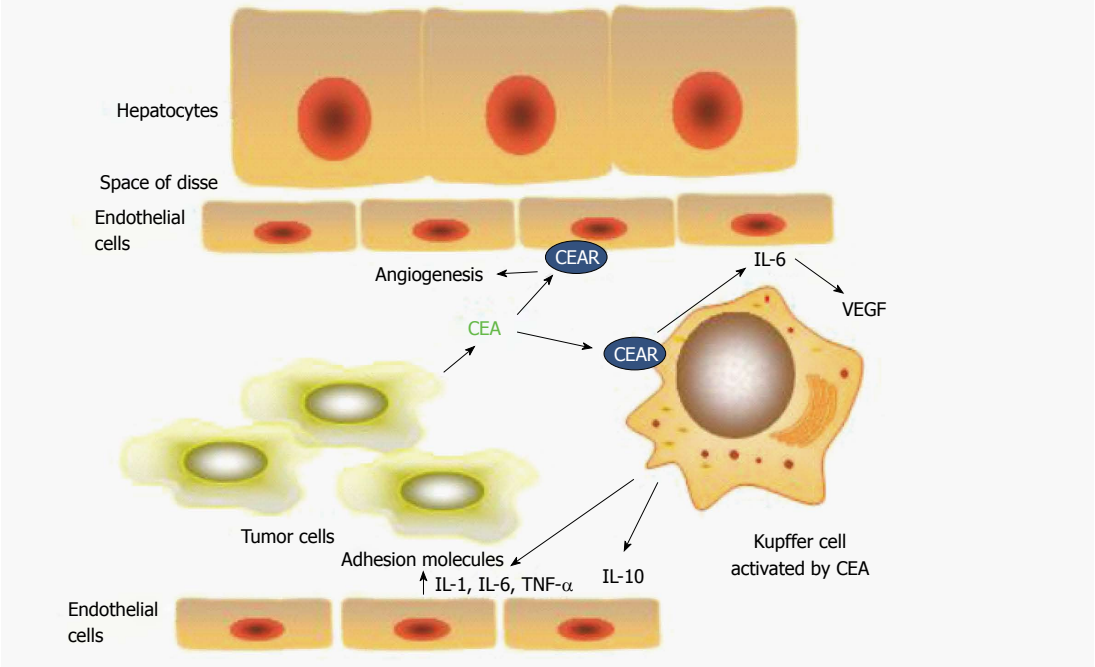

Figure 1 Schematic of the interactions of carcinoembryonic antigen in the hepatic sinusoid. Interactions of carcinoembryonic antigen (CEA) in the hepatic sinusoid. CEA released by the tumor cell binds with hnRNP M (CEAR) on the Kupffer cell surface resulting in release of the cytokines interleukin (IL)-1, IL-6, IL-10 and TNF-α. Effect of CEA induced cytokines on tumor cell interactions in the hepatic sinusoid. Cytokines IL-1, IL-6, IL-10 and TNF-α produced by Kupffer cells have a number of effects on the tumor cell microenvironment. These include up-regulation of adhesion molecules on hepatic sinusoidal endothelial cells. The most important of these seem to be E-selectin and ICAM-1. Cytokines such as IL-6 and IL-8 are pro-angiogenesis and they may also effect growth at the distant site[14,42].

CEA to secrete IL-6 and IL-8 both known pro-angiogenic factors[49]. Anti angiogenic factors are also produced (IL-10) and an imbalance between these two competing factions will tend towards pro-angiogenesis.

TARGETING CEA/CEAR INTERACTIONS AS A MEANS OF INHIBITING CYTOKINE PRODUCTION AS AN ANTI-METASTATIC THERAPY IN COLORECTAL CANCER

Because transformed cells home into tissues from the circulation in a highly selective way through complex molecular mechanisms, it provides a template for targeted therapy. Designing an effective mimetic-based therapy requires the identification of the responsible molecules and their mechanisms of action along with their regulation. The investigation of methods to block or modulate CEAR function *in vitro* and *in vivo* and relating this to tumor growth and development will increase our understanding of the molecular and biological mechanisms involved in the progression of gastrointestinal cancers. The investigation of methods to block or modulate CEAR function *in vitro* and *in vivo* and relating this to tumor growth and development has increased our understanding of the molecular and biological mechanisms involved in the progression of colorectal cancers. We have shown that the binding peptide YPELPK will induce cytokine responses in macrophages activated by CEA[50]. We have shown that the binding peptide YPELPK will induce cytokine responses in PMA activated THP-1 macrophages. These types of study are important for the development of rational therapies that will enhance those currently available.

OTHER POTENTIALLY PRO/ANTI-METASTATIC MOLECULES STUDIED

Fibulin-5

During the initial development of metastasis, the adhesion of tumor cells is mainly mediated through binding of extracellular matrix components to cell surface receptors[51]. Interestingly, one study[52] found an association of fibulin-5, the newest member of the fibulin family of extracellular matrix glycoproteins, with hepatic metastasis of colorectal carcinoma. Since fibulin-5 plays an important role in antagonizing angiogenesis[51-53] in humans, we sought to confirm the expression of fibulin-5 expression level in the CTT.

Avoidance hypotheses in the CTT

Our understanding of cancer and metastases in primates began when colon cancer was described in old world monkeys as early as 1914 and then in the new world in inter alia, Goeldi's monkey in the early 1960s. The latter, *Callimico goeldii* shares the Colombian habitat with the CTT and is a small black-furred New World lower order primate. At that time tens of thousands of CTT were imported by the pharmaceutical industry utilizing the "tamarin alarm reaction" to test the efficacy of anxiolytics although literature to document

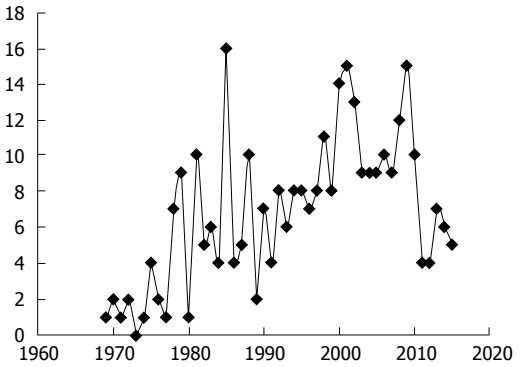

Figure 2 Graphic representation of published cotton top tamarin articles by year as estimated by a typical online search. The line chart is not necessarily inclusive of all cotton top tamarin (CTT) articles but merely confined to the search terms used. It is therefore used mainly to demonstrate the overall trends in annual publications devoted to this animal "CTT supermodel".

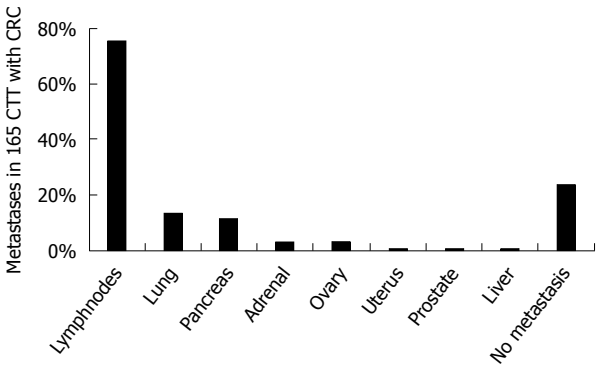

Figure 3 Schematic bar diagram depicting proportion of metastases in cotton top tamarin with colorectal cancer. The bar diagram shows the distribution of metastasis based on the ORAU colony cancer statistics. The paucity of liver metastases is remarkable. CTT: Cotton top tamarin; CRC: Colorectal cancer.

this is difficult to come by utilizing electronic search methods. Problems of CTT husbandry arose when the animals developed the "wasting marmoset syndrome"[54] postulated to be caused by a coronavirus but later effectively countered by improving the nutritional intake. The major challenge facing the CTT is the reduction of its numbers in its shrinking natural habitat in northwestern Colombia and most CTT are today found in zoos. As a result the species was listed as endangered in 1973 by the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES)[55]. Since that time, the numbers of articles on the CTT had increased exponentially. This attests to the interest of the scientific community in the CTT specifically. Despite this interest, primate research in the United States is in decline with the closures of the ORAU (Oak Ridge Associated Universities) facility and that of the NEPC (New England Primate Center) which housed a significant proportion of CTT. The waning of public support and therefore funding opportunities combined with the expense of operations has largely been the cause of this decline. This is regrettable as it is generally not appreciated that monkey publication numbers are an extensive part of the scientific literature (678000 Medline™-listed publications) attesting to their usefulness and their command of the attention of the scientific community. Now, specifically CTT publications which were formerly about one tenth of non-human primate-themed publications (398 total CTT per National Library of Medicine ticket 28045-54653), are in decline in the example depicted graphically (Figure 2).

In view of the importance of this animal model applicable to many models of human disease, a CTT symposium was convened resulting in the publication of numerous articles in a supplement published in the journal Digestive Diseases and Sciences in 1985[9]. Thereafter a comprehensive book on the CTT was edited by Dr. Neil K. Clapp and published in 1993 by CRC Press[2]. This review seeks to build on that experience specifically focusing on the ability of the CTT to avoid hepatic metastases even though a large proportion of the animals develop advanced colorectal cancer. This is a unique feature of the CTT not typically found in other marmosets. It would appear that CTT peritoneal metastasis and ascites is also not common as it is with terminal human disease.

CTT MODEL FOR HEPATIC METASTASIS

The CTT develops colitis in the juvenile years and about one third (34.5%) go on to develop colon cancer and die of the disease. An observational study showed only 1.2% of animals had hepatic metastases[56] (Figure 3).

The etiology of the cancer seems to be the de novo inflammation-dysplasia-cancer variety although animals with adenomas have been described[56]. The risk of cancer deaths shows an increase from 3-8 years and then a gradual decline after age 12 similar to the recently described risk in humans with positive family history of CRC[57]. The etiology is still unknown but stress and micro-organisms may play a role[3,4]. Over half have multiple primaries diffusely distributed but the descending colon has the greatest proportion similar to the rectosigmoid colon predominance in ulcerative colitis cancer location.

CTT COLON CANCER BIOLOGY VIS A VIS HUMAN

Despite similarities in cancer biology between CTT and humans it can be seen from Table 1 that the anticipated mutations are either not described or characterization has not been attempted.

When considering changes associated with metastases[63] no CXC, CXCR4, CXCL12 (fusins), CCR7 (chemokine receptor 7) chemokines thought to be important in metastases have been described as yet in the CTT. Interestingly CXCL12 has been described in the common marmoset[64] and thus likely to be expressed in the CTT but is not necessarily a given.

Table 1 Comparison of cancer genetics in the cotton top tamarin and human

Stage of neoplasia	Sporadic human CRC	Human IBD-associated CRC	CTT colitis-CRC
Normal-appearing mucosa	*APC* initiator	Inflammation leading to mutations listed below	No cell line mutations in APC exon 4 and 15[58]
Early adenoma/indefinite dysplasia	Aneuploidy, methylation, Sialyl Tn	Aneuploidy, methylation, Sialyl Tn, MSI	90%diploid; no methylation[59]
Early promotor	MSI; *kRAS*, COX-2	DCC	kRAS absent[60]
Intermediate adenoma/low-grade dysplasia	c-src	c-src	c-src ND
Intermediate promoter	DCC/DPC4	*kRAS*	DCC/DPC4 ND; kRAS absent[60]
Late promoter	p53	*APC*	p53 ND; as above for APC exon 4 and 15[58]

Adapted from Itzkowitz and Harpaz 2004[61]; and Mattar MC, Lough D, Pishvaian MJ, Charabaty A[62]. APC: Adenomatous polyposis coli gene; MSI: Microsatellite instability; DCC: Deleted in colon cancer; *kRAS*: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; COX-2: Cyclooxygenase 2 gene; c-src: Rous sarcoma gene that encodes for usual C-terminal inhibitory phosphorylation site (tyrosine-527); DPC4: Deleted in pancreatic carcinoma, locus 4; p53: Protein 53 kilodaltons; ND: Not done.

The human kallikrein zymogens (hK2) closely related to prostate specific antigen (PSA) have been studied in the CTT[65]. They concluded that the CTT has no functional hK2 or PSA. Since these moieties regulate cancer cell survival and growth the CTT may be a natural knock-out model for the study of this topic. We see in these findings yet another potential mechanism by which the CTT impedes cancer proliferation. Likely Toll-like receptors (TLR) also remain to be discovered in the CTT and may have likely similar functions to the human.

Since lectins[66] and adhesion molecules have been described in the CTT we have focused our attention on these molecules, particularly CEA. We also explored extracellular matrix proteins as the interaction between these proteins and cells play an important role in tumorigenesis and metastases. One such protein, fibulin-5[51-53] has calcium-binding EGF-like domains and associate with vascular and vascular structures. It is thought therefore to have anti-angiogenic actions and be an inhibitor of metastasis. It has not as yet been studied in the CTT.

CEA HOMOLOGUES IN THE CTT AND HUMAN

Cross reactive CEA species in monkeys were known when we published our seminal paper in 1994[67]. Haagensen *et al*[68] had reported CEA in higher order non-human primates, including the chimpanzee and gorilla. A mouse analogue had also been described by Abraham Fuks' group in 1989[69]. Our initial experiments used a number of antibodies to CEA including the T84.66 antibody developed by Dr. J. Shively's laboratory group at the Beckman Institute at the City of Hope which was used in the CEA kit commercialized by Roche™. This antibody when used in immunohistochemistry produced consistently negative results when using colon CTT fixed tissues and later native antigen in CTT colon tissue extracts in early experiments[70]. We tested other antigens such as organ specific neoantigen[71], thought to be the mediator of the lymphocyte adherence inhibition test for cancer and found reactivity[72]. In the early 1990's Karel Kithier of the Department of Pathology at Wayne State University was beta-testing a CEA kit produced by Tosoh Medics (CEA-AIA PACK kit™) which seemed to have a greater proportion of positive results when compared to another CEA kit marketed by Abbot. These results were expanded and confirmed and found to yield similar levels in CTT and humans with IBD[60,73] which opened a pathway to possible intervention[33] in addition to other cancer related moieties such as telomerase[60]. In order to show the CEA molecular moieties recognized by the kit we obtained both solid phase and tracer antibodies from Hybritech Inc., from Dr. Harry Rittenhouse. The resultant Western blotting[67] showed a specific 50 kDa band shared by CTT and humans (Figure 4).

A control blot using T84.1EC monoclonal which reacts with both NCA and CEA did not recognize the 50 kDa shared antigen in the same samples. Based on the known epitopes on the antibodies concerned we were able to speculate that the shared antigen included the Ig-like loop domains however the exact structure of this moiety remains unknown. In this publication we did hypothesize that "the smaller CTT CEA molecule might lack the critical peptides necessary for uptake and hence explain the paucity of liver involvement in CTTs with CRC".

EXPRESSION OF THE CEA GENE FAMILY OF PROTEINS IN THE CTT AND HUMANS

The next question we needed to answer was if an entire repertoire of CEA-family adhesion molecules were expressed by the CTT and if this subspecies was unique in this respect in the *Callitrichidae* family. In a later paper in 2000[73] we explored these questions by expanding the repertoire of antigens and extending our observations to include a close cousin of the CTT, the common marmoset (Callithrix jacchus - CM) which is

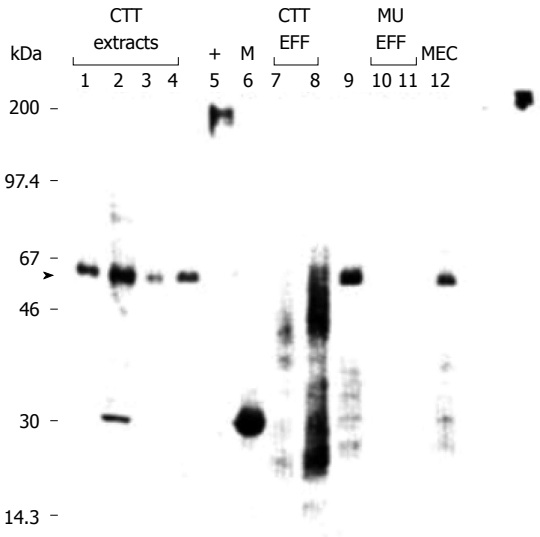

Figure 4 Specific carcinoembryonic antigen bands shared by cotton top tamarin and humans. Western blot using solid phase anti-CEA monoclonal antibody. Immunoblotting was performed using 5.6 pg protein/ml antibody after electrophoresis of a 12% SDS-polyacrylamide gel run under reducing conditions. Relative mobility (M) is shown on the left, and the type of samples loaded at 10 pg protein/lane are shown above the numbered lanes. Cotton-top tamarin extracts are in lanes 1 to 4 and effluent samples in 7 and 8. An extract from a patient with histologically proven rectal cancer and IBD is in lane 9, with human effluent samples in lanes I0 and 11 and human meconium in lane 12. Lane 5 contains the positive human CEA control, and the M, markers (M) are in lane 6. An arrow indicates the M, - 50000 band evident also in human extract (lane 5) likely a deglycosylated moiety. (Published with permission of Elsevier[67] and modified). CEA: Carcinoembryonic antigen.

not endangered but does develop colitis uncomplicated by cancer. Aware that the changes likely to influence CEA uptake were at the N-terminal end of the CEA molecule we used antibodies directed at epitope in that region, including the aforementioned Tosoh Medics™ kit and T84.66 antibody to quantify the CEA in colonic washings.

We found that the concentration of CEA in washings was sevenfold times higher than in washings from humans with IBD. T84.66 antibody was able to demonstrate a faint high MW band in a specimen of colonic washing from a CTT by immunoblot. In the Western blot using T84.66 we could now also demonstrate specific bands (immunohistochemistry had been consistently negative) in CTT washings and tissue extract of Mr $\geqslant$ 90 kDa which is the size of the non-glycosylated protein core of the CEA molecule[74]. CEA levels assayed by the Tosoh™ kit in CTT extracts were significantly higher than those from CM extracts ($P < 0.005$). Consistent with this finding was a high mean value in sera of CTT (134 ng/mL) compared to undetectable levels in the CM. NCA levels tended to be the lowest in the CTT as compared to humans and CM. Both animals' samples were low when reacted with a CEA-family antibody. In contrast binding with the anti-BGP was seen in both animals. We concluded that the similarity between human and tamarin native CEA is greater than previously believed and that it was likely that the N-terminal epitopes were conserved in the tamarins.

OTHER ANTIGENS SHARED BY CTT AND HUMANS

By the end of 2000 we published an expanded group of antibody assays involving 7 additional common epitopes representing most colonic cell types and blood group/carbohydrate antigens, some of them accepted tumor markers[60,70-76]. Mucin antigens, epidermal growth factor (EGFR) and telomerase were shared by CTT and humans but *k-ras* p21 and adenoma antigen (Adnab-9) were not. This supported the notion that carcinogenesis was likely de novo and did not progress through the adenoma-carcinoma sequence. Our finding of EGFR reactivity in the CTT provided a basis[76] for fibulin-5 role in the anti-metastatic armamentarium and OSN (by reactivity of BAC 18 monoclonal) suggesting similarities in anti-cancer immune responsiveness *via* the lymphocyte migration reaction. We were able to demonstrate also significant levels of EGFR epitope in the urine of 5 CTT (0.152 ± 0.053 absorbance OD-background at 405 μm) by ELISA. CEA reactivity with various antibodies in these samples were low (mean < 0.05). Blood Group Substances (BGS) were detectable [Span 0.063; FBB 0.103; CaCo 0.054) as were Bac18.1 (anti-OSN antibody) at 0.06]. Wild-type (Table 1) Ras p21; src, common membrane antigen (CMA), were negative corresponding to the data derived from CTT tissue. The only pieces of the puzzle missing were the questions of CEA molecular homology and how to tie the available data together to explain how the CTT dodges liver metastases.

DEFINITIVE STUDIES ON THE HOMOLOGY OF THE CEA MOLECULE

A comparative study of the C-terminus of the CEA molecule by the Stanners' group looked at multiple primate and prosimian species. The membrane linkage in CTT and other monkeys was found to be GPI anchorage rather than transmembrane (TM) seen in mice and rats[77]. This paper showed that the more versatile glycophosphatidyl inositol (GPI) linkage with a greater functionality constellation likely confers a tendency to tumorigenesis. Mutational packages evolved over time, one applicable to most primates including humans, and the second confined to the Cebidae radiation of New World monkeys leading to inhibition of cell differentiation. The rate of Ka (indicator of selective pressure acting on a protein-coding gene) nonsynonymous mutations for this radiation is 7X higher than the average Ka in primates. This led us to postulate that if such mutations occur at the C-terminus, it is quite feasible that similar mutations occur at the N-terminus where they may affect uptake of CEA, inhibiting hepatic metastases. Having

Table 2 Representative somatic sequences from the PELPK region of carcinoembryonic antigen in humans, cotton top tamarin and common marmoset

HCEA-A1 PELPKPSISSNNSKPVEDKDAVAFTCEPETQDA
CTT-WT PELPKPSISSNNSKPVEDKDAGAFTCEPETQDA
CM-WT PELPKPSISSINSKPVEDKDAGAFTCEPETQDA
M1-A1 (CTT) PE**V**PKPSISSNNSKP**GG**DKDAGAFT**W**EPETQDA
M2-A1 (CTT) PE**VS**KPSISSNNSKP**GG**DKDAGAFT**W**EPETQDA
M3-A1 (CTT) PE**VS**KPSISSNNSKP**V**GDKDAGAFT**W**EPETQDA
M4-A1 (CTT) PE**VS**KPSISSNNSKP**V**GDKDAGAFT**W**EPETQDA
M5-A1 (CTT) PE**VS**KP**Y**ISSNNS**N**PVEN**K**DAGAFT**W**EPETQDA
M6-A1 (CTT) PE**VS**KP**FI**FSNNSKP**GG**DKDAGAFT**W**EPETQDA
M8-A1 (CTT) PE**VS**KP**FI**F**R**NNSKP**GG**DKDAGAFT**W**EPGTQDA
M10-A1 (CTT) PELPKP**FI**F**S**NN**F**KPVEDKDA**V**AFTCEPETQDT
M14-A1 (CTT) PELPKP**FI**F**S**NN**F**KPVEDKDA**V**AFTCEPETQDA
M15-A1 (CTT) PELPKP**FI**F**S**NN**F**KPVEDKDA**V**AFTCEPETQDA
M16-A1 (CTT) PELPKPS**I**LSNNSKPVEDKDA**V**AFTCEPETQDA
M17-A1 (CTT) PELPKPS**I**P**S**NNSNPVEDKDA**VGL**TCEPDTQNT
M19-A1 (CM) PELPKPSIS**S**YNSKPVEDKDAGAFTCEPETQDA
M20-A1 (CM) PELPKPSIS**S**YNSKPVEDKDAGAFTCEPETQDA
M21-A1 (CM) PELPKP**FI**S**S**YN**F**KPVEDKDAGAFTCEPETQDA
M24-A1 (CM) PELPKPS**I**F**S**N**N**SKPVEDKDA**V**AFTCEPETQDA
M27-A1 (CM) PELPKPSISS**N**NSNPVEDKDA**V**AFTCEPETQDA
M31-A1 (CM) PE**V**SKP**FI**F**S**N**N**SKPV**G**DKDAGAFTCEPETQDA
M40-A1 (CM) P**G**VPKP**FI**F**R**IN**F**KPV**G**DKDAGAFTCEPETQDA

Sequences changes from those of wild type (germ line) are shown in bold. Published with permission of Springer[8].

also found common CEA-family and fibulin-5 ligand antigens we resolved to also investigate for parallel pathways of metastasis inhibition.

SUMMARY: SPECIFIC METASTASIS-TARGETED STUDIES IN CTT AND HUMANS

Using DNA extracted from the CTT tissues a Hot start PCR was performed and (1) sequences around the hinge region between the N-terminal and the first loop domain of CEA obtained using a Big Dye Terminator™ sequencing kit. We also immunolabeled human and CTT liver tissue using: (2) BGP (CEACAM1) antibody kindly supplied by Christophe Wagener (University of Freiberg, Germany); (3) CEAR (CEA receptor) polyclonal antibody; and (4) an antibody directed to fibulin-5 supplied by W. P. Schiemann (Department of Pharmacology, University of Colorado, Aurora, CO, United States). Finally, (5) we deglycosylated CTT CEA to determine the degree of N-glycosylation, as glycosylation may correlate with hepatic metastases. We thus examined 5 potential ways in which the CTT may evade hepatic metastases in 10 CTT and 25 humans.

To briefly summarize the observed[8] results (Table 2) we enumerate: (1) 63% of the CTT had non-synonymous PELPK pentapeptide mutations shown to be essential for hepatic uptake of numerous groups of protein including CEA and stromelysin, laminin, complement protein *etc.* CM had the PELPK mutations

Table 3 Biological attributes of the fibulin family[51-53]

Attribute	Fibulin-1	Fibulin-2	Fibulin-3	Fibulin-4	Fibulin-5
Invasion of endothelium	Decrease	Increase	Decrease	Unknown	Decrease
Binding of tropoelastin	Moderate	High	low	Moderate	High
Effect on angiogenesis	Unknown	Unknown	Reduced	Unknown	Reduce
MMP/ fibronectin change	Increase	Unknown	Reduced	Unknown	Reduced

to a lesser extent (73% *vs* 29%, $P < 0.05$). Sequence changes in the PELPK binding motif are found in the CTT that prevent binding to CEAR and do not allow activation of Kupffer cells and thus secretion of pro-inflammatory cytokines.

This could account for the high CEA serum levels in the CTT as compared to CM and humans and contribute to the lack of liver metastasis. Zimmer and Thomas in 2001 showed in a minority of patients with advanced colon cancer all of whom had high CEA levels, changes in the PELPK sequence. CEA from these patients could not bind to Kupffer cells. These patients did not have liver metastasis though peritoneal carcinomatosis was common[78]. Serendipitously: (2) Normal sections of human livers bearing metastatic disease (our intended "negative" controls) showed translocation of the BGP to the cytosol of the hepatocyte whereas CTT livers did not have detectable BGP except in the gallbladder wall. BGP is usually a structural protein found in the bile canaliculus but conceivably, if translocated to the cytosol, may act as a downstream mediator of VEGF, facilitating the progression of liver metastasis. Human cirrhotic and hepatitis livers demonstrated orthotopic BGP compared to human livers bearing metastases ($P < 0.02$) where the BGP had translocated to the cytoplasm (Figure 5).

This may answer the age-old clinical question as to why patients with cirrhosis rarely get hepatic metastases from extrahepatic primary cancers.We also observed: (3) a diminution of CEAR staining (Figure 6) in the CTT liver sections compared to humans ($P < 0.02$), a further barrier to CEA hepatic uptake in the CTT as compared to the human liver. Surprisingly: (4) Extremely robust fibulin-5 hepatocyte labeling was detected in CTT livers (Figure 7) and finally: (5) Minimal glycosylation of CTT CEA was demonstrated as opposed to human CEA. 80% of CTT *vs* 60% of CM were positive for fibulin-5 binding with functions of the various fibulins contrasted in Table 3.

Hepatocytes were the predominant cell types expressing fibulin-5 in both types of animals but tended to be more intense (Figure 6) in the CTT (0.75 ± 0.289 and 0.4 ± 0.418 respectively) and also seen to be of the same distribution and intensity in the human liver controls. Kupffer cells did not stain ($P = 0.033$ CTT and $P = 0.09$ CM) but occasional bile duct

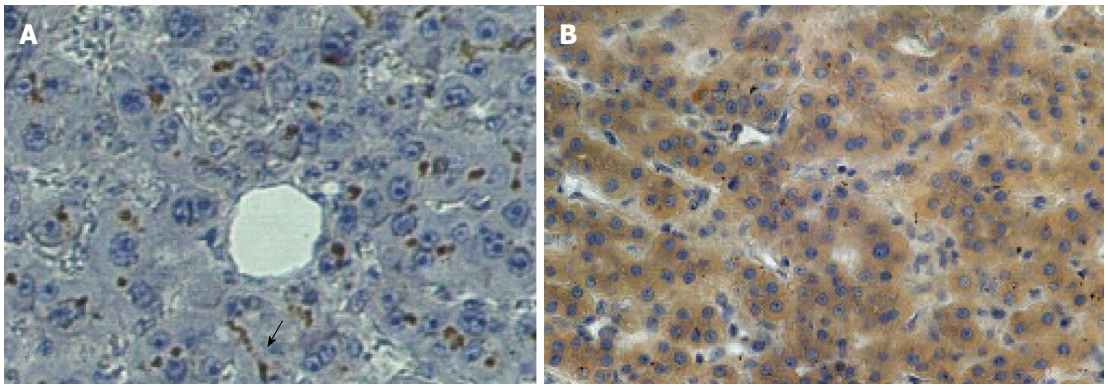

Figure 5 Distribution of CEACAM1 (BGP) in human liver. Photomicrographs of CEACAM1 staining with monoclonal antibody 4D1/C2 showing very intense brown staining mainly in the distribution of the biliary canaliculus in normal human liver (A).In contrast, in the normal portion of a liver from a patient with hepatic metastasis (B), dark staining is seen in the cytoplasm of the hepatocytes with no canalicular staining evident. Published with permission of Springer[8]. The arrow points to the typical distribution of bound ant-BGP in the bile canaliculus in the normal liver (A) at center, bottom.

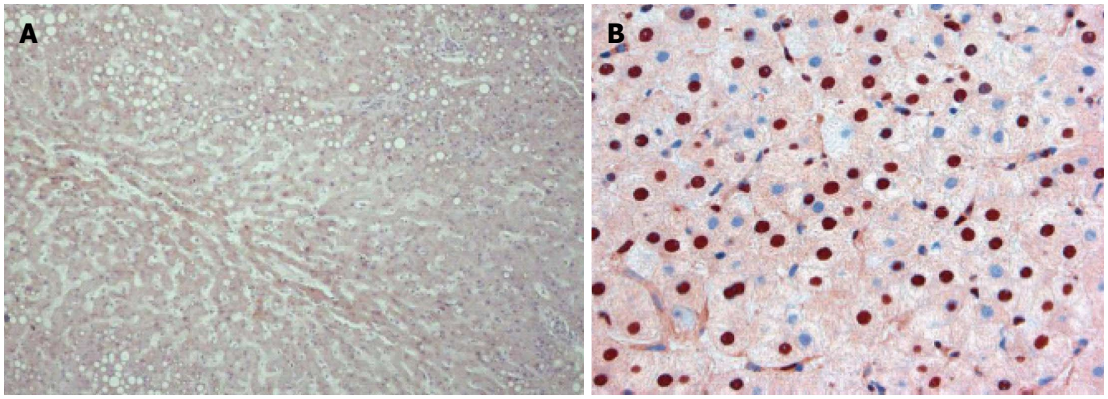

Figure 6 Distribution of carcinoembryonic antigen receptor in cotton top tamarin and human. The distribution of CEAR in the CTT (A) at low power can be seen in the cytoplasm of the hepatocyte by the light brown stain. In humans (B) at a higher power the increased intensity of staining in hepatocyte nuclei can be clearly seen. Published with permission of Springer[8]. CEA: Carcinoembryonic antigen; CTT: Cotton top tamarin; CEAR: Carcinoembryonic antigen receptor.

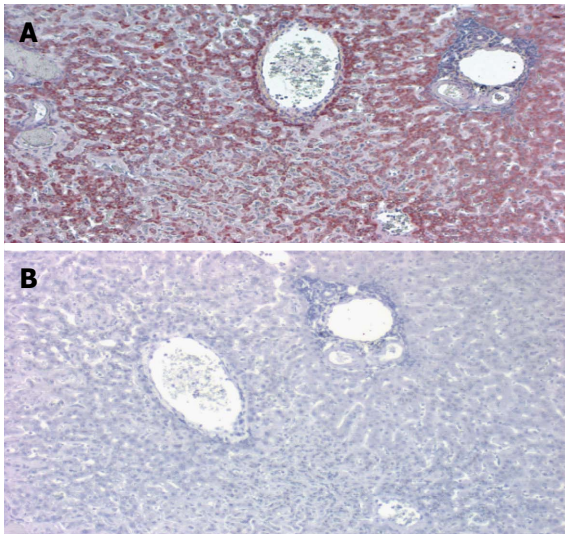

Figure 7 Immune labeling of CTT liver by anti fibulin-5 monoclonal antibody (A) and corresponding negative control (B). A: A central vein is seen in the upper center and a portal triad to the right. The dark red staining denotes the distribution of the antibody which is particularly intense surround the central vein. This is a low power magnification; B: In the negative control no staining is evident.

cells were positive but of lesser intensity in the CTT (0.125 ± 0.25 and 0.375 ± 0.479 respectively - Figure 8). Vascular staining was seen only in 2/5 CTT sections at a mean intensity of (0.3 ± 0.447). No appreciable uptake was noted in white blood cells in CTT and minimal in CM.

We therefore postulate 5 possible pathways of spontaneous hepatic metastasis evasion in the CTT, 4 of which we have previously reported[8], summarized in Table 4.

OTHER POTENTIAL ANTI-METASTATIC AND METASTOGENIC MOIETIES IN THE CTT

Lastly using the Gelco Diagnostics antibody kit to POA we determined a low level of POA[79] in extracts from CTT non-cancerous tissues 1.603 ± 1.656 μg/mL (mean ± SD). In contrast to serum in 13 humans with pancreatic cancer (9.622 ± 6.424) these levels were modest.Since normal serum levels in humans are < 15 U/mL even an order of magnitude increase in the

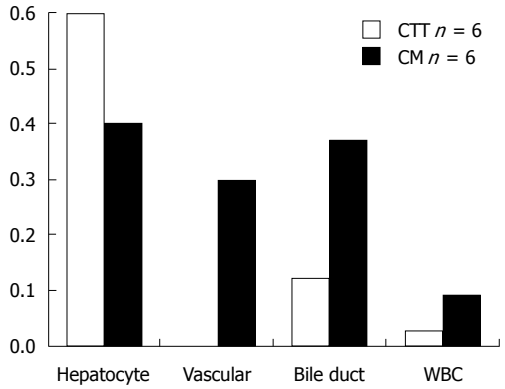

Figure 8 Summary of fibulin-5 immune labeling in cotton top tamarin, common marmoset and humans. Intensity of labeling: CTT and CM of Hepatocyte, Bile Ducts, Vascular Cells and White Blood Cells with Anti-fibulin-5. CTT: Cotton top tamarin; CM: Common marmoset.

CTT is unlikely to increase levels where they might influence the tumor invasiveness. In the study cited, a significant correlation was found between POA and CEA levels classified by stage of CRC (P = 0.003). Although further study is required it would appear that POA is not a major determinant in the CTT of the metastatic process to the extent that it is in humans making this another potential discouraging factor for the development of hepatic metastasis.

Ultimately, we would need to show that the cytokine environment milieu is similar in the CTT as described above for humans. Wilson *et al*[80] using 32 monoclonal antibodies has shown MHC Class 1 and Ⅱ proteins on lymphocytic B cells (CD20-21, 23) and CD16-56 on NK cells; CD2 and CD3 and CD4/8 helper T cells. In addition IL-2 receptor CD25 receptor is present as well as B chain LFA-1 CD 18. There was however poor reactivity for CD11a; and ICAM-1 (CD54) was negative. 14 years later with more reagents available, Kap *et al*[81] confirmed much of the above findings with IL1, 2, 4, 6, 10, 12, and 17a found. In addition they found all 4 clones against CD11a to be positive in contrast to the above paper. CD11a and CD18 and CD29 are important factors for adhesion and are expressed in the CTT. In addition, CD40 of the TNF family is present thus demonstrating that the CTT has almost all the necessary repertoire of proteins as is present in humans to develop hepatic metastasis. Although ICAM-1 was not included in the repertoire, by virtue of CTT colitis to respond to an anti-integrin monoclonal[82], we can conclude that selectins adhesion molecules are also likely expressed. This being the case, it would appear that the aforementioned inhibitory mechanisms are sufficient to abrogate this phenomenon almost completely.

We cannot rule out additional mechanisms and would encourage such research efforts. While ICAM1 may not have been confirmed in the above review (because it does not seem that it was included in the battery of monoclonals described above), E-selectin and integrins certainly have been convincingly demonstrated to be actionable by therapeutic intervention in the CTT[80-82].

Table 4 Summarization of our findings in this study

Parameter	CTT	CM	Human
PELPK Change	+ + + +	+ +	-
CEAR expression	±	±	+ + + +
CEA glycosylation	±	±	+ + + +
CEACAM1 expression	-	-	+ + + +
Fibulin-5 expression	+ + +	+ + +	+ +[1]

[1]Human data have limited staining data; Modified from and published with permission of Springer[8]. CTT: Cotton top tamarin; CM: Common marmoset; CEA: Carcinoembryonic antigen.

Certainly, the above hypotheses are not established scientific facts but they are testable and applicable to a particularly vexing problem presented by hepatic metastases. The ability to intervene using novel approaches based on these ideas will constitute the final section of this review and may represent an encouraging future vision of hope for myriads of "terminal" cancer patients.

In quoting the final chapter of the late Neal K. Clapp's book on this topic: "Our research opportunities using the cotton-top tamarins to study colonic diseases are truly only limited by our ingenuity".

HOW MIGHT WE EXPLOIT THESE ADVANCES FOR THE BETTERMENT OF THE CANCER PATIENT?

Hepatic metastasis of CRC is a very common clinical situation in oncology with prevalence at the time of diagnosis of approximately 20%-25%. The liver has been shown to be the most common site of metastatic spread of colorectal cancer. The prognosis of colorectal hepatic metastases has improved in the last few years owing to surgical resection of liver metastases for patients with no extrahepatic disease; however, liver metastases are resectable in only 15% of the cases[83,84]. Often, factors such as location, size and number of hepatic metastases make it difficult to resect these tumors[83,85]. Thus early prevention of metastasis is crucial in improving the prognosis of patients diagnosed with primary colorectal cancer. As earlier documented by Smedsrød *et al*[86] colorectal hepatic metastasis involve four steps which are interrelated; the cancer cells must establish micrometastasis in the liver by passing through microvasculature which is guarded by Kupffer acting in concert with NK cells. To ensure nutritional supply and establish clinically evident metastatic disease, these cells engage growth factors and adhesion molecules such as VEGF, VCAMs *etc.* As can be appreciated from both clinical and experimental evidence, Kupffer cells are involved with all the rate

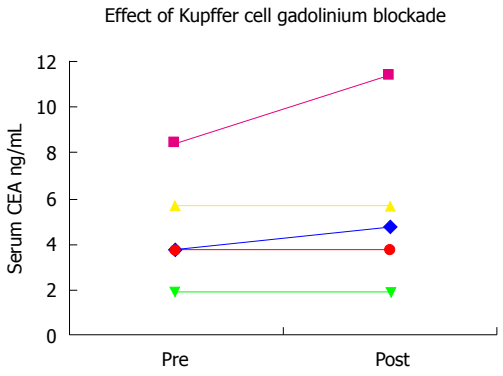

Figure 9 Carcinoembryonic antigen concentrations before and after gadolinium injection for magnetic resonance imaging. Five human patients had serial CEA levels measured before and after MRI with gadolinium contrast administration. No significant changes are seen after gadolinium for patients with normal or near-normal baseline levels. However the patient with a baseline CEA elevation showed a sizeable increase in the CEA level designated by the pink line. CEA: Carcinoembryonic antigen.

limiting step of this macrometastatic disease process which has been established in experimental systems but this role is not proven in human cancers[86]. These phases represent target points of recent therapeutic interventional designs.

USE OF GADOLINIUM BLOCKADE TO DEPLETE KUPFFER CELLS

In our earlier study using CTT[8], we showed that although they have CEA receptors similar to humans, they are able to evade CRC hepatic metastasis *via* two possible means; defective CEA receptors with much reduced receptor capacity or by sequence changes in the PELPK binding motif that is required for receptor activation (Table 2). We hypothesize exploiting methods to prevent CEA/CEAR binding in humans may prove effective in reducing the occurrence of hepatic and possibly lung metastasis. This hypothesis was tested earlier using gadolinium to block uptake of CEA by sinusoidal cells. Although the result was promising, only a transient blockage of CEA was achieved mostly due to the short wash out period for gadolinium (Figure 9). Therefore, using animal models this approach can be refined to optimize our earlier findings using gadolinium as blocking agent for Kupffer cells and the CEA/CEAR interaction.

As a novel approach to anti-metastatic therapy, gadolinium may be encapsulated in smart nano-devices that will prolong the residence time significantly. Using PEGylated-gellan-gum as the polymer of choice, gadolinium can be encapsulated in nanoparticles with average size of 250 nm[87].

Gellan gum is an exocellular microbial polysaccharide with a natural propensity to absorb biological fluid *in vivo* thus regulating the rate of drug/agent release. It consists of repeating tetrasaccharide units of glucose, glucuronic acid and rhamnose in a molar ratio of 2:1:1[88,89]. In addition to being used as a food additive, gellan gum has a wide range of applications in the pharmaceutical industry. In this area, its use has been mainly concentrated in ophthalmic drug delivery and oral sustained-release preparations, in which its ability to undergo cation-induced gelation is utilized as the main fabrication platform[87]. Gellan gum has a number of advantages as it can undergo temperature-dependent as well as cation-induced gelation, which may be of importance for *in vivo* slow release of encapsulated gadolinium. It also has proven non-toxicity in humans[90]. The FDA has also approved gellan gum as a food additive for human consumption[90], thus, its safety in humans is established. To improve the effectiveness of this targeting system, a three-dimensional matrix *via* covalent crosslinking with an end-reactive polyethylene glycol (PEG) through amide linkage can be used. Such modification provides a stronger gellan gum matrix that is nontoxic to normal cells and possesses the ability to deliver biological molecules such as gadolinium to target cells[91] in a controlled manner as illustrated in Figure 10. There is some experimental animal model work that suggests that gadolinium in the form of metallofullerenol nanoparticles inhibit cancer metastases[92] but the metastases observed were mainly pulmonary.

FIBULIN AS A TREATMENT FOR HEPATIC METASTASIS

The process of nanoprecipitation described by the schematics in Figure 10 can potentially be applied to protein encapsulation provided the formulation parameters are optimized as described earlier by our group[91]. Fibulin-5 plays an important role in antagonizing angiogenesis in humans; furthermore its overexpression has been shown to inhibit HCC cell migration and invasion *in vitro*[93], thus fibulin-5 could also be encapsulated in gellan gum nanoparticles to increase their *in vivo* residence time. Nanoparticles formulation should be optimized as described earlier[91] prior to encapsulation of fibulin-5. We would expect fibulin-5 to be the most effective of the fibulins in reducing angiogenesis but fibulin-3 may also be considered (Table 3). While fibulin-5 has been shown to suppress metastasis to the liver and lung[94], it has yet to be used directly in a nanotechnology formulation as we propose. A report that human oncogenic fibulin-5 may promote nasopharyngeal cancer metastasis[95] would suggest that we proceed cautiously with this approach with both agents.

TARGETING CEA/CEAR INTERACTIONS AS A THERAPEUTIC APPROACH TO LIVER METASTASIS

CEA has been shown to be a mediator of metastasis for colorectal cancer by causing changes in the tumor microenvironment that increase the potential for tumor

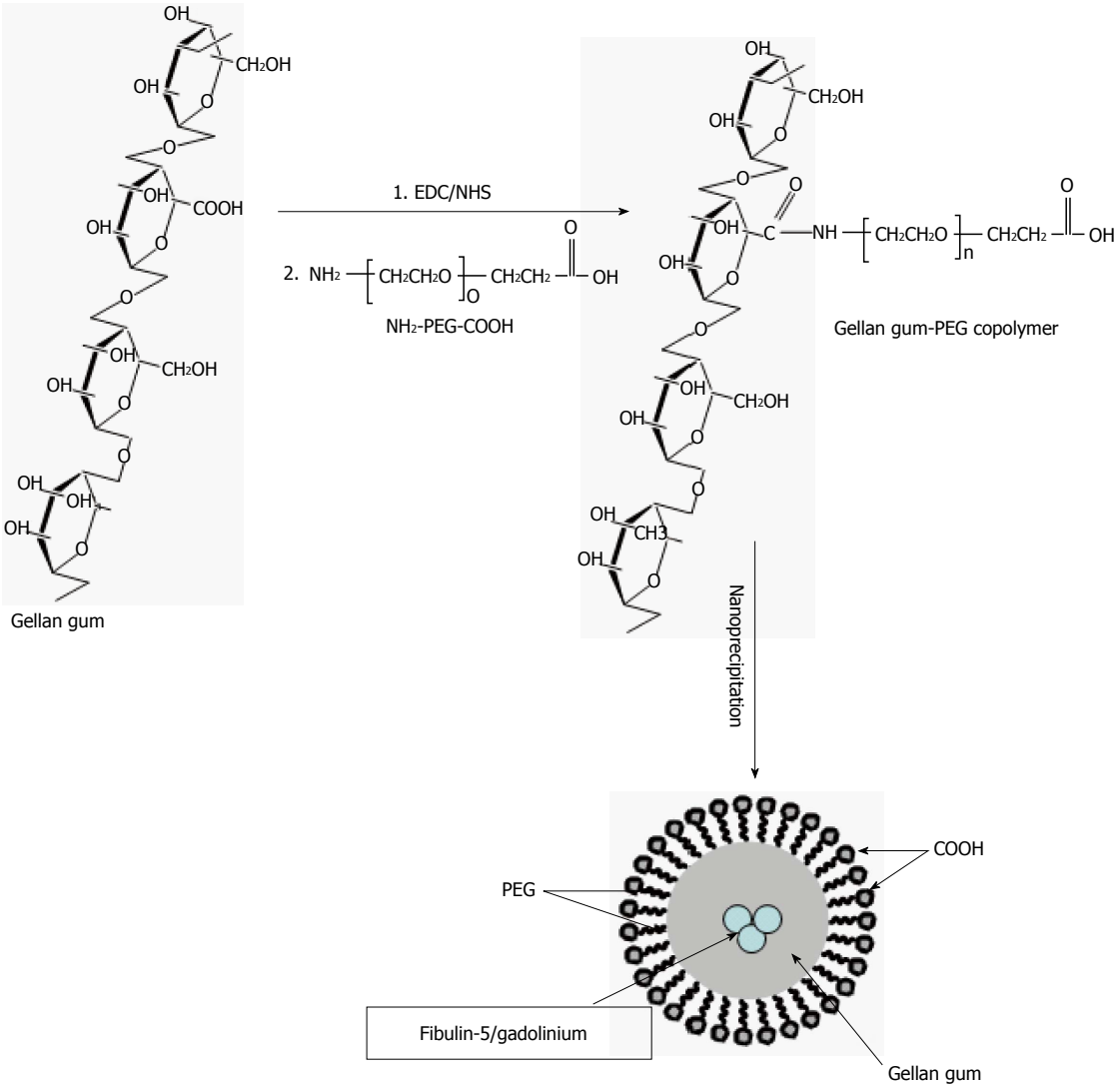

Figure 10 Schematic representation of the technique used for the synthesis of gadolinium encapsulated Gellan-gum-block-Polyethylene glycol co-polymeric nanoparticles. The above figure is a representation of the technical steps to generate nanoparticles of gadolinium encapsulated by Gellan-gum-block-Polyethylene glycol copolymeric micelle nanoparticles. This should ensure delivery and advantageous dwell time in order to block Kupffer functions of uptake of CEA and reduce the pro-metastogenic role of these cells. CEA: Carcinoembryonic antigen.

cell implantation and growth[14]. CEA interaction with its receptor therefore suggests a targeted approach to therapy. A number of methods could be used to achieve disruption of CEA/CEAR including the use of antibodies specific for CEA or its receptor. In general this type of approach has worked well against various cancers and growth receptors *e.g.*, EGFR. Other approaches using small molecule inhibitors of ligand receptor interactions are also being used, In particular RNA aptamers hold great promise as therapeutic agents[96]. These aptamers are now being used in studies involving CEA[97]. Lee *et al*[98] have described the use of a RNA aptamer directed against the PELPK motif of CEA to prevent liver metastasis from the mucinous colon cancer cell line LS174T. They suggest this occurred by disruption of CEA/CEAR interaction. LS174T produces large amounts of CEA and has a high metastatic potential to the liver[99]. Recently, aptamers have also been used against the homotypic adhesion sites of CEA to block tumor cell/cell interactions[100] further emphasizing the feasibility of this approach[101]. Aptamers are also being used for radiolocalization of CEA producing tumors and may even supplant the use of antibodies for this purpose[102]. The use of CEA antagonists to reduce the metastatic potential has now become feasible especially with the advent of small molecule inhibitors. More pre-clinical trials are needed before these approaches can be used in cancer patients at high risk for metastases.

CONCLUSION

We thus have the means to potentially interfere with the uptake of CEA from the hepatic sinusoid Kupffer cell receptor and hopefully disrupt the inflammatory cascade and well as intervening in the fibulin pathways in delivery of fibulin-5 to shore up hepatic defenses against tumor spread to the liver. In time, additional

interventions will be designed to make this a reality based on the lessons learned from the cotton top tamarin- the artful liver metastasis dodger.

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REVIEW

Current status of intragastric balloon for obesity treatment

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Abstract

Endoscopic bariatric therapy may be a useful alternative to pharmacological treatment for obesity, and it provides greater efficacy with lower risks than do conventional surgical procedures. Among the various endoscopic treatments for obesity, the intragastric balloon is associated with significant efficacy in body weight reduction and relief of comorbid disease symptoms. Anatomically, this treatment is based on gastric space-occupying effects that increase the feeling of satiety and may also affect gut neuroendocrine signaling. The simplicity of the intragastric balloon procedure may account for its widespread role in obesity treatment and its applicability to various degrees of obesity. However, advances in device properties and procedural techniques are still needed in order to improve its safety and cost-effectiveness. Furthermore, verification of the physiological outcomes of intragastric balloon treatment and the clinical predictive factors for treatment responses should be considered. In this article, we discuss the types, efficacy, safety, and future directions of intragastric balloon treatment.

Key words: Intragastric balloon; Obesity; Bariatric; Metabolic; Endoscopy

Core tip: Obesity is a complex metabolic illness that is associated with several comorbid diseases. There has been a constant demand for safe and more effective weight reduction interventions to fill the gap in the treatment of obesity. The intragastric balloon is a fascinating intermediate alternative solution between

medical obesity treatment and bariatric surgical procedures for obese patients that may provide better efficacy and have a more favorable risk profile.

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INTRODUCTION

Obesity is a complex metabolic illness that results from excess accumulation of body fat and may lead to negative health consequences. Obesity increases the prevalence of various diseases, including diabetes mellitus, hypertension, coronary heart disease, sleep apnea, stroke, gastroesophageal reflux disease, gall bladder disease, certain types of malignancy, and non-alcoholic fatty liver disease[1]. Moreover, it is also a major avoidable health detriment. Current therapeutic approaches to obesity are lifestyle changes, pharmacologic treatment, and bariatric surgery. Although intensive lifestyle modification was reportedly associated with only limited weight reduction[2-4], when it is combined with weight-loss drugs approved for long-term use, an additional weight reduction of 3%-9% can occur within 1 year[5]. Such drugs are said to improve several cardiometabolic risk factors, but they are also related to harmful adverse effects[5]. Although new obesity medications have recently been approved and introduced[6-8], they are associated with issues of safety and high costs. Weight-loss surgery provides the most sustained and effective therapeutic choice for obesity. Accessible methods include the adjustable gastric band, Roux-en-Y gastric bypass, or sleeve gastrectomy[9,10]. Regardless of its proven effectiveness, only 1% of obese patients eligible for the surgical procedure choose to undergo it[11]. The major issues with surgery are difficult accessibility, high costs, patient non-preference, and significant morbidity and mortality. Although its associated mortality has decreased considerably, the complication rate in the early and late stages of the bariatric procedure persist at 17% (95%CI: 11%-23%)[10].

Therefore, minimally invasive and effective methods are needed for the treatment of obesity. As such, endoscopic bariatric treatment was recently introduced. It includes intragastric balloons, gastroplasty techniques, aspiration therapy, and gastrointestinal bypass sleeves. Among them, the intragastric balloon has been the most frequently used in practice and the most studied for obesity treatment.

INTRAGASTRIC BALLOON

In 1985, the Garren-Edwards gastric bubble (GEGB) was the first intragastric balloon approved for obesity treatment and was introduced in the United States market. It was made of polyurethane, had a cylindrical design, and was filled with 200-220 mL of air[12]. However, several adverse events were associated with its use, including small bowel obstruction associated with spontaneous deflation and gastric mucosal injury. Although the GEGB is no longer used, considerable advancements to its design have led to the development of a more effective and safer intragastric balloon. It is now being used in numerous countries. Additionally, the United States Food and Drug Administration (FDA) recently approved two new intragastric balloons.

The increased prevalence of obesity has motivated experts in bariatric medicine to advance minimally invasive endoscopic treatment for obesity management as well as innovative techniques that address important features of treatments, such as their efficiency and safety. A new meta-analysis showed that endoscopic obesity treatment could be effective and of substantial value if combined with a multidisciplinary and comprehensive treatment plan[13].

The intragastric balloon technique has become an effective method of achieving significant weight reduction in obese people (Figure 1). One or more intragastric balloons can be placed in the stomach using endoscopic procedures under mild sedation in an outpatient setting. Intragastric balloons allow patients to sense fullness and ultimately reduce their food intake. It is hypothesized that the intragastric balloon facilitates satiety peripherally by being an obstacle to food consumption, decreasing intragastric volume, and delaying gastric emptying[14]. Additionally, signals transmitted centrally through the vagal nerves by activated gastric stretching receptors could affect satiety[14]. The intragastric balloon permits an early feeling of satiety, which is thought to be a consequence of gastric distention. The mechanical intragastric distention to a meaningful volume during mealtime significantly decreases the amount of food eaten[15,16].

The intragastric balloon may also act *via* its relationship with various neurohumoral factors. It affects hunger control and gastric emptying by altering gut hormones and peptides such as ghrelin, leptin, cholecystokinin, and pancreatic polypeptide[17,18] and may also be related to physiological adaptation to weight loss.

The intragastric balloon may play diverse roles in obesity treatment as a preemptive therapy, a metabolic therapy, or a primary therapy.

TYPES OF INTRAGASTRIC BALLOONS

Orbera intragastric balloon

The most frequently used balloon is the Orbera Intragastric Balloon (Apollo Endosurgery, Austin, TX, United States), which was known previously as the BioEnterics Intragastric Balloon (BIB). It is an elastic

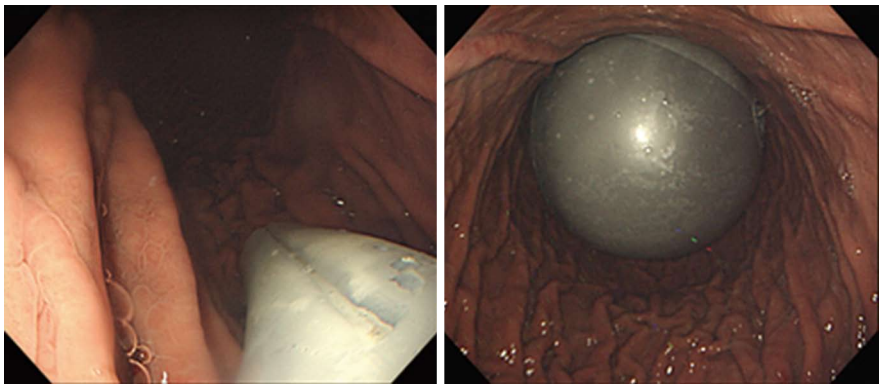

Figure 1 Intragastric balloon placement.

Table 1 Types of intragastric balloons

Balloon	Material	Volume	Weight loss[1]	Note
Fluid-supplied				
Orbera (Apollo Endosurgery)	Silicone	400-700 mL (saline)	16.9 ± 0.9 kg (at 6 mo)[58]	Most widely used and studied intragastric balloon Volume adjustment at the time of placement
Spatz Adjustable Balloon system (Spatz FGIA)	Silicone	400-600 mL (saline)	24 kg (at 12 mo)[64]	Totally adjustable balloon Approved for 12 mo of implantation
ReShape Duo® Integrated DualBalloon System (ReShape medical)	Silicone	900 mL (450 mL × 2) (saline)	25.1 ± 1.6 %EWL (at 6 mo)[62]	Two independent balloons connected to silicone shaft
The Elipse™ (Allurion Technologies)	N/A	450-550 mL (filling fluid)	2.4 kg (at 6 wk)[19]	Naturally swallowed, self-emptying, and naturally excreted
Air-supplied				
Obalon® Gastric Balloon (Obalon Therapeutics)	N/A	250 mL (air, nitrogen)	5 kg (at 12 wk)[66]	Can be swallowed A second or a third balloon can be swallowed depending on patient's progress
Heliosphere BAG® (Helioscopie)	Polyurethane and silicone	950 mL (air)	16 ± 7 kg (at 6 mo)[23]	Less than 30g

[1]Values extrapolated from representative reviews and clinical trials of each intervention. N/A: Not available; %EWL: Percentage of excess weight loss.

silicone balloon containing saline (450-700 mL) (Table 1). The positioning assembly, which comprises a balloon-filling tube and a catheter with the deflated balloon, is blindly advanced to the gastro-esophageal junction. An endoscopic device is inserted to ensure the precise deployment of the intragastric balloon, which is then filled with methylene-mixed saline under direct observation *via* the catheter. If an unexpected balloon rupture occurs, the methylene blue turns the urine green. The Orbera balloon is usually implanted for 6 mo, removed endoscopically by needle aspiration of the intragastric fluid, and retrieved with a snare or grasper. The FDA approved the use of the Orbera balloon on August 6, 2015. It is expected that the Orbera balloon could provide a valuable and less invasive therapeutic approach to bariatric treatment.

ReShape duo

The ReShape Duo® (ReShape Medical, San Clemente, CA, United States) aims to improve the space-occupying effects of intragastric balloons. This non-invasive device is delivered *via* the mouth through a 30-min endoscopic procedure. It is inflated with 900 mL of saline, which is equally distributed to each of two balloons. The balloon is inflated by a controller with methylene blue-mixed saline. This dual balloon system may reduce deflation-associated complications. If one of the balloons ruptures, the other balloon could sustain the location of the device in the stomach. It is recommended that the balloon be retrieved 6 mo after placement. The ReShape Duo was also approved by the FDA, on July 29, 2015.

Spatz

The Spatz Adjustable Balloon System (Spatz Medical, Great Neck, NY, United States) is a silicone balloon that is inflated with saline. It includes a filling catheter, which is extractable endoscopically, that permits an intragastric volume adjustment of 400-800 mL. The volume of the intragastric balloon can be modified to improve the patient's tolerance and increase weight reduction. The Spatz Adjustable Balloon System is allowed to be implanted in the stomach for 12 mo in locations outside the United States.

Elipse

The Elipse™ (Allurion Technologies, Wellesley, MA, United States) is a swallowable, self-draining, and naturally expelled intragastric balloon device for weight reduction. It is covered with a vegetarian shell and fixed to a flexible, slim tube. A resorbable substance inside the balloon degrades, allowing the balloon to empty naturally after a certain period. The deflated intragastric balloon is devised to be excreted *via* the digestive tract[19]. It is inflated with 550 mL of fluid and can be placed in the stomach for 4 mo. Endoscopic procedures are not needed for Elipse™ placement or removal.

Obalon

The Obalon Gastric Balloon® (Obalon Therapeutics Inc, Carlsbad, CA, United States) is a balloon filled with 250 mL of air. It has a self-sealing valve linked to a catheter and is set inside a gelatin capsule. The balloon is packaged in the capsule and swallowed, and the thin catheter is extended alongside the esophagus into the stomach. Fluoroscopy is used to define the location of the intragastric balloon, and the gelatin capsule disintegrates and releases the balloon. The balloon is then inflated using a gas-contained canister. The catheter is separated from the balloon and removed after balloon inflation.

Heliosphere bag

The Heliosphere Bag® (Helioscopie, Vienne, France) comprises a double-bagged polymer covered with an external silicone pouch. It is slowly filled with 960 mL of air for a final inflation volume of 700 mL[20,21]. The weight of the Heliosphere Bag is less than 30 g[22,23].

Silimed balloon

The Silimed Gastric Balloon is a spherical silicone balloon set inside a thin silicone sheath[24]. It is attached to the endoscope by a snare, advanced by an endoscope, and placed in the gastric fundus. It is filled with 650 mL of saline solution mixed with contrast dye and 10 mL of methylene blue.

Adjustable totally implantable intragastric prosthesis

The adjustable totally implantable intragastric prosthesis (ATIIP) is a polyurethane intragastric balloon that is rugby-shaped, 12 cm long, and has a volume of 300 mL when inflated with air[25]. This balloon is placed with an endoscopic percutaneous gastrostomy technique followed by the deployment of a subcutaneous totally implantable system through a surgical procedure. This method may reduce the likelihood of device dislocation and allows for balloon volume adjustment. The proximal balloon position in the gastric fundus-corpus lesion could affect gastric accommodation, neurohormonal process, and electrical action, thus modifying various control processes related to satiety[14].

INTRAGASTRIC BALLOON TREATMENT FOR OBESITY

Indications

The intragastric balloon may offer a minimally invasive and valuable method for managing obesity and related conditions. It is used to achieve weight loss in obese people, generally those with a body mass index (BMI) > 35 kg/m^2, or 30 kg/m^2 with certain comorbidities. Intragastric balloon treatment may play a different role in bariatric treatment based on the grade of obesity.

Preemptive therapy

Early intervention and preemptive therapy for weight reduction can be performed in obese patients (BMI $\geqslant$ 30 kg/m^2) at risk for disease development, at high risk for all-cause mortality, and with a high cardiovascular risk profile[26].

The objective of preemptive treatment is to achieve modest weight reduction, and, therefore, the overall risk/benefit ratio could validate the standards for procedures with this indication. Depending on the circumstances, indications for placement of intragastric balloons could also be extended to manage overweight people with a BMI < 30 kg/m^2 who desire weight reduction but who cannot achieve body weight loss with a controlled dietary program or with pharmacotherapy[27].

Metabolic therapy

Body weight loss achieved with intragastric balloon placement is associated with improvements in obesity-related metabolic illness. The intragastric balloon for metabolic therapy may be performed in patients with mild obesity (BMI $\geqslant$ 30 kg/m^2), where recovery from metabolic disease is the primary concern[28]. Co-existing illnesses, such as hyperlipidemia, type Ⅱ diabetes mellitus, and hypertension, could be particularly improved or resolved with even a modest reduction in body weight[29,30]. Treatment for metabolic issues should be relatively low-risk and have superior stability.

Primary therapy

The goal of intragastric balloon treatment is to achieve weight reduction in severely obese people, generally those with a BMI > 35 kg/m^2 with or without comorbidities, and who could not achieve long-term weight loss with a weight-control regimen. In addition, intragastric balloon therapy could be performed in patients with a BMI $\geqslant$ 40 kg/m^2, primarily as a preparation for bariatric treatment or in patients with increased surgical risks. Obese patients who reject bariatric surgical procedures or who do not have an approach for surgery can also opt for it. Intragastric balloon therapy used as a primary therapy could induce weight reduction and improve obesity-related comorbidities with a level of safety and efficiency

comparable to that of bariatric surgery[28]. However, lower efficiency is also acceptable because of the lower risk profile of intragastric balloon therapy.

Exclusion criteria

Exclusion criteria include any situation that could increase the risks related to intragastric balloon insertion, such as a large hiatal hernia (> 5 cm), active ulcer in the stomach or duodenum, previous surgical resection of the stomach, inflammatory bowel disease, gastrointestinal neoplasm, oropharyngeal abnormalities, active gastrointestinal bleeding, coagulative disorder, variceal disease, alcoholic disease or drug abuse, psychiatric disease, pregnancy, use of anti-coagulants or anti-inflammatory drugs, and cardiovascular, pulmonary or cerebrovascular diseases[31].

EFFECTS OF INTRAGASTRIC BALLOONS

The purpose of intragastric balloon treatment is to stimulate weight loss and assist with recovery from associated comorbidities with adequate safety. Gastric capacity restriction is an essential factor in surgical bariatric management.

Surgical gastric restriction could induce early satiety and potentiate gastric mechanical and chemical stimulation through a relationship with various gastric or exogenic factors. It affects hunger control and gastric emptying through alterations in gut hormones and peptides[32-34]. The intragastric balloon attempts to mimic surgical weight loss procedures by restricting the effective gastric volume.

Body weight loss

Results from previous studies indicated that the mean weight loss associated with intragastric balloon therapy ranged between 10.5 and 13.7 kg after 3 mo and between 12 and 26.3 kg after a 6-mo placement of the Orbera intragastric balloon[22,23,35-49]. After balloon removal at 6 mo, the achieved weight reduction endured to some extent. The excess weight loss (EWL) at the 12-mo implantation mark (6 mo after removal) was 14% to 50.9%[37,40,42,44,50-54]. The EWL at 12 mo after balloon extraction ranged from 14.2% to 27.2%[51,55-57]. The Orbera intragastric balloon was most effective during the first 3 mo of therapy. During that time, average weight loss of obese patients was 12.9 kg, or 80% of the total achieved weight reduction[58]. Additionally, the initial body weight loss (BWL) following intragastric balloon placement was associated with significant long-term weight maintenance. The percentage of BWL 1 mo after intragastric balloon placement was significantly associated with weight loss after 6, 12, and 18 mo (Pearson correlation coefficient = 0.77, 0.65, and 0.62, respectively, $P < 0.001$ for all)[59].

A study reported that insertion of a second balloon is not difficult and achieved good results. The mean %EWL was 31.5 ± 23.2 after the second balloon removal[43]. Some studies showed long-term results after intragastric balloon placement. A multi-center European study presented results for weight loss 3 years after balloon removal, which accounted for 29.1% of mean EWL[54]. A study that included 195 patients who were followed up for 5 years after intragastric balloon insertion demonstrated a %EWL of 12.97 ± 8.54[44].

Additional limited studies have utilized other intragastric balloons. A Spanish study with 60 patients showed a total body weight reduction of 16.6 ± 9.33 kg 6 mo after implantation of the ReShape Duo double-balloon system[60]. A prospective study with a double-balloon system showed a mean EWL of 18.3% in the control group compared to 31.8% in the treatment group 6 mo post-balloon implantation[61]. Furthermore, 64% of the reduced body weight was maintained at 12 mo post-implantation (6 mo after removal). In the REDUCE Pivotal Trial, a prospective randomized trial with 326 patients, patients assigned to the double-balloon system plus exercise and diet showed significantly superior EWL compared to the sham endoscopy plus exercise and diet alone group at 6 mo post-implantation (25.1% *vs* 11.3%, $P < 0.05$) in an intent-to-treat analysis[62].

A pilot trial showed 15.6 kg of mean weight loss at 24 wk and 24.4 kg at 52 wk after Spatz adjustable balloon placement[63]. A small observational investigation in 73 obese patients showed 45.7% of EWL 12 mo after deployment of the Spatz adjustable balloon[64].

Two preliminary studies with the Silimed Gastric Balloon reported results after the completion of 6 mo of treatment. The mean weight loss was 11.3 kg[24] and 8.1 kg[65]. A total of 57 morbidly obese patients underwent ATIIP placement. Mean EWL was 28.7% at 6 mo (38 patients) and 39.2% at 12 mo (20 patients)[25]. The Obalon (orally ingestable intragastric balloon) showed median weight losses after 4 weeks, 8 weeks, and 12 wk as 2.2 kg, 4.0 kg, and 5 kg, respectively[66]. A prospective study with 50 obese patients compared the effects of intragastric balloon therapy or pharmacotherapy on weight reduction. At 6 mo, patients in the intragastric balloon group had lost more weight than had patients in the pharmacotherapy group (percent of initial weight lost, %IWL = 14.5 ± 1.2; percent of excess BMI lost, %EBL = 37.7 ± 3.2 *vs* %IWL = 9.1 ± 1.5, %EBL = 25.3 ± 4.1, respectively, $P < 0.005$)[67].

Although the intragastric balloon has been shown to be effective in causing a meaningful weight loss, several studies have reported that the results were short-lasting, with most patients regaining weight following intragastric balloon removal[31,36,51,55,68-72].

Improvement in metabolic diseases

Obesity in patients is related to several comorbidities that are significant targets for obesity treatment. A

Table 2 Plasma ghrelin and leptin level after intragastric balloon treatment

Ref.	Balloon	Weight loss at 6 mo	Ghrelin (at 0 mo)	Ghrelin (after 3 mo)	Ghrelin (after 6 mo)	Ghrelin (after 12 mo)	Leptin (at 0 mo)	Leptin (after 3 mo)	Leptin (after 6 mo)	Leptin (after 12 mo)
Mathus-Vliegen *et al*[17]	Orbera	17.4 ± 7.8 kg	722.3 ± 151.5 pg/mL	791.5 ± 239.0 pg/mL	743.7 ± 115.2 pg/mL	N/A	N/A	N/A	N/A	N/A
Fuller *et al*[48]	N/A	14.2%	414.1 pmol/L	448 pmol/L	452.4 pmol/L	379.4 pmol/L	23.4 ng/mL	18.5 ng/mL	11.7 ng/mL	19.7 ng/mL
Bužga *et al*[85]	MedSil®	18.4 ± 8.2 kg	240.5 ± 101.5 μg/L	378.1 ± 155.8 μg/L	335.8 ± 149.2 μg/L	N/A	30.4 ± 17.2 μg/L	18.2 ± 15.8 μg/L	14.9 ± 15.5 μg/L	N/A
[1]Nikolic *et al*[86]	Orbera	N/A	958.3 pg/mL	1346.2 pg/mL	1050.1 pg/mL	922.6 pg/mL	25.1 ng/mL	14.3 ng/mL	10.5 ng/mL	17.5 ng/mL
Konopko-Zubrzycka *et al*[39]	Orbera	17.1 ± 8.0 kg	621.9 ± 182.4 pg/mL	903.9 ± 237 pg/mL (1 mo)	N/A	N/A	61.3 ± 36.7 ng/mL	39.9 ± 17.5 ng/mL (1 mo)	N/A	N/A
Martinez-Brocca *et al*[79]	Orbera	12.7 ± 5.6 kg (4 mo)	934.4 ± 199.2 pg/mL	947.1 ± 195.1 pg/mL (1 mo)	N/A	N/A	31.9 ± 16.4 ng/mL	22.4 ± 15.1 ng/mL (1 mo)	N/A	N/A
Mion *et al*[78]	Orbera	8.7 kg	3.2 ± 0.4 ng/mL	N/A	1.9 ± 0.1 ng/mL	N/A	27.8 ± 3.7 ng/mL	N/A	18.7 ± 2.7 ng/mL	N/A

[1]Data from patients with body mass indexes < 40 kg/m^2. Data are presented as mean ± SD or median. N/A: Not available.

study with 143 obese patients described the effects of the Orbera intragastric balloon at the 12-mo follow-up[51]. The incidence of metabolic syndrome declined from 34.8% (before balloon insertion) to 14.5%, 13%, and 11.6% at the time of removal, at the 6-mo follow-up, and at the 1-year follow-up, respectively. The incidence of type 2 diabetes mellitus decreased from 32.6% to 20.9%, 22.5%, and 21.3%, respectively. Likewise, the occurrence of hyperuricemia, hypertriglyceridemia, and hypercholesterolemia decreased from 26.1%, 37.7%, and 33.4% to 25.4%, 14.5%, and 16.7%, respectively, at the time of removal, 25.9%, 15.2%, and 16.7%, respectively, at the 6-mo follow-up, and 26.4%, 17.4%, and 18.9%, respectively, at the 1-year follow-up. A multi-center study presented data following treatment with the Orbera intragastric balloon in overweight populations[54]. The percentage of patients with co-morbidities at baseline and at the 3-year follow-up was 29% and 16% for hypertension, 15% and 10% for diabetes mellitus, 20% and 18% for dyslipidemia, 32% and 21% for hypercholesterolemia, and 25% and 13% for osteoarthropathy, respectively. A study with 119 obese patients assessed the effects of the Orbera intragastric balloon on obesity-associated diseases and quality of life[42]. Six months after placement of the intragastric balloon, the rate of metabolic syndrome in the patients decreased from 42.9% to 15.1% (P < 0.0005). Cholesterol, triglycerides, fasting glucose, C-reactive protein levels, and blood pressure also improved after balloon treatment (P < 0.005). In patients with diabetes mellitus, the HbA1c level was decreased at 6 mo compared to that at baseline (7.4% to 5.8%; P < 0.0005). In addition, the quality of life of patients increased.

Obesity is one of the risk factors for nonalcoholic fatty liver disease (NAFLD). About 27% of patients with NAFLD can develop fibrosis, and 19% can develop cirrhosis[73]. A randomized controlled study showed that intragastric balloon therapy improved the histology of nonalcoholic steatohepatitis[74]. Six months after implantation, a significant reduction in the median NAFLD activity scores was observed in the intragastric balloon-treated group (2 *vs* 4, P = 0.030) compared to that in the control group. Additionally, the median steatosis scores displayed a trend toward improvement in the balloon-treated group compared to those in the control group (1 *vs* 1, P = 0.075).

Alterations in gastrointestinal hormones

Body weight is controlled by multifaceted coordination of both central and peripheral factors. It is now obvious that a physiologic change in gut neurohumoral signaling is one important contributor to weight reduction and improvement in related diseases obtained by anatomic surgical manipulation of the gastrointestinal tract[75]. Moreover, intragastric balloon treatment may affect weight changes through an interaction of gastric neurohumoral factors (Table 2). These factors include several gut peptides and hormones, such as ghrelin, leptin, cholecystokinin, peptide YY, pancreatic polypeptide, and glucagon-like peptide-1.

These factors are interrelated and participate in the peripheral mediation of satiety[76,77]. Ghrelin is a hormone that has been known to influence energy balance. However, several studies presented varying results regarding ghrelin concentrations after balloon treatment. A study with 40 obese patients who underwent balloon placement indicated no effect on ghrelin levels when patients were fasting or meal-suppressed[17]. In another study, 17 patients with non-morbid obesity underwent balloon placement, and fasting plasma ghrelin concentrations significantly decreased (3.2 to 1.9 ng/mL; P = 0.021) as a result[78]. Martinez-Brocca *et al*[79] reported that fasting and meal-suppressed plasma ghrelin levels did not

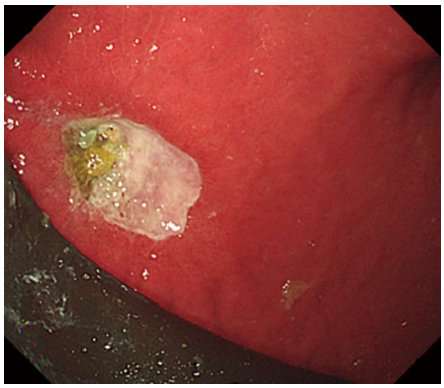

Figure 2 Gastric ulcer induced by intragastric balloon placement.

differ significantly between groups in morbidly obese patients. Konopko-Zubrzycka *et al*[39] reported that body weight reduction after balloon treatment is related to a transient elevation in plasma ghrelin levels and a decrease in plasma leptin levels.

Another study evaluated fasting and postprandial cholecystokinin and pancreatic polypeptide secretion after 13 wk of balloon treatment in obese patients. Baseline and meal-stimulated cholecystokinin levels were decreased[18].

Maintenance of weight loss is controlled by the collaboration of several processes, including environmental, behavioral, and homeostatic factors[80]. Physiological adaptation to weight reduction and weight regain show interplaying alterations in the stability of the levels of hunger hormones and energy homeostasis, in addition to changes in subjective appetite and nutrient metabolism. Appetite-related gastrointestinal hormones may play a crucial function in body weight regain after weight reduction[81]. Bariatric procedures may prove to be effective methods to aid in altering obese patients' physiology and may provide a good opportunity for long-term maintenance. Procedures that affect the physiology of weight loss and regain may eventually fortify future approaches for obesity treatment.

COMPLICATIONS OF THE INTRAGASTRIC BALLOON

Adverse events rates following Orbera intragastric balloon placement showed pooled incidences of pain (33.7%) and nausea (29%) as common adverse side effects[82]. The incidence of GERD, gastric ulcers (Figure 2), and balloon migration was 18.3%, 2%, and 1.4%, respectively. Serious adverse events with the Orbera balloon are uncommon, with prevalences of small bowel obstruction, perforation, and death as 0.3%, 0.1% and 0.08%, respectively[82]. Similarly, the REDUCE pivotal trial (n = 264), which evaluated the efficacy and safety of the ReShape Duo intragastric balloon, showed that symptoms such as nausea, abdominal pain, and vomiting were common, but decreased rapidly with medical management. Spontaneous intragastric balloon deflation occurred in 6% of patients, but balloon migrations did not occur concurrently. Early balloon removal occurred in 9.1% of study participants for non-ulcer-related intolerance. In 35% of the study participants, a gastric ulcer presented initially, but ulcer incidence and size decreased following subsequent device modification[62].

FUTURE DIRECTIONS FOR THE INTRAGASTRIC BALLOON

New emerging technology trends in endoscopic treatment for obesity require an extensive and meticulous research plan to promote finding and recognize their optimal role in managing obese patients and their applications for clinical practice[83]. Intragastric balloon placement can be performed through a simple endoscopic method and is easily reversible. This simplicity offers an expansive role in obesity treatment based on the degree of obesity. It is important to establish an appropriate method for intragastric balloon treatment by classifying the degree of obesity. Although serious complications with intragastric balloon treatment are rare, safety is an issue that cannot be overlooked. Various factors are associated with balloon safety, such as its durability and the simplicity of the procedure. Advances in device properties and procedural techniques could offer more cost-effectiveness with this outpatient procedure[84]. There is also uncertainty about the ideal filling medium and content. A preference for fluid other than air has been mentioned[14], but a debate still exists, with no established guidelines to date. There is a need to establish standards of intragastric balloon treatment practice, which could comprise pre- and post-procedural guidelines and long-term schedule management. In addition, collaboration with dietary counseling and an exercise program could offer quality assurance with intragastric balloon treatment.

Intragastric balloon treatment might produce only short-lasting effects in obesity treatment. Thus, it is important to maintain weight loss following intragastric balloon removal. Long-term management for weight reduction after intragastric balloon removal can also comprise intensive lifestyle modification, alone or with pharmacotherapy, and could be suggested to protect against weight regain.

Material and safety issues regarding the intragastric balloon should be further investigated. More research should be performed to investigate a pathophysiologic pattern of obesity, the uncertain role of gut hormones, potential predictive factors for the efficacy of the intragastric balloon in obesity treatment, and individualized treatment-induced changes.

CONCLUSION

Effective and safe weight reduction is very important

for the treatment of obesity, which is responsible for about 5% of all obesity-related deaths globally. Intragastric balloon treatment is more than just a transient curiosity. It shows promise in improving the quality of life and health status for obese patients. It offers a minimally invasive and effective method for managing obesity and associated conditions. Although there remains the possibility of improvement with research and development, gastroenterologists should maintain attention and interest in determining satisfactory outcomes and verifying standards of intragastric balloon treatment for the obesity epidemic.

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MINIREVIEWS

Role and mechanisms of action of *Escherichia coli* Nissle 1917 in the maintenance of remission in ulcerative colitis patients: An update

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory disease, whose etiology is still unclear. Its pathogenesis involves an interaction between genetic factors, immune response and the "forgotten organ", Gut Microbiota. Several studies have been conducted to assess the role of antibiotics and probiotics as additional or alternative therapies for Ulcerative Colitis. *Escherichia coli* Nissle (EcN) is a nonpathogenic Gram-negative strain isolated in 1917 by Alfred Nissle and it is the active component of microbial drug Mutaflor® (Ardeypharm GmbH, Herdecke, Germany and EcN, Cadigroup, In Italy) used in many gastrointestinal disorder including diarrhea, uncomplicated diverticular disease and UC. It is the only probiotic recommended in ECCO guidelines as effective alternative to mesalazine in maintenance of remission in UC patients. In this review we propose an update on the role of EcN 1917 in maintenance of remission in UC patients, including data about efficacy and safety. Further studies may be helpful for this subject to further the full use of potential of EcN.

Key words: Ulcerative colitis; *Escherichia coli* Nissle; Metanalysis; Probiotic; Randomized trial; Inflammatory bowel disease

Core tip: *Escherichia coli* (*E. coli*) Nissle is a nonpathogenic Gram-negative strain used as a probiotic with very good quality paper assessing its bio-equivalence to mesalazine in maintaining remission in ulcerative colitis. Mechanisms of actions of this compound include immune-modulatory properties, reinforcement of intestinal barrier and inhibitory effect towards other pathogenic *E. coli*.

Scaldaferri F, Gerardi V, Mangiola F, Lopetuso LR, Pizzoferrato M, Petito V, Papa A, Stojanovic J, Poscia A, Cammarota G, Gasbarrini A. Role and mechanisms of action of *Escherichia coli* nissle 1917 in the maintenance of remission in ulcerative colitis patients: An update. *World J Gastroenterol* 2016; 22(24): 5505-5511 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5505.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5505

INTRODUCTION

Ulcerative colitis (UC) is a chronic remitting and relapsing disease, characterized by a continuous inflammation which can stretch from the rectum up to the entire colon, often resulting in mucosal ulceration, rectal bleeding, diarrhea, abdominal pain. Its etiology is still unclear, and it is multifactorial. Several factors have been identified as major determinants for induction or relapses and, among these, the imbalanced gut microbiota has become more crucial in recent years. The main hypothesis is that it is due to an excessive immune response to endogenous bacteria, in genetically predisposed individuals[1].

The human microflora, known as "microbiota", includes more then thousand different species and higher than 15000 different bacterial strains, for an average total weight of 1 kg. In recent years several studies investigated the correlation between dysbiosis and intestinal and extra-intestinal diseases, including inflammatory bowel disease (IBD) and so UC[2].

Probiotics are viable agents conferring benefits to the health of the human host[3]. They can provide a beneficial effect on intestinal epithelial cells in numerous ways. Some strains can block pathogen entry into the epithelial cell by providing a physical barrier or by creating a mucus barrier; other probiotics maintain intestinal permeability acting on tight junctions. Some probiotic strains produce antimicrobial factors, other strains modulate the immune response[4].

The role of microbiota in UC was supported by several evidences: inflammation is greatest in intestinal tracts with high concentration of bacteria, surgical reduction of the bacterial load is associated with improvement of inflammation and inflammation does not occur in germ free animals[5].

The treatment goal of UC is the induction and the maintenance of remission. 5-aminosalicylic acid (ASA) compounds, azathioprine/6 mercaptopurine, corticosteroids, cyclosporine, methotrexate, and anti-TNFα agents are conventional therapies used to control the disease. There are also other therapies, used as addition or alternative to conventional therapies in UC, in particular antibiotics and probiotics, which modulate gut microbiota.

Escherichia coli (*E. coli*) Nissle (EcN) 1917 is a non-pathogenic Gram-negative strain used in many gastrointestinal disorder including diarrhea[6], uncomplicated diverticular disease[7] and IBD, in particular UC[8].

STRUCTURE AND MECHANISMS OF ACTION OF EcN 1917

EcN 1917 (O6:K5:H1) was isolated by Prof. Alfred Nissle from Freiburg, Germany, in 1917 from the intestinal microflora of a young soldier. This soldier - unlike his comrades - did not develop infectious diarrhea, when stationed during World War I in Southeastern Europe (Dobrudja/Balkan peninsula), endemic for Shigella at that time. The strain was named *E. coli* strain Nissle 1917.

Using this *E. coli* strain Prof. Nissle developed the probiotic drug Mutaflor® and introduced it into medical practice in the same year. Since 1917, Mutaflor® is available in the German pharmaceutical market without interruption and recently also in Italy as EcN (cadigroup).

The lack of defined virulence factors (alpha-hemolysin, P-fimbrial adhesins, *etc.*) combined with the expression of fitness factors such as microcins, different iron uptake systems (enterobactin, yersiniabactin, aerobactin, salmochelin, ferric dicitrate transport system, and the *chu* heme transport locus), adhesins, and proteases may support its survival and efficacious colonization of the human gut, and contribute to the probiotic character of EcN 1917.

It exhibits a semi-rough lipopolysaccharide (LPS) phenotype and serum sensitivity and does not produce known toxins[9]. EcN colonizes the intestine within few days and it remains as colonic flora for months after administration[10].

EcN has an intestinal anti-inflammatory effect, but also systemic effects[11], and there are many theories about its mechanism of action (Figure 1): (1) It has direct antimicrobial effects: it inhibits EHEC (*E. coli* EDL933) colonization in animal models[12] and synthesis of Shiga-Toxins in co-cultivation experiment with STEC (Shiga-Toxin producing *E. coli*)[13]. (2) It is involved in the bacterial-epithelial crosstalk ("Host cell signaling") by biofilm formation: it expresses F1C Fimbria. This is very important in the formation of biofilm, adherence to epithelial cells and persistence in infant mouse colonization[14]. Its flagellum is the major "propulsor" *in vivo*, which allow this probiotic strain to efficiently compete with pathogens for binding sites on host tissue[15]. It directly stimulates

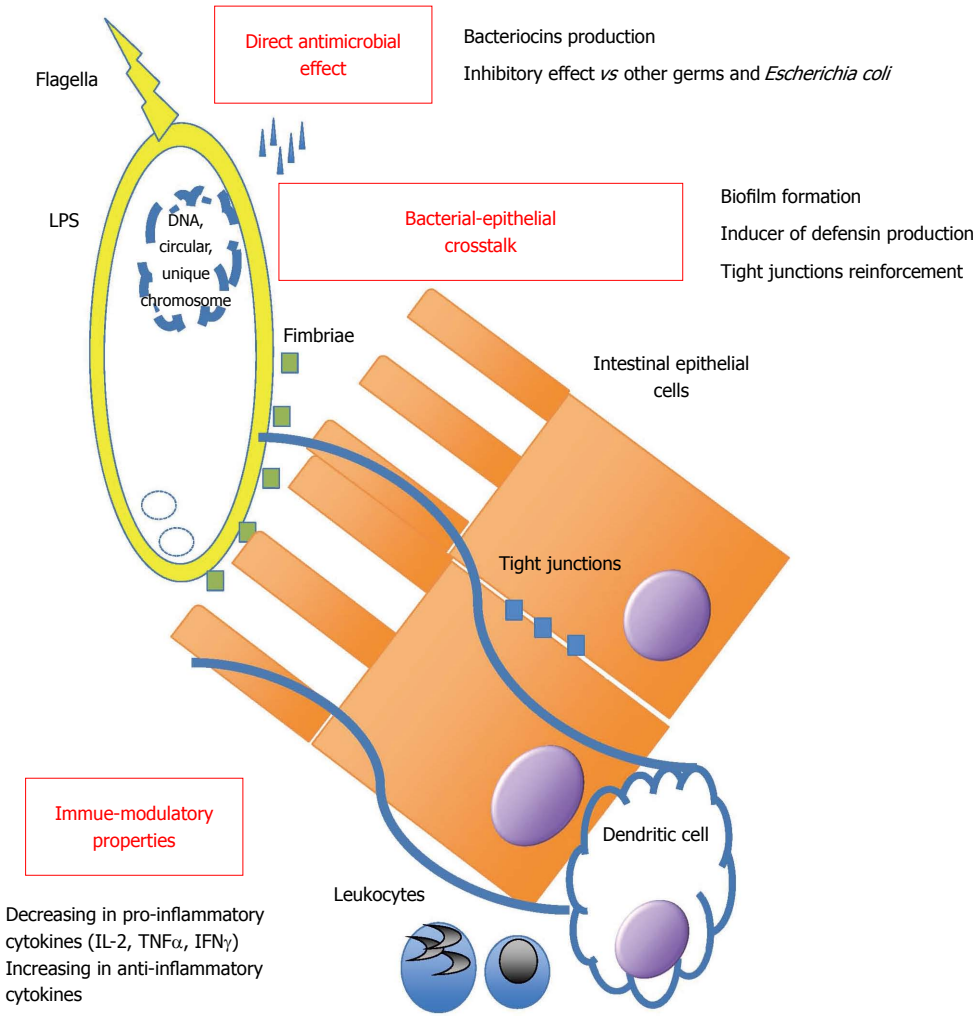

Figure 1 Structure and mechanisms of action of *Escherichia coli* Nissle 1917. LPS: Lipopolysaccharide; IL-2: Interleukin-2; TNF: Tumor necrosis factor; IFN: Interferon.

defensin production by intestinal epithelial cells, such as the human beta-defensin that inhibits adhesion and invasion of intestinal cells by pathogenic adherent invasive *E. coli*, which play a key role as trigger in immune response in IBD patients[16-18]. It strengthens tight junctions of intestinal epithelial cells, by up-regulating the expression of the mRNA for the zonula occludens proteins ZO-1 and ZO-2, so it has an effect on the repair of the "leaky gut"[19-21]. (3) It interacts with immune system by causing decreasing in pro-inflammatory cytokines (IL-2, TNF-α, IFNγ) and increasing in anti-inflammatory cytokines by peripheral blood mononuclear cells *in vitro*. It may reduce the expansion of newly recruited T cells into the intestinal mucosa and decrease intestinal inflammation, but it doesn't affect activated tissue-bound T cells, which may eliminate deleterious antigens in order to maintain immunological homeostasis[22,23]. Furthermore EcN has a specific LPS that is responsible for its immunogenicity, without major immunotoxic properties at doses suggested and that provide, together with the other described features, a powerful effect on intestinal immune function[24].

Efficacy of EcN in animal models of colitis

Schultz *et al*[25] conducted a study on animal models of acute and chronic colitis. Acute colitis was induced by administration of dextran-sodium sulfate (DSS) in drinking water and chronic colitis was induced by transferin $CD4^+$ $CD62L^+$ T lymphocytes from BALB/c mice in SCID mice. These studies have shown that administration of EcN ameliorates intestinal inflammation (measured by histological scores) in chronic models but not in acute models, in accordance with clinical observations. Therefore, it was shown that EcN reduced secretion of pro-inflammatory cytokines, measured by enzyme-linked immune-sorbent assay[25].

Grabig *et al*[5] demonstrated that EcN ameliorates experimental colitis induced by administration of 5% DSS in mice *via* TLR-2 and TLR-4 dependent pathway.

Decreasing of symptom scores and differences in body mass loss were shown in animal models of DSS colitis in BALB/c mice treated with EcN in the study conducted by Kokesová *et al*[26].

Table 1 Main results from trials on *Escherichia coli* Nissle on ulcerative colitis

Efficacy of EcN 1917 in maintanance of UC remission
Results from major randomized controlled clinical trials
EcN 200 mg/d is equivalent to Mesalazine 1000 mg/d in mantainance of UC remission[27]
EcN 400 mg/d is equivalent to to Mesalazine 2400 mg/d in maintanance of UC remission following an acute flare[28]
EcN 200 mg/d is equivalent to Mesalazine 1500 mg/d in mantainance of UC remission[10]
Results from minor studies
Rectal admistration of EcN 40 mL/d is effective in moderate distal active UC[30]
EcN 200 mg/d is equivalent to Mesalazine 1500 mg/d in maintanance of UC remission[6]

EcN: *Escherichia coli* Nissle; UC: Ulcerative colitis.

CLINICAL ROLE OF EcN 1917 IN MAINTANANCE OF UC

There are three major double-blind RCTs (Table 1), which compare EcN to mesalazine in prevention of relapse in UC patients, all of them designed to demonstrate equivalence of two treatment according to Schuirmann's two-one side test or "non inferiority trials".

The first trial was conducted by Kruis *et al*[27] in 1997. It was a randomized, double-blind, double-dummy study conducted on 120 out-patients in Germany, Czech Republic and Austria. Patients had a confirmed diagnosis of ulcerative colitis in remission. In particular patients had to be in remission for a maximum of 12 mo, with clinical activity index (CAI) < 4, no endoscopic or histological signs of acute inflammation. Each patient had to have had at least 2 relapses prior inclusion.

Patients received 500 mg mesalazine t.d.s. and a placebo form of EcN preparation or 200 mg/d of a preparation containing EcN in a single dose ("Mutaflor", Ardeypharm GmbH, Herdecke, Germany. 100 mg contains 25×10^9 viable *E. coli* bacteria) and a placebo form of mesalazine. The duration of the study was 12 wk. Study objectives included the assessment of the equivalence of the CAI under the two treatment modalities and the comparison of the relapse rates, relapse-free times and global assessment.

Study population was homogeneous into the two groups, with a prevalence in left sided colitis and small prevalence of active use of steroids (less then 25 % in both groups).

From the results of this study, no significant difference was observed between the two groups, although a low statistical power and a minor trend towards a slightly higher CAI in the EcN group. No serious adverse events reported for both groups.

The second trial was conducted by Rembacken *et al*[28] in 1999. This was a single-center, randomised, double-dummy study involving 116 patients, which were treated with mesalazine 800 tds (Asacol formulation) or EcN 2 cp per 2 times daily ("Mutaflor", Ardeypharm GmbH, Herdecke, Germany. 100 mg contains 25×10^9 viable *E. coli* bacteria).

Inclusion criteria were: 18-80 years of age, clinical active (mild-moderate and severe) ulcerative colitis ("Leeds-Index") defined by number of 4 or more liquid stools/day for the last 7 d, with or without blood, erythema on sigmoidoscopy and histological confirmation of active ulcerative colitis.

The study populations were comparable. In particular: the median clinical activity index on study entry was 11 in the mesalazine group and nine in the EcN group (up to 30% of patients had a severe disease). The median sigmoidoscopy score was four in both groups. All patients received rectal or oral steroids at different doses together with a 1-wk course of oral gentamicin. At baseline both groups had an high active usage of steroids, around for 50% of the cases; furthermore, 2% in mesalazine group and 18% in EcN. In both groups the proportion between proctitis, left sided and pancolitis was similar (1/3 per each condition). No significant differences between the 2 groups were found.

Active treatment, started at the enrolment, was hydrocortisone enema twice daily, prednisolone 30 mg a day in moderate colitis and prednisolone 60 mg a day in severe colitis (according to Truelov Witts criteria). Only people in remission at 12 wk were enrolled in the follow up part of the trial assessing maintenance of remission.

Starting from remission, patients were maintained on either mesalazine or *E. coli* but the doses were reduced respectively at 1.2 g per day for the mesalazine group and 2 capsules per day for the EcN group. The follow up was at 12 mo. 59 were randomised to mesalazine and 57 to EcN. Of them 75% and 68% reached remission. Of those in remission, 73% of patients in the mesalazine group and 67% in the EcN group, relapsed by 12 mo. In the mesalazine group, the mean duration of remission was 206 d (median 175) compared with 221 (median 185) in the group given *E. coli*, ($P = 0.0174$). The treatment with EcN was proved safe, acceptable, and clinically equivalent to mesalazine in maintaining remission after an acute relapse of ulcerative colitis. Finally, this study is characterized by a very high rate of relapse: this is however not un-expected as the study population comprehends moderate and also severe patients.

The third trial was conducted by Kruis *et al*[10] in 2004. This was a double-blind, double dummy study in which 327 patients affected by ulcerative colitis in remission phase were recruited. 162 patients received Mutaflor 200 mg/d and 165 received Mesalazine 500 mg three times daily for 12 mo. Inclusion criteria were: age between 18-70 years, diagnosis of UC in remission [CAI $\leqslant$ 4, endoscopic index (EI) $\leqslant$ 4, and no signs of acute inflammation on histological examination]. Furthermore, within inclusion criteria there was at

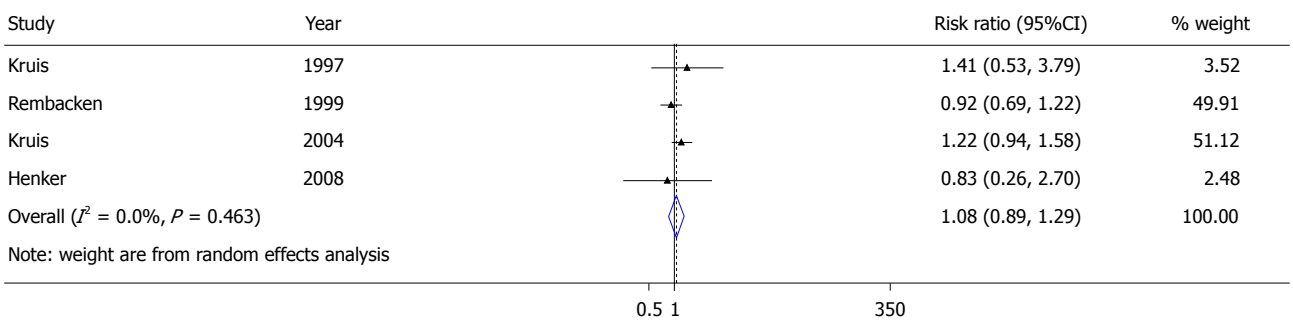

Figure 2 Metanalysis on randomized controlled trials assessing role of *Escherichia coli* Nissle on maintenance of remission in ulcerative colitis.

least two acute attacks of UC prior to the study and duration of the current remission of no longer than 12 mo. Primary objective of the study was meant to compare the number of patients experiencing a relapse during the 12 mo observation time between the two treatment groups. Secondary aims included efficacy variables like physician's and patient's assessment of general well being and calculation of a quality of life index. Additionally, time to relapse, CAI, EI, and histological findings were also evaluated. In the EcN group 36.4% of patients relapsed compared to 33.9% in the mesalazine group and statistical tests showed equivalence of the two treatments. A subgroup analyses showed no difference in terms of duration and localization of disease or pre-trial treatment. No difference of quality of life was shown in the two groups.

Overall same results on tolerance were found: it was very good or good in the EcN group in 80.0% and in the mesalazine group in 86.0%. According to the physician's assessment, the respective values were 85.1% and 90.3%. No unexpected drug reactions occurred during the study.

This is perhaps the best study based on the quality of data and also the large number of patients enrolled. Furthermore clinical outcomes were assessed by well-established endoscopic and histological activity indices, like in modern trials for more powerful drugs.

In addition to the above-described trials, there is a multicentric placebo-controlled study on 90 patients with moderate distal active UC conducted by Matthes *et al*[29] Patients in EcN groups received EcN 40, 20 or 10 mL (amount of bacteria 10E8/mL) enema once daily for at least 2 wk. A clinical DAI was recorded after 2, 4 and/or 8 wk.

The majority of patients also received concomitant medical treatment such as oral mesalazine. Remission rates and improvement of the histological score was showed particularly in the EcN 40 mL group, but further studies on largest population are required.

This study has shown that rectal administration of EcN is an effective treatment, with a dose dependent efficacy as shown in the Per Protocol analysis. Unfortunately the ITT analysis did not show significant results[29].

Many meta-analysis present in the literature support the role of ECN in the therapy of ulcerative colitis[6,10,27,28,30]. In particular, a very recent published meta-analysis, performed by Losurdo *et al*[31] (Figure 2), showed a non-significant inferiority of EcN in relapse prevention compared to mesalazine in preventing disease relapse, thus confirming current guideline recommendations[31], despite a novel randomized double-blinded placebo controlled trial conducted in Denmark and published very recently[32], with negative results. One hundred patients with active UC defined by CAI-score $\geqslant$ 6 and with calprotectin higher than 50 mg/kg, were enrolled and randomized into four groups of treatment: Ciprofloxacin (for 1 wk) followed by EcN (for 7 wk), Ciprofloxacin (for 7 wk) followed by placebo (for one week), placebo (for one week) followed by EcN (for 7 wk) and placebo (for one week) followed by placebo (for 7 wk). Aim of the study was the induction of the remission in ulcerative colitis and Kaplan-Meier curves were used to compare groups. In this study, the 54% of patients in the placebo/EcN group reached remission, compared to 89% of patients in the placebo/placebo group (P < 0.05), 78% of Ciprofloxacin/placebo group and 66% Ciprofloxacin/EcN group. Furthermore, the placebo/EcN group had the largest number of withdrawals. These impressive results, which would exclude a role of EcN in treatment of active ulcerative colitis, display several limitations, which make this study really weak. First of all this is a monocenter study, with a very not homogeneous population as showed in the table of patients characteristics. Mean CAI score at baseline was 10.5, 8.9, 9.3 and 8.9 in the Cipro/EcN, Cipro/placebo group, placebo/EcN group, placebo/placebo group, respectively. Furthermore patients clearly differed in concomitant medications use, in particular for use of active use of topical drugs and steroids as well as immunosuppressant. Taken together, these data suggest that this trial display major limitations regarding groups homogeneity. We confirmed the data from the published metanalysis, which we performed independently before discovering that it was just published. In the present paper we report an extract of the recent published metanalysis on the equivalence of the treatment between ECN and mesalazine[31],

Table 2 Main potential clinical indications for *Escherichia coli* Nissle in gastroenterology

Maintenance of remission in ulcerative colitis[6,10,27,28]
Irritable bowel syndrome[34-37]
Constipation[38,39]
Acute diarrhea[6,40]
Collagenous colitis[41]
Uncomplicated diverticular disease[7]

starting from the major trials available. An equivalence between EcN and mesalamine on maintenance of remission in UC is still detectable (Figure 2).

Other studies assessing the use of EcN in ulcerative colitis

There is also an open-label multicenter pilot study that investigate the clinical benefit of EcN 1917 for maintenance therapy in young patients with UC. In this study 34 patients with UC in remission aged between 11 and 18 years were allocated either to EcN (2 capsules daily n = 24) or 5-ASA (median 1.5 g/d, n = 10), and observed over one year[33]. Inclusion criteria were: 11-18 years of age, ulcerative colitis in remission for a maximum of 12 mo, at least 2 relapses prior inclusion, active therapy with mesalazine. Taking into account the low statistical power of the study, relapse rate was 25% (6/24) in the EcN group and 30% (3/10) in the 5-ASA group. Data on the patients' global health and development were favorable and no serious adverse events were reported[33].

CONCLUSION

EcN is a well known probiotic, used in several countries for GI diseases (Table 2)[6,10,27,28,34-41], registered as a drug in certain European countries, and it is the only one approved for maintenance of remission in UC patients by ECCO guidelines, based on data discussed also in the present paper. Trials designed to be non-inferiority/equivalence trials, comparing EcN to mesalazine, have reported equivalent rates of relapse between the two treatments, demonstrating that EcN is equivalent to mesalazine in the maintenance of remission in UC. Of the 3 major trials demonstrating these findings, the best and larger trial is the one conducted by Kruis *et al* and published on 2004. Finally, EcN showed a robust safe profile in UC patients. Further studies may be helpful to further dissect mechanisms of actions and perhaps optimize dose and newer indication of EcN.

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ORIGINAL ARTICLE

Basic Study

Transient receptor potential vanilloid 4-dependent calcium influx and ATP release in mouse and rat gastric epithelia

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Abstract

AIM: To explore the expression of transient receptor potential vanilloid 4 (TRPV4) and its physiological meaning in mouse and rat gastric epithelia.

METHODS: RT-PCR and immunochemistry were used to detect TRPV4 mRNA and protein expression in mouse stomach and a rat normal gastric epithelial cell line (RGE1-01), while Ca^{2+}-imaging and electrophysiology were used to evaluate TRPV4 channel activity. ATP release was measured by a luciferin-luciferase assay. Gastric emptying was also compared between WT and TRPV4 knockout mice.

RESULTS: TRPV4 mRNA and protein were detected in mouse tissues and RGE1-01 cells. A TRPV4-specific agonist (GSK1016790A) increased intracellular Ca^{2+} concentrations and/or evoked TRPV4-like current activities in WT mouse gastric epithelial cells and

RGE1-01 cells, but not TRPV4KO cells. GSK1016790A or mechanical stimuli induced ATP release from RGE1-01 cells while TRPV4 knockout mice displayed delayed gastric emptying *in vivo*.

CONCLUSION: TRPV4 is expressed in mouse and rat gastric epithelium and contributes to ATP release and gastric emptying.

Key words: Transient receptor potential vanilloid 4; Stomach; Gastric emptying; ATP

Core tip: A mechano-sensitive ion channel, transient receptor potential vanilloid 4 (TRPV4), is expressed in gastric epithelium and contributes to ATP release and gastric emptying. These findings suggest that gastric distension stimulates TRPV4 on gastric epithelium and released ATP stimulates sub-epithelial nerve fibers or acts on visceral smooth muscles. TRPV4 might be a promising novel diagnostic and therapeutic target for functional gastric disorders.

Mihara H, Suzuki N, Boudaka AA, Muhammad JS, Tominaga M, Tabuchi Y, Sugiyama T. Transient receptor potential vanilloid 4-dependent calcium influx and ATP release in mouse and rat gastric epithelia. *World J Gastroenterol* 2016; 22(24): 5512-5519 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5512.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5512

INTRODUCTION

The transient receptor potential vanilloid 4 channel (TRPV4) is a non-selective cation channel that is involved in various cellular functions[1] and is activated by several physical and chemical stimuli, including mechanical stimuli, endogenous arachidonic acid metabolites (epoxyeicosatrienoic acids)[2], and heat. TRPV4 is also activated by the specific agonist GSK1016790A that elicits whole-cell currents in mouse and rat TRPV4-expressing cells with EC_{50} values of 18.5 and 10 nmol/L, respectively[3]. TRPV4 is widely expressed throughout the body, including gastrointestinal tract epithelium and the esophagus[4]. Although the physiological function of TRPV4 expression in intestinal epithelial cells is unknown, TRPV4 activation in these cells causes increases in intracellular calcium concentrations, chemokine release, and incidence of colitis[5], as well as increased paracellular permeability[6]. Furthermore, TRPV4 antagonists are promising therapeutic options for colitis[7,8]. However, TRPV4 expression in the gastric epithelium awaits evaluation.

In addition to its function as an intracellular energy donor, ATP is recognized as an important signaling molecule that mediates diverse biological effects *via* cell surface receptors: the purinergic receptors[9]. ATP is released by neurons of the central, peripheral, and enteric nervous system[10,11], and acts as a non-adrenergic non-cholinergic (NANC) neurotransmitter that causes different responses or effects (either excitatory or inhibitory depending on the P2 receptor subtype upon which they act as well as the animal species under study). Several studies showed that purinergic neurotransmission (assuming that gut neurons are the sole source of released ATP) affects gastric motility[12]. Recent reports showed that ATP is also released from non-neuronal tissues and has an effect on tissue function. Moreover, we found that ATP release in the esophagus and urothelium was mediated by TRPV4 stimulation[4,13,14]. However, there are no data concerning whether TRPV4 is expressed in the stomach and, if so, whether TRPV4 stimulation plays a role in mediating ATP release. Therefore, this study explored the morphological (RT-PCR and immunostaining) and functional (Ca^{2+}-imaging, patch clamp and gastric emptying) expression of TRPV4 in mouse and rat stomach with special focus on gastric epithelium.

MATERIALS AND METHODS

Animals

Eight week-old male C57BL/6NCr (SLC) and TRPV4-knockout (TRPV4KO) mice[15] weighing between 23 and 25 g were housed in a controlled environment (12-h light/12-h dark cycle; room temperature, 22-24 ℃; 50%-60% relative humidity) with free access to food and water. All procedures involving the care and use of animals were approved by The Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences.

Cell lines

RGE1-01 is an immortalized rat gastric mucosal cell line that shows distinct cell differentiation types and preserves some epithelial cell characteristics. RGE1-01 cells were maintained at 34 ℃ in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin with the addition of ITES (see reference[16] for details).

Acute isolated mouse gastric epithelium

WT and TRPV4KO mice were sacrificed by cervical dislocation. The stomachs were washed in cold (4 ℃) PBS (-) and then incubated in trypsin solution (Invitrogen) at 4 ℃ for 1 h. Gastric epithelial cells were harvested and plated on CELL-TAK (BD Biosciences)-coated glass cover slips and used for Ca^{2+}-imaging and patch clamp experiments.

Reverse transcription PCR analysis

RT-PCR was performed as previously described[4,17]. Total RNA (1 μg) was isolated using the RNeasy Mini

Table 1 Primer sequences for RT-PCR

Primer name	Sequence (5'→3')
mTRPV4-F	ACAACACCCGAGAGAACACC
mTRPV4-R	CCCAAACTTACGCCACTTGT
mGAPDH-F	TGAAGGGTGGAGCCAAAAGG
mGAPDH-R	GGAAGAGTGGGAGTTGCTGTTG
rTRPV4-F	CCTGGCAGGGATCGAGGCCT
rTRPV4-R	GGATGGTGGTGGCCCACTGC
rGAPDH-F	GCCAAGGCTGTGGGCAAGGT
rGAPDH-R	GAGCAATGCCAGCCCCAGCA

Kit (Qiagen, Courtaboeuf, France) and measured with a NanoDrop device (Thermo Fisher Scientific Inc., Wilmington, United States). Genomic DNA was eliminated in the process of reverse transcription (QuantiTect Reverse Transcription Kit, QIAGEN). PCR was performed using rTaq DNA polymerase (TaKaRa) in an iCycler (Bio-Rad) with specific primer sets (Table 1).

Immunochemistry

Immunochemistry was performed as previously described[4] using the antibodies summarized in Table 2. For section preparation, mouse stomachs were fixed at 4 ℃ for 6 h. Tissues were placed in PBS-sucrose and embedded in OCT compound (Tissue Tek, Elkhart, IN, United States). Non-specific antibody binding was reduced by incubation in BlockAce (Yukijirushi, Sapporo, Japan) for 1 h at room temperature prior to antibody exposure. Preparations were analyzed using a confocal laser scanning microscope (LSM 700, Carl Zeiss). For immunocytochemistry, RGE1-01 cells were fixed at 4 ℃ for 20 min with the same fixative. Bovine serum albumin (3% BSA; Sigma) was used as a blocking solution.

Ca^{2+}-imaging

Fura-2 fluorescence was measured in primary mouse gastric epithelial cells and RGE1-01 cells with a standard bath solution containing 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose at pH 7.4 (adjusted with NaOH) at 25 ℃. Results are presented as ratios of fluorescence intensities obtained with fura-2 emissions at 340 nm and 380 nm. GSK1016790A[3] and ionomycin (both from Sigma) were used as a TRPV4 agonist and a positive control, respectively. F_{340}/F_{380} was calculated and acquired with an image processing system (IP-Lab, Scanalytics Inc., Rockville, MD or AQUA COSMOS, Hamamatsu Co., Japan) and ImageJ software (http://rsb.info.nih.gov/ij/). Changes in ratio (Δ) were calculated by subtracting the mean basal values from peak values. Since the degree of responses (strong, weak, or no response) to GSK varied from cell to cell in WT gastric epithelial cells, we expressed the observed changes in all ionomycin-responsive cells as a ratio between WT and TRPV4 knockout mice (Δ). We evaluated 53 and 41 ionomycin-responsive cells from six WT mice and five TRPV4 knockout mice, respectively. Given the variations in response times, and that some WT cells responded 30 s after GSK application, we decided to incubate cells with GSK for 90 s.

Table 2 Primary and secondary antisera for immunochemistry

Tissue antigen/host	Dilution	Source
TRPV4/rabbit	1:500	B. Nilius, or Abcam
Goat anti-rabbit IgG-Alexa488	1:1500	Invitrogen, Inc.

Electrophysiology

The standard bath solution was the same as that used in the Ca^{2+}-imaging experiments. Pipette solutions for whole-cell recordings contained 140 mmol/L KCl, 5 mmol/L EGTA and 10 mmol/L HEPES, pH 7.4. Whole-cell recording data on primary gastric epithelial cells three hours after insolation and RGE1-01 were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pCLAMP software, Molecular Devices, Foster City, CA, United States). Voltage ramp-pulses from -100 mV to +100 mV (500 ms) were applied every 5 s to generate an I-V curve.

ATP release measurement

ATP concentrations released from RGE1-01 rat gastric epithelial cells cultured in 12-well plates or stretch silicon chambers (STB-10-04 from STREX Inc., Osaka, Japan) were measured by a luciferin-luciferase assay (ATP Bioluminescence assay kit CLS II, Roche Diagnostics) and a luminometer (Lumat LB 9507, Berthold Technologies, Japan), using a previously described method that was slightly modified[4]. For chemical stimuli, cells cultured to 70%-80% confluence and incubated in 500 μL bath solution for 30 min at room temperature (25 ℃) were used to measure basal ATP release. The superfusate was collected and replaced gently with another 500 μL of bath solution with or without the TRPV4 agonists GSK1016790A or 5,6-EET. The superfusate was collected after 15 min and the ratio of released ATP (15 min stimulation/30 min basal condition) was calculated. An aliquot (200 μL) of superfusate was then mixed with 200 μL luciferin-luciferase reagent for luminometric ATP measurements. For mechanical stimuli, stretching was quantitatively applied with a STB-10 stretch machine (STREX Inc.) to RGE1-01 cells cultured on a silicon chamber. Three minutes after chamber placement in the stretch machine, the superfusate was washed away to exclude artificial ATP release and replaced with 500 μL of bath solution for basal ATP measurement. After a further 3 min, the superfusate was collected and replaced, whereupon mechanical stimuli was applied for 3 min and the ratio of released ATP (3 min stimulation/3 min basal condition) was calculated. To block TRPV4 channels, cells were pre-treated with the specific TRPV4 antagonist HC 067047 (Sigma, 1 μmol/L) for 3 min[18].

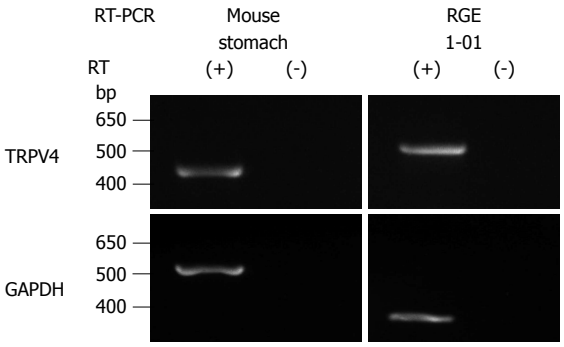

Figure 1 *TRPV4* mRNA expression in mouse stomach and RGE1-01 cells. *TRPV4* and *GAPDH* mRNA levels were examined with (+) and without (-) RT reaction. The expected sizes of the amplified fragments for *TRPV4* and *GAPDH* were 404 and 545 bp for mouse and 524 and 268 bp for rat, respectively. *TRPV4* mRNA was detected in mouse stomach and RGE1-01 cells (RGE1-01: normal rat gastric epithelial cell line). Band positions differed due to the use of different primers.

Gastric emptying

Eight-week-old WT and TRPV4KO male mice were used with a modified version of previously reported methods[17]. Gastric emptying was determined by transit of a test meal containing phenol red. Mice were fasted for 14 h with *ad libitum* water before the experiment. Five mg/kg (200 μL) of the test meal was administered into the stomach using a feeding needle. Fifteen minutes later, the mice were euthanized by cervical dislocation and the gastrointestinal tract was removed. The stomach was minced and the remaining phenol red concentration was measured. Gastric emptying was expressed as mean ± SEM for each group.

Data analysis

Values for Ca^{2+}-imaging, patch-clamp experiments, ATP measurements, and gastric emptying are presented as mean ± SEM from three or more independent experiments. A Student's *t*-test or non-parametric Bonferroni-type multiple comparison was used. Significance was accepted for $P < 0.05$.

RESULTS

TRPV4 expression in mouse and rat gastric epithelia

Given that TRPV4 was shown to be expressed in the esophagus and intestinal epithelia[4-6,19,20], we examined *TRPV4* mRNA expression in mouse stomach and a rat gastric epithelial cell line, RGE1-01. *TRPV4* mRNA was detected in mouse stomach and RGE1-01 cells (Figure 1). We next examined TRPV4 protein expression in mouse and the RGE1-01 cells. A strong homogenous immunofluorescent signal was confined to the epithelial cell layer of the WT mouse gastric corpus and antrum but not in cells from TRPV4KO mice (Figure 2A). Meanwhile, Z-stack images obtained by confocal microscopy of RGE1-01 cells displayed apical TRPV4 expression (Figure 2B).

TRPV4-mediated increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in mouse primary gastric epithelial cells

To confirm functional TRPV4 expression in primary gastric epithelial cells and RGE1-01 cells, we examined the response to the reported specific TRPV4 agonist, GSK1016790A (GSK)[3], using a fluorescent Ca^{2+}-imaging system (10 μmol/L, fura-2/AM). Response traces of $[Ca^{2+}]_i$ for WT and TRPV4KO gastric epithelial cells in the presence of GSK (100 nmol/L) showed that almost all cells isolated from WT stomach responded to GSK (Figure 3A) and the $[Ca^{2+}]_i$ increases were significantly larger in WT cells compared to TRPV4KO cells (Figure 3B). This finding suggests that the majority of gastric epithelial cells expressed TRPV4 and $[Ca^{2+}]_i$ responses to GSK were TRPV4 specific.

TRPV4-mediated current responses in mouse primary gastric epithelial cells and RGE1-01 cells

We next performed patch-clamp experiments with acute isolated mouse gastric epithelial cells in the presence of GSK (300 nmol/L) and observed inward current responses with an outwardly rectifying IV-relationship in WT but not TRPV4KO cells (Figure 4A)[3]. Current responses were observed in all 5 trials with WT gastric epithelial cells but were completely absent with TRPV4KO cells, which indicates that the majority of gastric epithelial cells expressed TRPV4. Similar chemical stimulation with GSK (300 nmol/L) induced TRPV4-like current responses in the rat gastric epithelial cell line, RGE1-01 (Figure 4B). These data strongly indicated functional expression of TRPV4 in mouse and rat gastric epithelial cells.

TRPV4 activators induced ATP release from RGE1-01 cells

Mechanical stimuli reportedly activate TRPV4 expressed in esophageal keratinocytes that in turn leads to increased ATP release[4]. To examine whether TRPV4 stimulation has a similar effect in gastric epithelium, we measured ATP release in chemically- or mechanically-stimulated RGE1-01 cells using a luciferin-luciferase assay. TRPV4 agonists GSK1016790A (GSK, 100 nmol/L) or 5,6-EET (500 nmol/L)[2] significantly increased ATP release in RGE1-01 cells (Figure 5A, 2- to 3-fold higher *vs* control, $P < 0.05$). Additionally, 120% lateral stretch applied for 3 min to RGE1-01 cells cultured on a silicon chamber induced significantly higher amounts of ATP release (Figure 5B, about 2-fold *vs* without stretch (control), $P < 0.05$), and these responses were inhibited by the specific TRPV4 inhibitor HC 067047 (1 μmol/L) (stretched cells showed no detachment over the 3-min stretch period). These results suggested that chemical and mechanical stimuli-induced ATP release in RGE1-01 cells was mediated by TRPV4 channel activation.

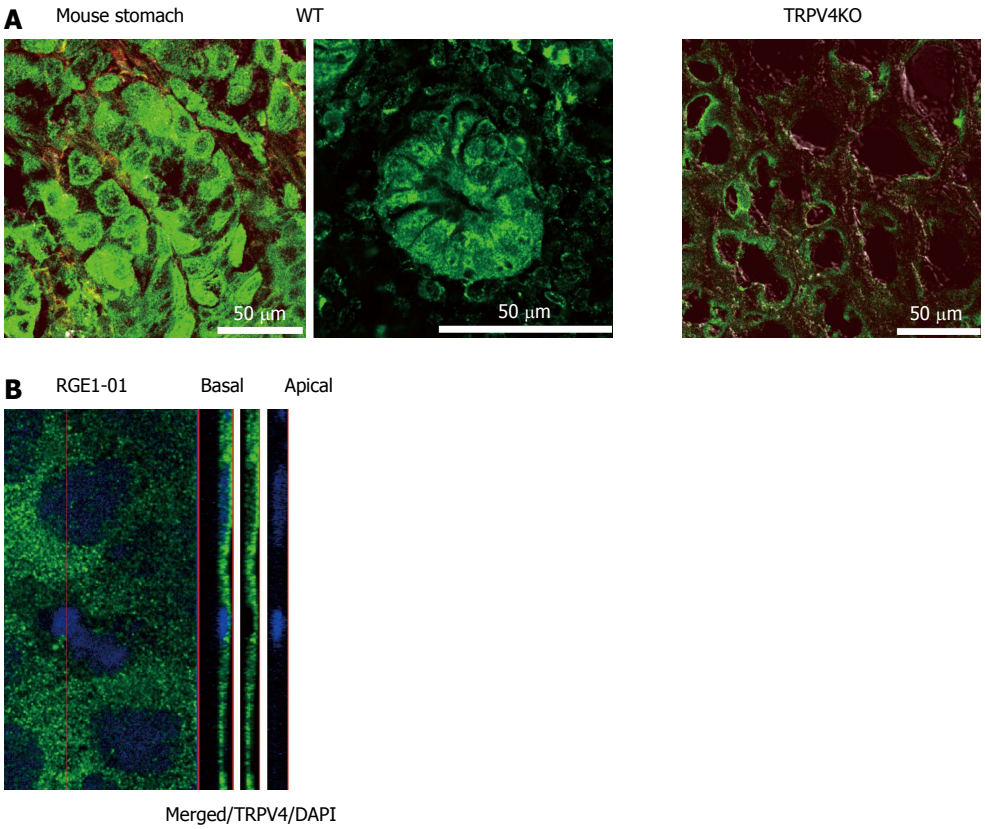

Figure 2 TRPV4 protein expression in mouse stomach and RGE1-01 cells. A: TRPV4 expression was homogeneously observed in WT but not TRPV4KO mouse gastric epithelium. Bars indicate 50 μm; B: Z-stack image of RGE1-01 cells demonstrated apical TRPV4 expression.

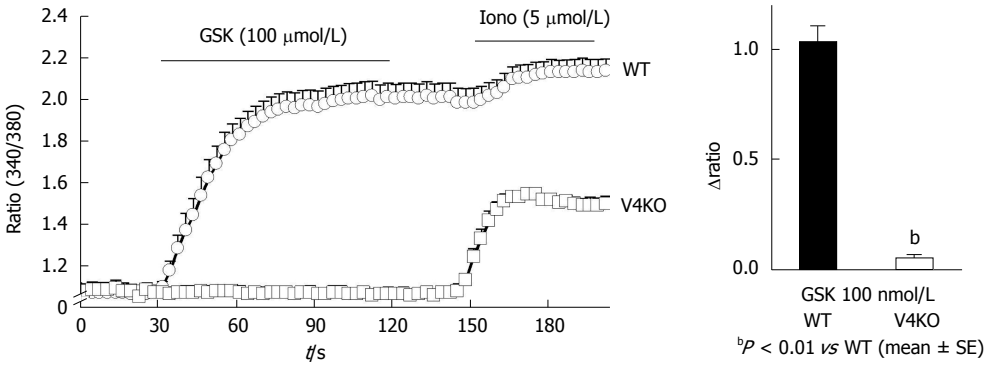

Figure 3 TRPV4-mediated increases in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in mouse primary gastric epithelial cells. A: $[Ca^{2+}]_i$ changes (340/380 ratio) in response to the TRPV4 specific agonist GSK1016790A (GSK, 100 nmol/L) in WT or TRPV4KO (V4KO) primary gastric epithelial cells (mean ± SEM). Ionomycin (iono, 5 μmol/L) was used as a positive control. Bars indicate the period of chemical application; B: GSK significantly increased $[Ca^{2+}]_i$ in WT cells (means ± SD; 1.03 ± 0.07, n = 20) compared to TRPV4KO cells (0.05 ± 0.01, n = 20) ($^bP < 0.01$ *vs* WT).

Delayed gastric emptying in TRPV4KO mice

Since TRPV4 has been shown to sense chemical and mechanical stimuli and contribute to ATP release from gastric epithelial cells, we hypothesized that TRPV4KO mice would exhibit altered gastric motility. To evaluate the physiological role of TRPV4 expressed in the gastric epithelium, we performed an *in vivo* experiment to compare gastric emptying rates of WT and TRPV4KO mice. Gastric emptying rates in TRPV4KO mice were about 2/3 of those in WT (Figure 6), suggesting that TRPV4 contributes to gastric motor function.

DISCUSSION

We identified morphological and functional TRPV4 expression in mouse gastric epithelial cells as well as the rat gastric epithelial cell line RGE1-01 (Figures 1-4). Furthermore, we demonstrated that chemical and mechanical stimuli can induce TRPV4-dependent ATP release from RGE1-01 cells (Figure 5), and that stimulation of gastric epithelial TRPV4 enhances gastric emptying *in vivo* (Figure 6). Using immunohistochemistry, Ca^{2+} imaging,

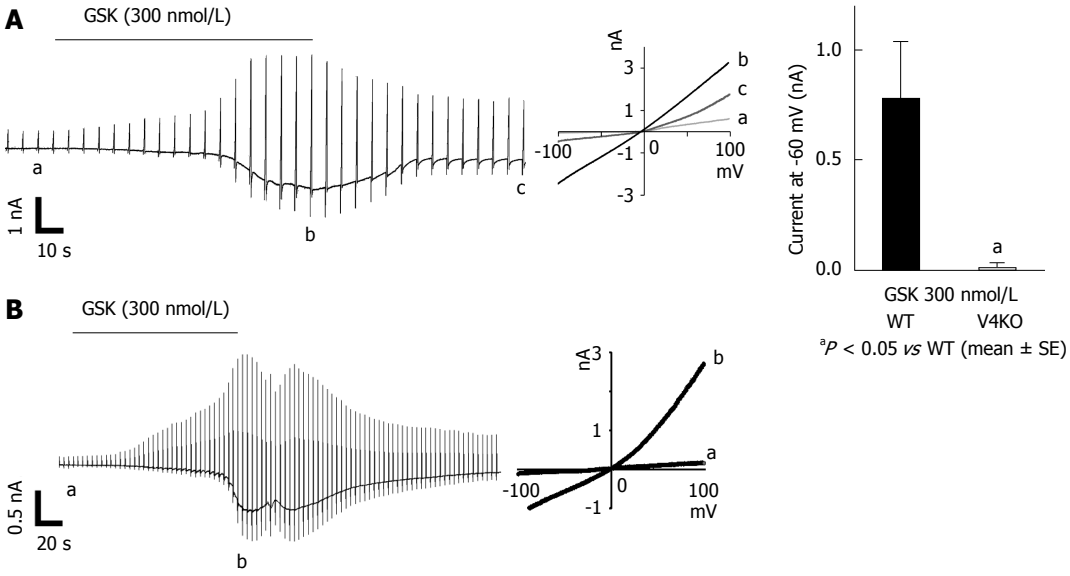

Figure 4 TRPV4-mediated current responses in mouse primary gastric epithelial cells and RGE1-01 cells. A: GSK (300 nmol/L) evoked inward current responses in WT primary gastric epithelial cells. Currents in response to ramp-pulses at points a, b and c (left in panel B) are shown (middle), with a strongly outwardly rectifying current-voltage relationship. Significantly larger inward currents at -60 mV were obtained from WT cells (means ± SEM; 0.76 ± 0.27 nA, n = 5) than in TRPV4KO cells (0.01 ± 0.00 nA, n = 5) ([a]P < 0.05 *vs* WT). B: Similar current responses were also obtained in RGE1-01 cells.

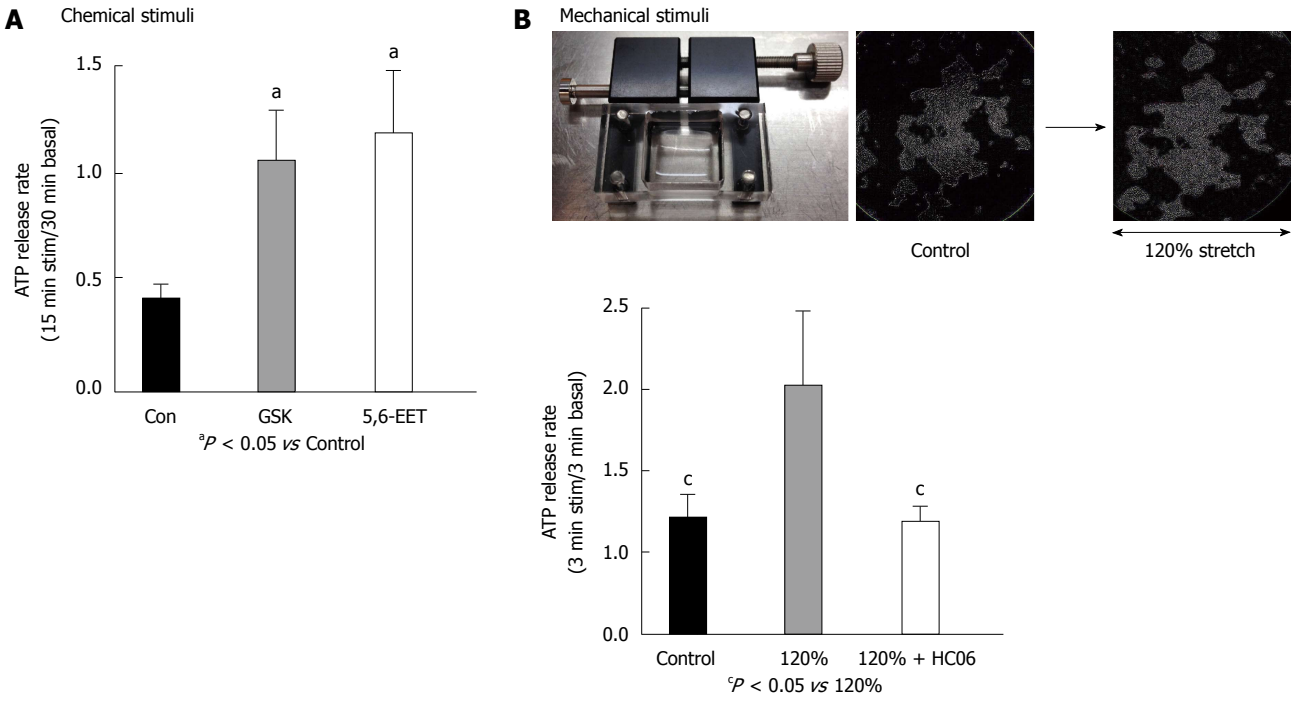

Figure 5 TRPV4 activator-induced ATP release in the RGE1-01 cells. A: GSK1016790A (GSK, 100 nmol/L) or 5,6-EET (500 nmol/L) induced significantly higher ATP release in RGE1-01 cells ([a]P < 0.05 *vs* Control). B: Mechanical stimuli were quantitatively applied with a stretch apparatus. Microscopy images demonstrated that cells were stretched laterally without detachment. A 120% stretch induced significantly higher amounts of ATP release from RGE1-01 cells [[c]P < 0.05 *vs* 0% stretch (control)] that could be inhibited by pre-treatment with specific TRPV4 antagonist HC 067047 (1 μmol/L). TRPV4: Transient receptor potential vanilloid 4.

and electrophysiology, we found that the majority of mouse gastric epithelial cells exhibited abundant TRPV4 expression and responded to TRPV4 agonists, suggesting that TRPV4 is a candidate mechanoreceptor in gastric epithelial cells. In fact, ATP release was several hundred nmol/L in our *in vitro* study, suggesting that the corresponding ATP concentration *in vivo* might be estimated to be several μmol/L, which would be sufficient to activate the P2 receptor present in the wall of mouse stomach[21,22]. These results suggested the hypothesis that luminal distension stimulates TRPV4 on gastric epithelial cells that in turn release ATP. The released ATP either stimulates subepithelial sensory nerve fibers that form the afferent limb of short or long gastrointestinal reflex arcs or acts directly on visceral smooth muscles expressing

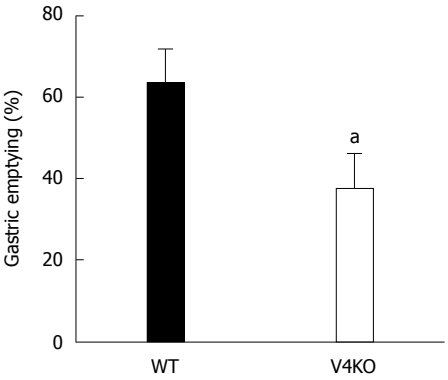

Figure 6 Delayed gastric empting in TRPV4 knockout mice. Gastric emptying rates *in vivo* in TRPV4KO mice were significantly delayed relative to WT mice ($^{a}P < 0.05$, *vs* WT, $n = 7$-9).

purinergic receptors. The first possibility is supported by morphological evidence showing that purinergic receptors, mainly P2X2 and P2X3, were identified on putative gastric mechanosensing structures, including the vagal afferent intraganglionic laminar endings that are located in close proximity to the epithelium[23,24]. These vagal afferents form the afferent limb of the central vago-vagal reflex (long reflex arc) and are known to increase gastric motility following stimulation[25]. The hypothesis is also supported by findings from a previous study wherein P2X3-knockout mice show a blunted neural response to gastric distension and no differences in distension-evoked ATP release between knockout and control mice[26]. The released ATP could also trigger a local reflex arc intrinsic to the stomach wall, which is supported by results from a previous study wherein ATP was shown to induce tetrodotoxin-sensitive contraction responses mediated by neuronal P2X receptors in an *in vitro* whole-stomach preparation[27].

The possibility that ATP released from gastric epithelium could directly stimulate purinergic receptors expressed on gastric visceral smooth muscles is rather unlikely considering the short half-life of ATP and the distance that ATP must cross while diffusing from the gastric epithelium to visceral smooth muscles. Moreover, gastric smooth muscles are known to functionally express P2Y receptors that mediate relaxation in response to ATP[27] and would be expected, upon stimulation, to delay gastric emptying, which is opposite to our current findings. This outcome further decreases the likelihood of a direct effect for ATP released from gastric epithelium on smooth muscles. However, the purinergic signaling pathway that mediates gastric distension-induced epithelial TRPV4 stimulation requires further future characterization.

In conclusion, TRPV4 is morphologically and functionally expressed in mouse and rat gastric epithelia and contributes to ATP release and gastric emptying. Our results suggest that TRPV4 could be a promising novel diagnostic and therapeutic target for functional gastrointestinal disorders.

ACKNOWLEDGMENTS

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COMMENTS

Background

The transient receptor potential vanilloid 4 channel (TRPV4) is a non-selective cation channel that is activated by mechanical stimuli. ATP has been recognized as an important signaling molecule *via* cell surface ATP receptors. This study explored TRPV4 expression in mouse and rat stomach and whether the stimulation mediated ATP release.

Research frontiers

The authors have reported that ATP release in the esophagus and urothelium is mediated by TRPV4 stimulation.

Innovations and breakthroughs

This is the first study showing TRPV4 expression and ATP release by its stimulation in the mouse stomach and/or rat gastric epithelial cells.

Applications

These data suggested the hypothesis that luminal distension stimulates TRPV4 on gastric epithelial cells that in turn release ATP. However TRPV4 expression in human gastric epithelium and the purinergic signaling pathway requires further evaluation.

Terminology

TRPV4 is a non-selective cation channel, that is activated by several physical and chemical stimuli, including mechanical stimuli. The channel activation increases intracellular Ca^{2+} concentration and elicits whole-cell currents in TRPV4-expressing cells.

Peer-review

The manuscript describes an original research performed in mouse stomach and in rat epithelium cell line focusing on the expression and function of TRPV4 ion channels. The study concept is based on previous findings of the authors regarding TRPV4-mediated ATP release on the esophasgus. The series of experiments in this manuscript demonstrated delayed gastric emptying in TRPV4 knockout mice compared to their WT littermates, as well as TRPV4 expression in mRNA and protein levels in both mouse and rat gastric epithelial cell line. In general, the idea is interesting, the various morphological and functional methods are sophisticated, the figures are demonstrative.

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ORIGINAL ARTICLE

Basic Study

Intravoxel incoherent motion diffusion-weighted imaging for monitoring chemotherapeutic efficacy in gastric cancer

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Author contributions: Song XL and Jeong YY designed the studies; Song XL, Kang YJ and Moon CM performed the majority of experiments; Song XL, Ahn KY, Kang YJ and Cho HJ contributed to the analysis and interpretation of imaging date and histological examination; Song XL wrote the first draft of the manuscript; Jeong GW and Kang HK have approved the final manuscript and completed manuscript; Also, all authors agree with the content of the manuscript.

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Animal care and use statement: The animal protocol was designed to minimize pain or discomfort to the animals according to Institutional Animal Care and Use Committee Guidelines. All mice, fed with a standard diet with water ad libitum, were maintained at appropriate laboratory conditions (a photoperiod of 12 h light and darkness, 50% humidity, 23 ℃). After MRI examination, mice were euthanized by overdose of isoflurane (over than 5%) for tissue collection.

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Abstract

AIM: To assess intravoxel incoherent motion diffusion-weighted imaging (IVIM-DWI) for monitoring early efficacy of chemotherapy in a human gastric cancer mouse model.

METHODS: IVIM-DWI was performed with 12 *b*-values (0-800 s/mm^2) in 25 human gastric cancer-bearing nude mice at baseline (day 0), and then they were randomly divided into control and 1-, 3-, 5- and 7-d treatment groups (n = 5 per group). The control group underwent longitudinal MRI scans at days 1, 3, 5 and 7, and the treatment groups underwent subsequent MRI scans after a specified 5-fluorouracil/calcium

folinate treatment. Together with tumor volumes (TV), the apparent diffusion coefficient (ADC) and IVIM parameters [true water molecular diffusion coefficient (D), perfusion fraction (f) and pseudo-related diffusion coefficient (D^*)] were measured. The differences in those parameters from baseline to each measurement (ΔTV%, ΔADC%, ΔD%, Δf% and ΔD^*%) were calculated. After image acquisition, tumor necrosis, microvessel density (MVD) and cellular apoptosis were evaluated by hematoxylin-eosin (HE), CD31 and terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining respectively, to confirm the imaging findings. Mann-Whitney test and Spearman's correlation coefficient analysis were performed.

RESULTS: The observed relative volume increase (ΔTV%) in the treatment group were significantly smaller than those in the control group at day 5 ($\Delta TV_{treatment}$% = 19.63% ± 3.01% and $\Delta TV_{control}$% = 83.60% ± 14.87%, P = 0.008) and day 7 ($\Delta TV_{treatment}$% = 29.07% ± 10.01% and $\Delta TV_{control}$% = 177.06% ± 63.00%, P = 0.008). The difference in ΔTV% between the treatment and the control groups was not significant at days 1 and 3 after a short duration of treatment. Increases in ADC in the treatment group ($\Delta ADC\%_{treatment}$, median, 30.10% ± 18.32%, 36.11% ± 21.82%, 45.22% ± 24.36%) were significantly higher compared with the control group ($\Delta ADC\%_{control}$, median, 4.98% ± 3.39%, 6.26% ± 3.08%, 9.24% ± 6.33%) at days 3, 5 and 7 (P = 0.008, P = 0.016, P = 0.008, respectively). Increases in D in the treatment group ($\Delta D\%_{treatment}$, median 17.12% ± 8.20%, 24.16% ± 16.87%, 38.54% ± 19.36%) were higher than those in the control group ($\Delta D\%_{control}$, median -0.13% ± 4.23%, 5.89% ± 4.56%, 5.54% ± 4.44%) at days 1, 3, and 5 (P = 0.032, P = 0.008, P = 0.016, respectively). Relative changes in f were significantly lower in the treatment group compared with the control group at days 1, 3, 5 and 7 follow-up (median, -34.13% ± 16.61% *vs* 1.68% ± 3.40%, P = 0.016; -50.64% ± 6.82% *vs* 3.01% ± 6.50%, P = 0.008; -49.93% ± 6.05% *vs* 0.97% ± 4.38%, P = 0.008, and -46.22% ± 7.75% *vs* 8.14% ± 6.75%, P = 0.008, respectively). D* in the treatment group decreased significantly compared to those in the control group at all time points (median, -32.10% ± 12.22% *vs* 1.85% ± 5.54%, P = 0.008; -44.14% ± 14.83% *vs* 2.29% ± 10.38%, P = 0.008; -59.06% ± 19.10% *vs* 3.86% ± 5.10%, P = 0.008 and -47.20% ± 20.48% *vs* 7.13% ± 9.88%, P = 0.016, respectively). Furthermore, histopathologic findings showed positive correlations with ADC and D and tumor necrosis (r_s = 0.720, P < 0.001; r_s = 0.522, P = 0.007, respectively). The cellular apoptosis of the tumor also showed positive correlations with ADC and D (r_s = 0.626, P = 0.001; r_s = 0.542, P = 0.005, respectively). Perfusion-related parameters (f and D^*) were positively correlated to MVD (r_s = 0.618, P = 0.001; r_s = 0.538, P = 0.006, respectively), and negatively correlated to cellular apoptosis of the tumor (r_s = -0.550, P = 0.004; r_s = -0.692, P < 0.001, respectively).

CONCLUSION: IVIM-DWI is potentially useful for predicting the early efficacy of chemotherapy in a human gastric cancer mouse model.

Key words: Gastric cancer; Microvessel density; Nude mouse model; Intravoxel incoherent motion diffusion-weighted imaging; Terminal-deoxynucleoitidyl transferase mediated nick end labeling

Core tip: Intravoxel incoherent motion diffusion-weighted imaging (IVIM-DWI) is useful for monitoring changes of molecular diffusion and microcirculation in gastric cancer at the early stage of chemotherapy. The apparent diffusion coefficient (ADC) and IVIM parameters of true water molecular diffusion coefficient (D) could be reliable marker to detect the necrosis and cellular apoptosis, while perfusion-related IVIM parameters of perfusion fraction (f) and pseudo-related diffusion coefficient (D^*) are capable of noninvasive assessment of angiogenesis activity in gastric cancer undergoing chemotherapy.

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INTRODUCTION

Gastric cancer (GC) remains the fourth most common malignancy and the second-leading cause of cancer deaths in the world[1]. Despite many advances in cancer diagnosis and treatment, approximately two-thirds of patients are diagnosed with advanced gastric cancer (AGC) in many countries, excluding some trial results in Japanese and South Korean patients[2,3]. For many years, 5-fluorouracil (5-FU)-based chemotherapy has been considered a standard chemotherapy regimen for AGC[2-4]. Unfortunately, not all patients benefit from this regimen. If an ineffective therapy can be identified at an early stage of treatment, there will be an opportunity to change the therapy approach and clinical management in the individual patient quickly. The response evaluation criteria in solid tumors (RECIST) is widely adopted standard for evaluating therapy response based on the change in tumor size in clinical practice. However, this often takes several weeks to months to develop, and its evaluation period is too long to adjust patient management[5]. Therefore, increasing demands are being placed on imaging modalities to identify early

and reliable surrogate markers for the evaluation of therapeutic effect in patients with GC[6].

Diffusion-weighted magnetic resonance imaging (DWI) is capable of providing an apparent diffusion coefficient (ADC), which is a measure magnitude of diffusion (of water molecules) within tissue, and has become a favorite choice for oncologic studies[7]. It is well known that a lower ADC is a characteristic of most tumors compare with native tissues because of their high cellularity. An increase in tissue diffusivity, which is induced by an enlarged extracellular space, cell swelling or a loss of membrane integrity under an effective therapy, makes it possible to use ADC to identify the treatment response[8-11]. However, the ADC value derived from DWI based a mono-exponential model does not sufficiently demonstrate the characteristics of tissue behavior. The *b*-value, which represents the strength and timing of the gradients, determines DWI sensitivity to water motion. When a lower *b*-value is applied ($\leqslant$ 100 s/mm^2), microcirculation related protons have relative large diffusion distances, and are capable of changing diffusion signal intensities. In 1986, Le Bihan *et al*[12] first described the concept of intravoxel incoherent motion (IVIM), which can be used to estimate molecular diffusion and microcirculation in the capillaries separately through bi-exponential fitting of the DWI data using multiple *b*-values. In recent years, there has been increasing interests in IVIM-DWI, which allows acquisition of quantitative parameters that reflect tissue diffusivity and microcirculation perfusion simultaneously. The true water molecular diffusion coefficient (D), perfusion fractional (f) volume reflective of capillary blood volume and pseudo-related diffusion coefficient associated with capillary network blood flow (D*) can be measured using IVIM-DWI. The blood microcirculation within capillaries can be considered as a type of "pseudo-diffusion" because it has no specific orientation[13].

It has been determined that IVIM-DWI can provide a new opportunity to gain an insight into the perfusion of a tumor without contrast agent administration for preclinical and clinical applications[12,14,15]. Koh *et al*[16] reported that the calculated f values were lower in colorectal liver metastases, which are characterized by their hypovascular nature, than in the normal liver. In addition, DWI with 10 *b*-values between 0 and 700 s/mm^2 enables the measurement of diffusion and microcirculation contributions in renal allografts as early as 5-d after transplantation[17]. Moreover, IVIM-DWI has been used to evaluate the treatment responses to radiofrequency ablation[18] or a vascular disrupting agent of CKD-516[19] in rabbit model with VX2 liver tumors, and to neoadjuvant chemotherapy in human locoregionally advanced nasopharyngeal carcinoma. Although DWI is increasingly being applied in the body, few studies have focused on gastric lesions due to the limitations of modality of the gastric[20]. Recently, Cheng *et al*[21] applied IVIM with *b*-values of up to 1500 s/mm^2 to evaluate chemotherapeutic efficacy in a gastric cancer xenograft model. In that study, f increased after chemotherapy treatment, which refutes currents theories regarding angiogenesis activity within tumors after treatment[22]. A previous IVIM study in prostate cancer also found that f in tumors significantly increased compared to that in normal prostate tissue when a *b*-value of less than 750 s/mm^2 was used, while f decrease or became indistinguishable from the normal prostate tissue when high *b*-values were employed[23]. This phenomenon could be explained by the following theory: The departure of molecular diffusion at very high *b*-values may have an influence on perfusion-related parameters because both water diffusion and microcirculation contribute to the signal attenuation observed at lower *b*-values ($\leqslant$ 100 s/mm^2)[13,15]. It should be noted that higher b values may affect the accuracy of the IVIM-derived parameters, which depend heavily on the *b*-value selection.

Here, we investigate IVIM-DWI with *b*-values below 800 s/mm^2 as a potential imaging marker for assessing the early chemotherapy response in term of tissue diffusion and microvascular perfusion, by comparing the tissue cellularity and microvascular density (MVD) properties revealed by histopathological analysis during the full course of treatment using a mouse human gastric cancer xenograft model.

MATERIALS AND METHODS

Animal model

This study included 25 male adult (6 weeks old) nude mice (BALB/c-nu/nu, Orient Bio, Gwangju, South Korea), each weighing 20-25 g. All mice, fed with a standard diet with water ad libitum, were maintained in appropriate laboratory conditions (a photoperiod of 12 h light and darkness, 50% humidity, 23 ℃). After a two-week adaption period, 1 × 10^7 human gastric adenocarcinoma AGS cells (ATCC®, CRL-1739™) suspended in 100 μL cold PBS were subcutaneously injected into the lower right hind limbs of the nude mice with a 31 gauge syringe. To evaluate the therapeutic response, the tumor growth curves in each group were estimated based on the morphologic T2-weighted images by using the following formula: TV = π/6 × L × W × H (L, length, W, width, H, height of the tumor).

Study design

Following a baseline (day 0) MRI examination, human gastric cancer-bearing mice were randomly divided into control and 1-d, 3-d, 5-d, and 7-d treatment groups (n = 5 per group). 5-fluorouraci (5-FU) (15 mg/kg)/calcium folinate (5 mg/kg) was used for chemotherapy in the treatment groups, while same volume of saline was administered in the control group. 5-FU/calcium folinate was intraperitoneally injected on a bi-daily basis. Mice in the control group underwent longitudinal MRI at days 1, 3, 5 and 7. Each

treatment group underwent a second scan with same MRI protocol at days 1, 3, 5, or 7 after treatment. After the MRI examination, mice were euthanized by an overdose of isoflurane, and the tumor was stripped for further analysis. The short-term 7-d 5-FU treatment was performed, because this study was aimed to investigate the potential of IVIM-DWI for monitoring the early tumor diffusion and perfusion response to treatment.

MRI protocol

All MRI scans were performed using 3T MRI (GE Healthcare, Waukesha, WI, United States) with a wrist coil. Mice were placed on a heated pad and anesthetized with 2% isoflurane in oxygen (at a rate of 1.0 L/min) to void movement during imaging. After acquisition of the routine images for localization, a transverse T2-weighted image was obtained using fast spin echo (FSE) sequence [repetition time/echo time (TR/TE), 2000/99.6 ms; section thickness, 3 mm; matrix, 512 × 358; number of excitations (NEX), 4]. Subsequently, IVIM-DWI with 12 *b*-values (0, 10, 15, 20, 25, 30, 60, 75, 100, 200, 400 and 800 s/mm^2) were acquired using a free-breathing single-shot echo-planar imaging (EPI) sequence with application of three diffusion-gradients directions (TR/TE, 2500/66.5; section thickness, 3 mm; number of sections, 8; field of view, 10 × 10 cm^2; matrix, 128 × 128; NEX, 4) with an acquisition time of 7 min and 30 s for each study.

MR imaging analysis

The acquired datasets were transferred to a GE workstation (Advance Workstation 4.6) and analyzed using an in-house software. The ADC value was calculated by using a liner fit (least-squares fit) mono-exponential model based on the following equation: $S_b/S_0 = \exp(-b \times ADC)$[15], where S_0 is the signal intensity at a *b* value of 0 and S_b is the signal intensity at higher *b* values. IVIM parameters was calculated by a nonlinear fit (Levenberg-Marquardt fit)bi-exponential model, and the equation is shown as follow: $S_b/S_0 = (1\text{-}f) \times \exp(-bD) + f \times \exp[-b(D + D^*)]$[15], where D represents true water molecular diffusion coefficient, f and D* represent perfusion fraction and pseudo-related diffusion coefficient, respectively. A technician with 8 years of experience in MRI measured the tumor sizes and values of ADC, D, f, and D*. The technician was blinded to the information regarding the treatment and control groups.

Regions of interest (ROIs) were drawn by outlining the tumor border on ADC maps, which showed the largest cross-section of the tumor. The ROIs on ADC map were copied and pasted on the corresponding D, f, and D* maps. In each mouse, the change in tumor volume (TV) relative to the baseline was quantified to determine the treatment response as follows: $\Delta TV\% = [(TV_{given\ time} - TV_{baseline})/TV_{baseline}] \times 100$, where $TV_{given\ time}$ is the tumor volume on day 1, 3, 5, or 7 and $TV_{baseline}$ is the tumor volume on day 0. For the ADC and IVIM parameters, the percentage changes in values compared to the baseline were recorded by using the following formula: $\Delta Value\% = [(Value_{given\ time} - Value_{baseline})/Value_{baseline}] \times 100$, where $value_{given\ time}$ is the value on day 1, 3, 5, or 7 and $Value_{baseline}$ is the value on day 0.

Histopathological analysis

The tumor tissue was paraffin-embedded, and sliced in the transverse plane at 0.6 μm intervals to match the corresponding MR image, was selected. The necrotic fraction (NF) of the tumor was evaluated through hematoxylin-eosin (HE) staining. CD31 (Dako, Carpinteria, CA, United States; mouse anti-human; used at 1:200) staining was carried out on pathological specimens by immunohistochemical methods, to analyze the angiogenesis activity of the tumor. The paraffin-embedded sections were incubated with the primary antibody at 4 ℃ overnight. Secondary antibody (Stem cell Technologies; rabbit anti-mouse; used at 1:300) was applied at room temperature for 1 h and then sections were rinsed with PBS. Positive reaction was visualized by DAB chromogen (Dako, Carpinteria, CA, United States) according to standard methods. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Roche Applied Science, Penzberg, Germany) was performed to evaluate cellular apoptosis of the tumor by following the manufacturer's instructions.

Histopathological analysis was performed by using Image J software (http://rsb.info.nih.gov/ij). Tumor necrosis was scored according to the following formula: $NF = Area_{necrosis}/Area_{total\ tumor}$. Cellular apoptosis of the tumor was defined as the percentage of positive TUNEL-stained cells among 200 nuclei from five randomly selected fields at a high magnification (× 200). The mean MVD of the tumor was defined by the CD31-stained vessels[24], where any distinct area of positive for CD31 staining was defined as a single vessel, from five hot spots with higher vascular density compared to the remaining tissue in a high-power field (× 200; 0.578 mm^2).

Statistical analysis

SPSS 21.0 (SPSS, Chicago, IL, United States) was used for statistical analysis. The differences in relative changes in the ADC and IVIM parameters, and tumor volumes between the control and treatment groups were determined by the Mann-Whitney test. Spearman's correlation coefficient was used to determine the correlations between histological features, including NF, MVD and TUNEL and the corresponding ADC and IVIM parameters. Spearman's coefficient was considered to be satisfactory with a critical value of $r_s = 0.415$ at P value < 0.05 (two-tailed test). A spearman's coefficient of 0.90-1.00 indicated almost perfect agreement; 0.70-0.90 indicated high

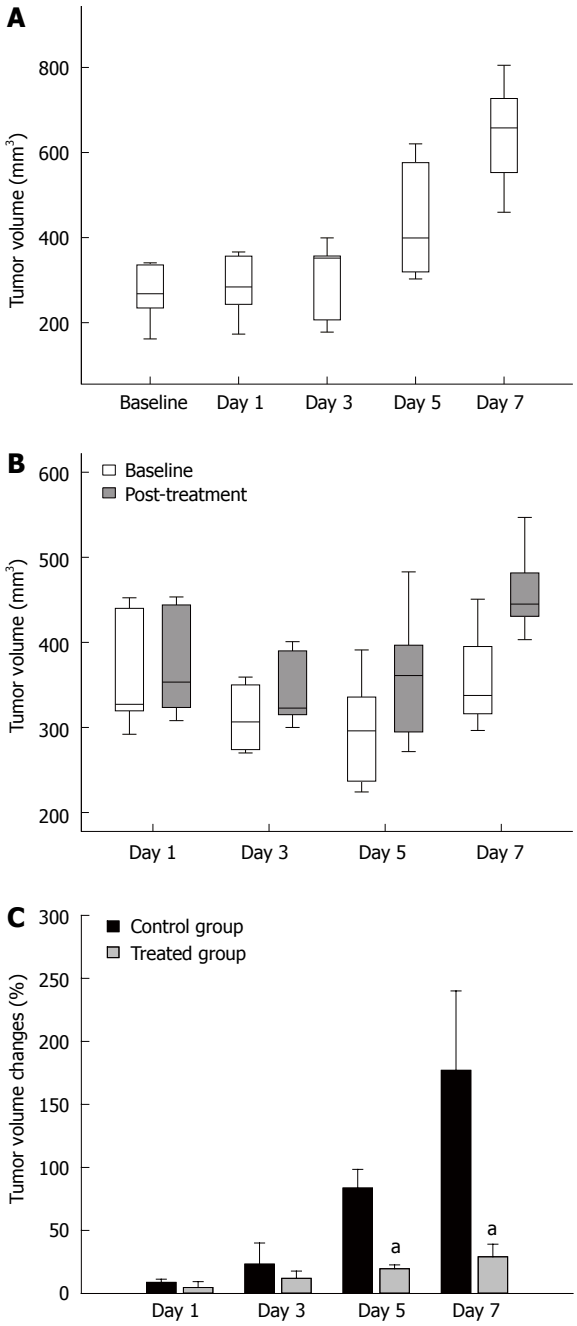


Figure 1 Anti-tumor effects of 5-fluorouracil therapy in mouse gastric cancer xenografts. A: Actual tumor volume changes in the control group; B: Actual tumor volume changes in the treatment groups; C: The comparison of the change in tumor volumes relative to baseline (day 0) between the control and treatment groups. The control group showed increases in tumor volume, while the treatment group showed as significant tumor growth delay from day 5. Center line = median; upper and lower margins of the box = 25th to the 75th percentile, respectively; whiskers = data from the minimum to the maximum. Error bars denote standard errors. $^{a}P < 0.05$ *vs* control group, $n = 5$ in each group.

agreement; 0.50-0.70 indicate moderate agreement; 0.30-0.50 indicate low agreement; and 0.00-0.30 indicate negligible agreement[25]. A two-tailed *P* value < 0.05 was considered as significant difference.

RESULTS

Effects of chemotherapy on tumor growth

Twenty-five tumors with a mean volume of 298.07 ± 103.44 mm^3 (range: 194.32-432.34 mm^3) before treatment were analyzed 20 to 25 d of implantation. The tumor volume in the control and treatment groups is shown in Figure 1A and B. 5-FU induced a significant growth delay, as assessed by the MRI-derived tumor volume measurements in comparison with that in the control group from day 5. As shown in Figure 1C, the observed relative volume increase (ΔTV%) in the therapy groups were significantly lower than in the control group at day 5 ($\Delta TV_{treatment}\%$ = 19.63% ± 3.01% and $\Delta TV_{control}\%$ = 83.60% ± 14.87%, $P = 0.008$) and day 7 ($\Delta TV_{treatment}\%$ = 29.07% ± 10.01% and $\Delta TV_{control}\%$ = 177.06% ± 63.00%, $P = 0.008$). The difference in ΔTV% between the treatment and the control groups was not significant at day 1 ($\Delta TV_{treatment}\%$ = 4.97% ± 4.59% and $\Delta TV_{control}\%$ = 8.08% ± 2.47%, $P = 0.841$) or day 3 ($\Delta TV_{treatment}\%$ = 15.36% ± 5.75% and $\Delta TV_{control}\%$ = 23.28% ± 16.76%, $P = 0.310$) after a short treatment duration.

IVIM-DWI assessment of a human gastric cancer xenograft

Table 1 summarizes the ADC, D, f, and D* values of the tumors in the control and treatment groups, that were measured at baseline and at days 1, 3, 5 and 7. Figure 2 shows the mean percentage changes in the DWI parameters relative to baseline at each time point in each group. In the control group, all ADC values and IVIM parameters of the tumor remained relatively constant over the 7-d experiment. ADC increases in the treatment group ($\Delta ADC\%_{treatment}$, median, 30.10% ± 18.32%, 36.11% ± 21.82%, 45.22% ± 24.36%) were significantly higher compared with the control group ($\Delta ADC\%_{control}$, median, 4.98% ± 3.39%, 6.26% ± 3.08%, 9.24% ± 6.33%) at days 3, 5 and 7 ($P = 0.008$, $P = 0.016$, $P = 0.008$, respectively) (Figure 2A). Increases in D in the treatment group ($\Delta D\%_{treatment}$, median 17.12% ± 8.20%, 24.16% ± 16.87%, 38.54% ± 19.36%) were higher than in the control group ($\Delta D\%_{control}$, median -0.13% ± 4.23%, 5.89% ± 4.56%, 5.54% ± 4.44%) at days 1, 3, and 5 ($P = 0.032$, $P = 0.008$, $P = 0.016$, respectively) (Figure 2B). The relative changes in f were significantly lower in the treatment group than in the control group at days 1, 3, 5 and 7 follow-up (median, -34.13% ± 16.61% *vs* 1.68% ± 3.40%, $P = 0.016$; -50.64% ± 6.82% *vs* 3.01% ± 6.50%, $P = 0.008$; -49.93% ± 6.05% *vs* 0.97% ± 4.38%, $P = 0.008$, and -46.22% ± 7.75% *vs* 8.14% ± 6.75%, $P = 0.008$, respectively) (Figure 2C). D* in the treatment group decreased significantly compared with the control group at all time points (median, -32.10% ± 12.22% *vs* 1.85% ±

Table 1 Summary of the apparent diffusion coefficient and intravoxel incoherent motion parameters at baseline (day 0) and on days 1, 3, 5, and 7 in each groups (n = 5 per group)

	ADC (10^{-3} mm^2/s)	IVIM parameters		
		D (10^{-3} mm^2/s)	f (%)	D^* (mm^2/s)
Control group				
Day 0	0.514 ± 0.050	0.480 ± 0.049	32.424 ± 6.647	0.112 ± 0.017
Day 1	0.520 ± 0.056	0.497 ± 0.052	33.010 ± 7.121	0.114 ± 0.016
Day 3	0.537 ± 0.029	0.506 ± 0.025	33.606 ± 8.113	0.115 ± 0.020
Day 5	0.545 ± 0.047	0.504 ± 0.021	32.602 ± 6.684	0.117 ± 0.018
Day 7	0.561 ± 0.053	0.524 ± 0.064	35.100 ± 7.858	0.120 ± 0.016
1-d treatment group				
Day 0	0.555 ± 0.028	0.524 ± 0.028	34.076 ± 7.247	0.171 ± 0.049
Day 1	0.597 ± 0.064	0.612 ± 0.065	21.912 ± 5.032	0.115 ± 0.040
3-d treatment group				
Day 0	0.528 ± 0.012	0.510 ± 0.019	37.066 ± 7.331	0.147 ± 0.025
Day 3	0.686 ± 0.107	0.636 ± 0.121	18.596 ± 5.766	0.083 ± 0.029
5-d treatment group				
Day 0	0.594 ± 0.069	0.567 ± 0.065	34.664 ± 8.337	0.173 ± 0.068
Day 5	0.804 ± 0.136	0.777 ± 0.107	17.344 ± 4.352	0.070 ± 0.045
7-d treatment group				
Day 0	0.560 ± 0.060	0.535 ± 0.081	37.598 ± 5.852	0.148 ± 0.082
Day 7	0.823 ± 0.221	0.710 ± 0.236	20.040 ± 3.042	0.065 ± 0.018

ADC: Apparent diffusion coefficient; IVIM: Intravoxel incoherent motion.

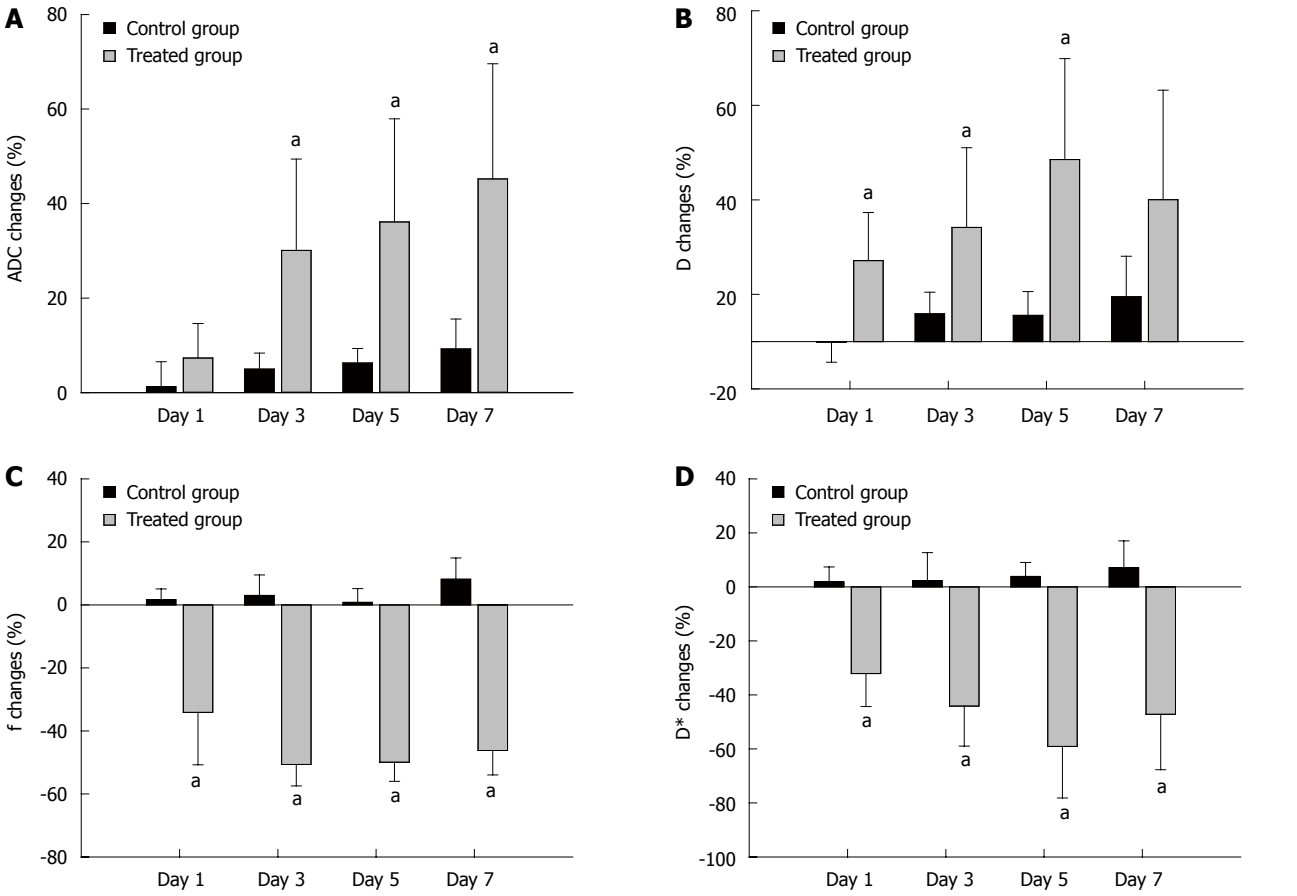


Figure 2 Comparison of the mean percentage changes from baseline in the intravoxel incoherent motion diffusion-weighted imaging derived values between the control (dark) and the 1-, 3-, 5- and 7-d treatment groups (grey). A: ADC value; B: D value; C: f value; D: D* value. Standard deviations are represented by vertical bars. Relative changes were determined by comparing the values at baseline and those in follow-up. $^aP < 0.05$ *vs* control. n = 5 in each group. ADC: Apparent diffusion coefficient.

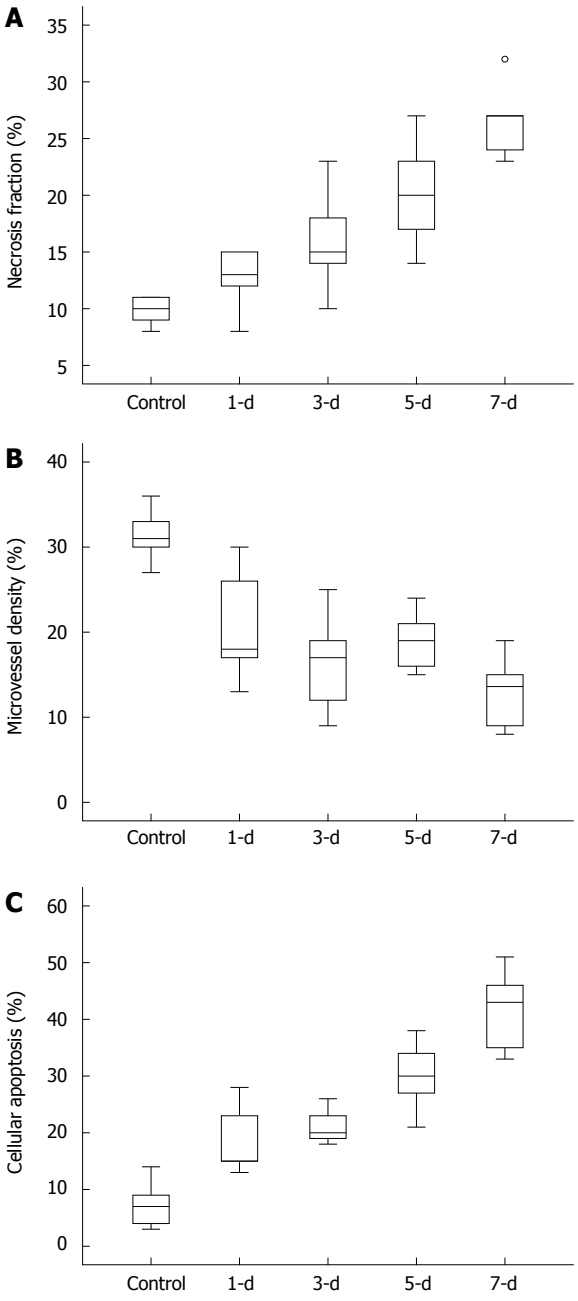


Figure 3 Box-and-Whisker plots show the results of histopathological analysis in the control and the 1-, 3-, 5- and 7-d treatment groups, respectively (*n* = 5 per group). A: Necrosis fraction of tumor; B: Microvessel density of tumor; C: Cellular apoptosis of tumor. Center line = median; upper and lower margins of box = 25th to the 75th percentile, respectively; whiskers = data from the minimum to the maximum; ○ = outlier.

5.54%, P = 0.008; -44.14% ± 14.83% *vs* 2.29% ± 10.38%, P = 0.008; -59.06% ± 19.10% *vs* 3.86% ± 5.10%, P = 0.008, and -47.20% ± 20.48% *vs* 7.13% ± 9.88%, P = 0.016, respectively) (Figure 2D).

Histopathological assessment of tumor response and its correlation with MR images

HE, CD31 and TUNEL staining were performed to confirm the tissue and vessel changes in the tumor. Figure 3 shows the quantification of the NF, MVD, and cellular apoptosis in all animals of the control and the treatment groups (n = 5 per group). The MVD scores in 5-FU treatment tumors decreased significantly compared with the control group. The 5-FU treated tumors displayed a time-dependent increase in NF and cellular apoptosis compared to those in the control group, indicating an effective therapeutic response.

Table 2 summarizes the relationship among ADC, the IVIM parameters, MVD, cellular apoptosis and necrosis of tumors (n = 25) determined by Spearman's correlation coefficient analysis. ADC and D were positively correlated with NF (r_s = 0.720, P < 0.001; r_s = 0.522, P = 0.007, respectively) (Figure 4A, B) and the cellular apoptosis of the tumor (r_s = 0.626, P = 0.001; r_s = 0.542, P = 0.005, respectively) (Figure 4E, F).There is no significant correlation among ADC, D, and MVD. Perfusion-related parameters f and D* shows positive correlations with MVD (r_s = 0.618, P = 0.001; r_s = 0.538, P = 0.006, respectively) (Figure 4C, D), and a negative correlation with the cellular apoptosis of the tumor (r_s = -0.550, P = 0.004; r_s = -0.692, P < 0.001, respectively) (Figure 4G, H). There is no significant correlation between the perfusion parameters and NF. Figure 5 shows the calculated ADC, D, f, and D* maps and the correspondent histopathologic images from the control and 3 d treated mice.

DISCUSSION

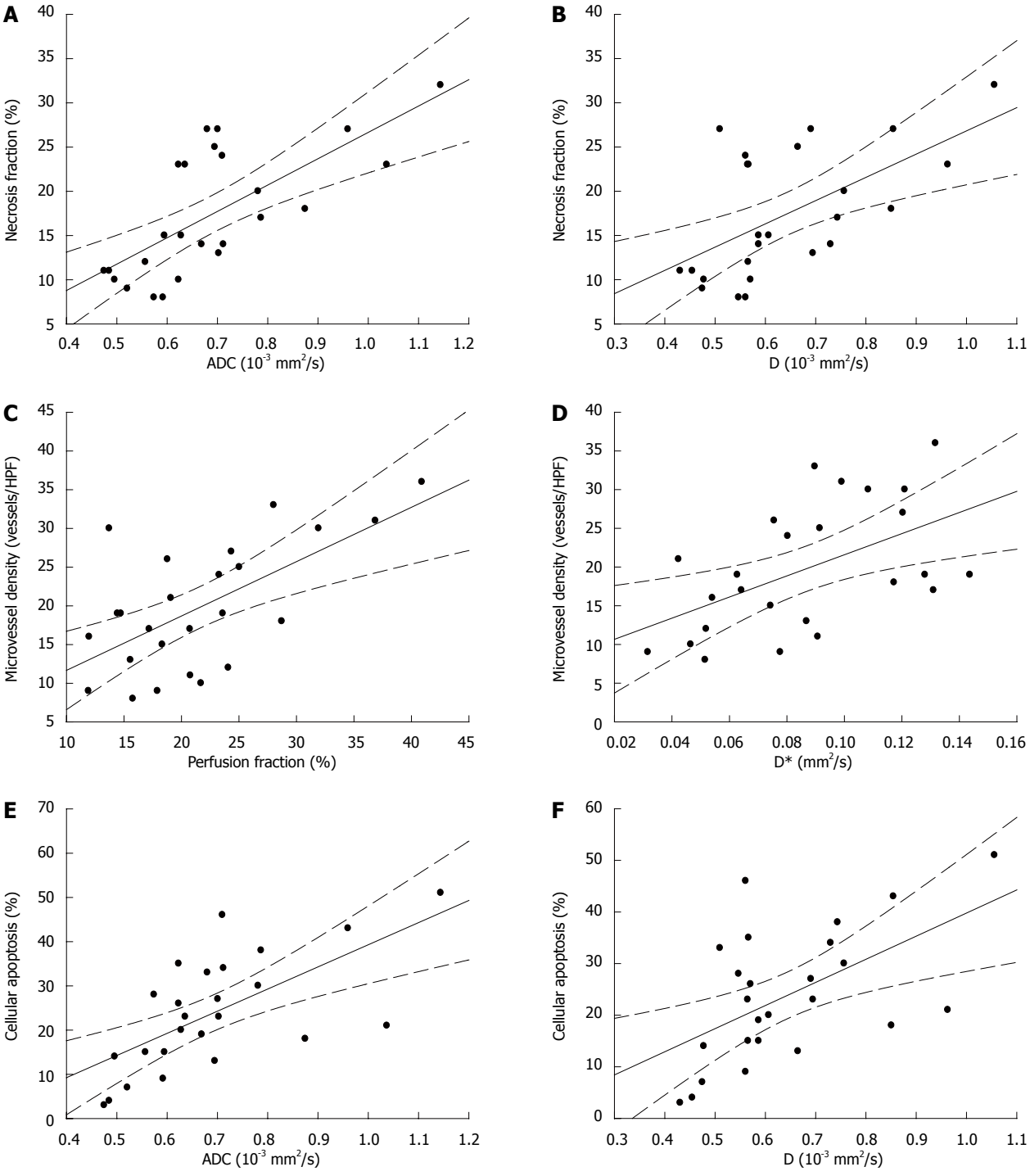
The ADC obtained from conventional DWI has been widely accepted as a marker to monitor the therapeutic efficacy of chemotherapy, radiotherapy or combined therapy with target medicine[26,27]. In recent years, there has been a resurgent interest in IVIM studies, which allows to measure tissue diffusion and perfusion simultaneously. In this study, we performed IVIM-DWI using 12 *b*-values less than 800 s/mm^2 to monitor the efficacy of chemotherapy in a mouse model of human gastric cancer. A histopathological analysis was carried out to evaluate the tissue cellularity and MVD properties of the tumor.

Our results demonstrated that conventional ADC significantly increased after 3-d of treatment and it showed a positive correlation with the 5-FU induced intratumoral necrosis and cellular apoptosis of the tumor. Papaevangelou *et al*[28] found that ADC changes in the tumor were associated with the induction of a mixture of necrosis and apoptosis after irinotecan treatment. Therefore, ADC could be a reliable marker for detecting the necrotic and apoptotic cell death in gastric cancer patients during treatment. IVIM derived D values that showed a similar trend to ADC, were significantly increased as early as after one day treatment compared with the baseline values. Moreover, HE and TUNEL stain showed that D values were positively correlated with intratumoral necrosis and cellular apoptosis, indicating the possibility of

Table 2 Correlation between apparent diffusion coefficient and intravoxel incoherent motion parameters, microvessel density, cellular apoptosis and necrosis fraction of tumors (n = 25)

	NF (%)		MVD (vessels/HPF)		Apoptosis (%)	
	r_s	*P* value	r_s	*P* value	r_s	*P* value
ADC ($\times 10^{-3}$ mm^2/s)	0.720	< 0.001	-0.395	0.051	0.626	0.001
IVIM parameters						
D ($\times 10^{-3}$ mm^2/s)	0.522	0.007	-0.201	0.335	0.542	0.005
f (%)	-0.347	0.089	0.618	0.001	-0.550	0.004
D^* ($\times$ mm^2/s)	-0.378	0.062	0.538	0.006	-0.692	< 0.001

P < 0.05 is considered to be statistically significant. ADC: Apparent diffusion coefficient; IVIM: Intravoxel incoherent motion; MVD: Microvessel density; NF: Necrosis fraction.



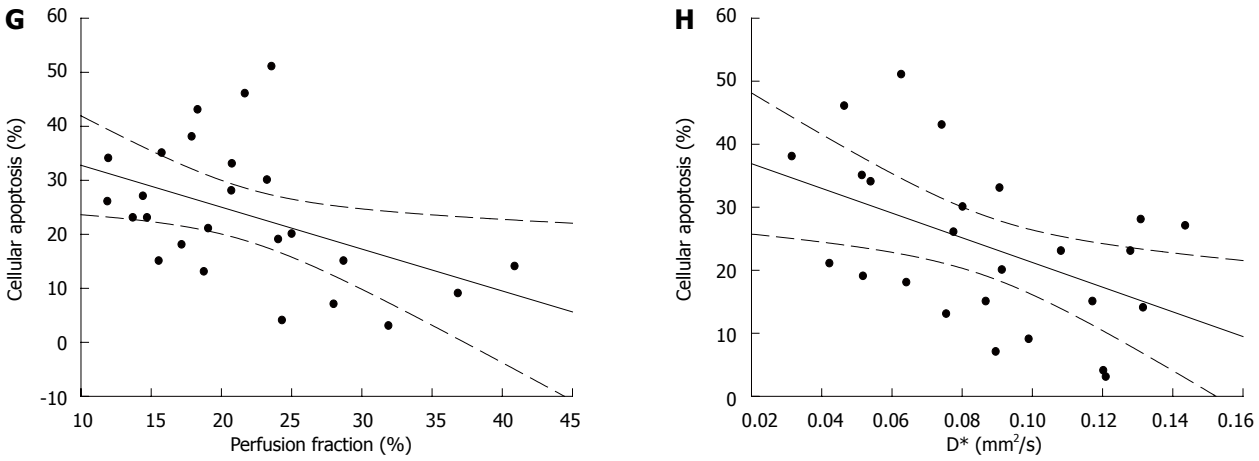


Figure 4 Representative scatter diagrams showing the relationships between intravoxel incoherent motion diffusion-weighted imaging derived parameters and the histological features (*n* = 25). A and B: ADC and D were positively correlated with tumor necrosis fraction; C and D: f and D* were positively correlated with microvascular density of tumor; E and F: ADC, D were positively correlated with tumor cellular apoptosis; G and H: f and D* were negatively correlated with tumor cellular apoptosis. *P* values are shown in Table 2. ADC: Apparent diffusion coefficient.

a noninvasive evaluation of necrosis or apoptotic. Our finding regarding the D value is consistent with previous studies which showed that an increase in D value can predict chemotherapeutic responsiveness in locoregionally advanced nasopharyngeal carcinoma[20] and advanced cervical cancer[29]. There is no denying that accurate quantification of the D value, which associates with the intra-to-extracellular spaces ratio and represents the true molecular diffusion, would eventually translate into a reliable marker to evaluate the tumor therapy response.

The f and D* significantly decreased during the early phase of chemotherapy. In addition, the f and D* were well correlated with the decrease in MVD revealed by the endothelial cell marker CD31 staining, which indicated that f and D* have the potential to assess tumor angiogenesis activity noninvasively. There are experimental and clinical dates in body tissues with IVIM-DWI which support the claim that the signal attenuation is related to microcirculation in tissue when *b*-values less than 100 s/mm^2 were applied. Wang *et al*[30] found an increase in the tumor blood flow induced by IV hydralazine injection was accompanied by an increase in the IVIM-derived D* in a mammary adenocarcinoma rat model. In a human brain study, f and D* were correlated separately with the relative blood volume and blood flow derived from dynamic susceptibility contrast enhancement imaging[31]. Joo *et al*[19] reported that IVIM-DWI has the potential to evaluate the early therapeutic effect induced by a vascular disrupting agent named CKD-516 in rabbit VX2 liver tumors. In addition, f and D* also showed a weak negative correlation with the cellular apoptosis of the tumor, indicating that the decreased cellularity also contributed to the changes in f and D*. This may reconfirm the finding that tissue water diffusion also contributes to the observed signal attenuation at low *b*-values. After effective chemotherapy, extracellular spaces would expand, resulting in less restriction of migration of water molecules and weakening the process of pseudo-diffusion. This would then yield a higher D value and lower f and D* than those obtained pre-treatment. Our results and the conclusions from previous studies[13,32] indicated the usefulness of IVIM-DWI for depicting the therapeutic efficiency in gastric cancer before changes in tumor size are evident.

The option of a bi-exponential model for extracting molecular diffusion and microcirculation perfusion information from DWI remains controversial. A previous IVIM study with the *b*-values up to 1500 s/mm^2 found that f increased after treatment, which contradicts our finding[21]. Pang *et al*[23] found that f obtained from *b*-values below 750 s/mm^2 is more in keeping with the increased perfusion in prostate cancer tissue, while f decrease or became indistinguishable from the normal level of prostate tissue when high *b*-values employed. Both tissue microcirculation and water diffusion in the tissue contributed to signal attenuation at a low *b*-value; hence, departure of molecular diffusion at very high *b*-values may influence the perfusion-related parameters[33,34]. Moreover, the IVIM method suffers significantly from the variations in the signal-to-noise ratio and is prone to generate measurement errors when *b*-values are lower than 100 s/mm^2[13]. To date, no consensus has been reached on the magnitude and number of *b*-values that ought to be applied in preclinical and clinical studies. Therefore, more studies are required to optimize the process of image collection and image post-processing for deriving sufficiently accurate parameters based on the original IVIM model.

There are limitations to this study. First, the observation endpoint in this study is too short. Secondly, because of the small sample sizes, serial relative changes in ADC and the IVIM parameters of the tumor were not evaluated in the treatment groups. Therefore, we need further studies with larger sample size and long-term observations to clarity the limitation of this study.

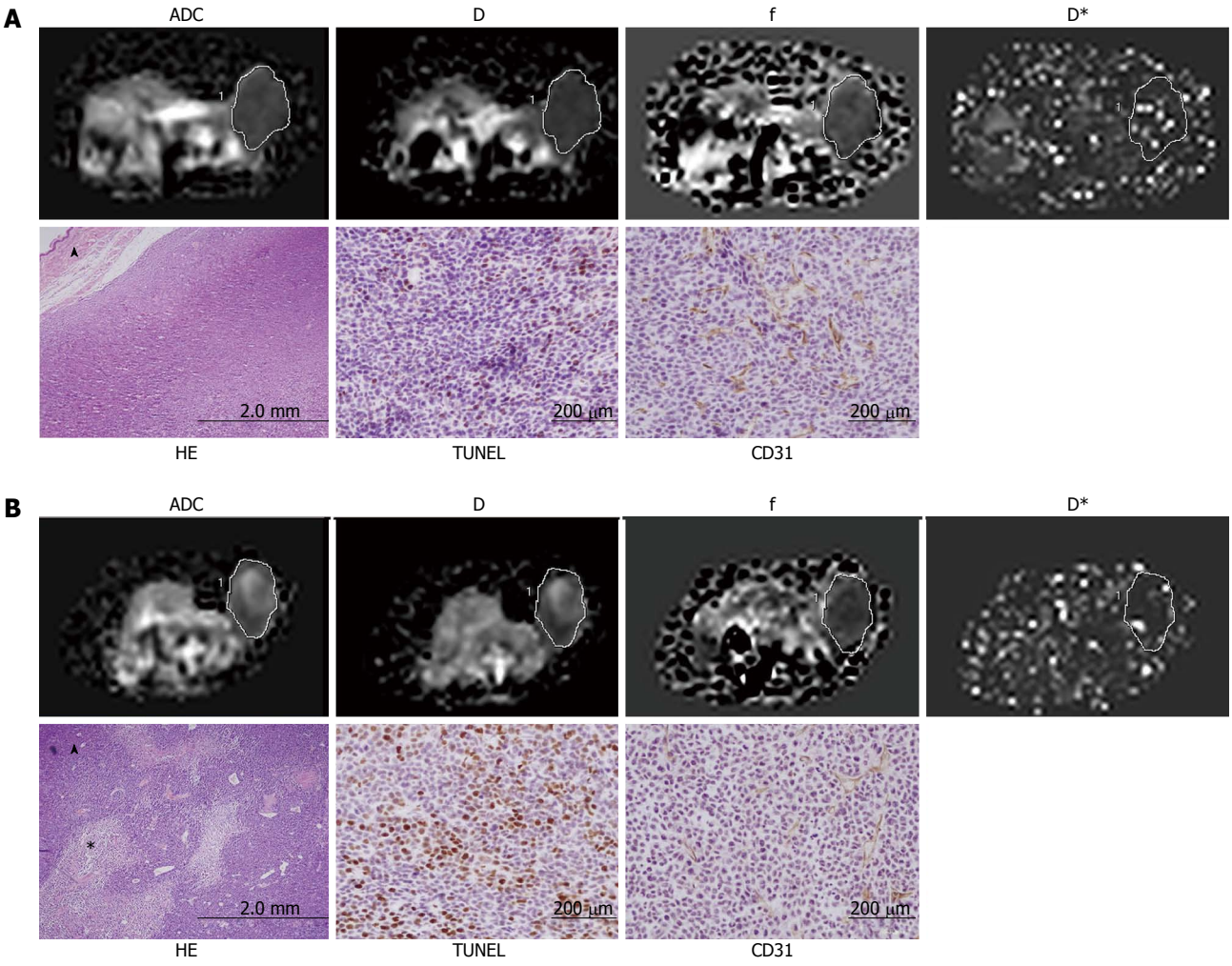


Figure 5 Calculated maps of intravoxel incoherent motion diffusion-weighted imaging parameters and the histopathological images. A: The lower ADC (0.521 × 10^{-3} mm^2/s) and D values (0.475 × 10^{-3} mm^2/s) and the higher f (40.92%) and D* (0.131 mm^2/s) values, which correspond to low necrosis (10%) and cellular apoptosis (7%) and high MVD (36) in the control group; B: Increased ADC (0.875 × 10^{-3} mm^2/s) and D (0.851 × 10^{-3} mm^2/s) values and reduced f (17.20%) and D* (0.098 mm^2/s) values, which correspond to the increased necrosis (18%) and cellular apoptosis (23%) and decreased MVD (17) in the 3-d treatment group. Note high signal intensity within tumor suggesting necrosis. asterisk means necrosis area; The triangle symbol means skin of the mouse. ADC: Apparent diffusion coefficient; MVD: Microvessel density.

In conclusion, IVIM-DWI raises the possibility of an effective, multi-parametric (D, f, D*) imaging method without requirement of gadolinium enhancement. The IVIM method could potentially be used to assess tissue diffusivity changes in addition to evaluate the microcirculatory perfusion of gastric cancer in response to chemotherapy.

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COMMENTS

Background

Chemotherapy is a standard treatment for advanced gastric cancer. Imaging modalities that help to identify early and reliable surrogate markers for the evaluation of chemotherapy response are important in patients with gastric cancer. Intravoxel incoherent motion diffusion-weighted imaging (IVIM-DWI), which allows acquisition of quantitative parameters that reflect tissue diffusivity and tissue microcapillary perfusion, maybe a useful tool that can monitor the early chemotherapy response in terms of gastric cancer tissue diffusion and microvascular perfusion.

Research frontiers

IVIM-DWI has been used to monitor the treatment responses to radiofrequency ablation or a vascular disrupting agent of CKD-516 in rabbit model with VX2 liver tumors, and the response of human locoregionally advanced nasopharyngeal carcinoma to neoadjuvant chemotherapy. DWI is increasingly being applied in the human body, but few studies focused on the gastric lesions due to the limitation of the modality.

Innovations and breakthroughs

In this study, IVIM-DWI with 12 *b*-values less than 800 s/mm^2 was performed to avoid the potential impact of higher *b*-values on the accuracy of the IVIM-derived parameters.

Applications

IVIM-DWI raises the possibility of an effective, multi-parametric (D, f, D*) imaging method that does not require gadolinium enhancement. The IVIM

method could potentially be used to assess tissue diffusivity changes in addition to measuring the microcirculatory perfusion of gastric cancer in response to chemotherapy.

Terminology

DWI is capable of providing a parameter of apparent diffusion coefficient (ADC). The measured ADC, which represents tissue diffusivity, has become a favorite choice in oncologic studies. IVIM-DWI allows acquisition of multi-quantitative parameters that reflect tissue diffusivity and microcirculation perfusion.

Peer-review

The authors have demonstrated the usefulness of IVIM-DWI for monitoring early chemotherapeutic efficacy in a human gastric cancer xenograft model with nude mouse. The manuscript presents interesting and novel findings. The data are well presented and important. However, noted by the authors, the limitation of this study is the short observation endpoint.

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ORIGINAL ARTICLE

Basic Study

MicroRNA-21 promotes phosphatase gene and protein kinase B/phosphatidylinositol 3-kinase expression in colorectal cancer

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Author contributions: Sheng WZ and Chen YS contributed equally to this study, Sheng WZ, Chen YS and Gao WD designed research; Sheng WZ, Chen YS, Gao WD, Tu CT, He J and Zhang B performed research; Gao WD, Tu CT, He J and Zhang B contributed new reagents or analytic tools; Sheng WZ and Chen YS analyzed data; Sheng WZ and Gao WD wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Fudan University affiliated Zhongshan Hospital Institutional review board.

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

Data sharing statement: No additional data are available.

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Abstract

AIM: To explore the regulatory mechanism of the target gene of microRNA-21 (miR-21), phosphatase gene (PTEN), and its downstream proteins, protein kinase B (AKT) and phosphatidylinositol 3-kinase (PI3K), in colorectal cancer (CRC) cells.

METHODS: Quantitative real-time PCR (qRT-PCR) and Western blot were used to detect the expression levels of miR-21 and PTEN in HCT116, HT29, Colo32 and SW480 CRC cell lines. Also, the expression levels of PTEN mRNA and its downstream proteins AKT and PI3K in HCT116 cells after downregulating miR-21 were investigated.

RESULTS: Comparing the miR-21 expression in CRC cells, the expression levels of miR-21 were highest in HCT116 cells, and the expression levels of miR-21 were lowest in SW480 cells. In comparing miR-21 and PTEN expression in CRC cells, we found that the protein expression levels of miR-21 and PTEN were inversely correlated ($P < 0.05$); when miR-21 expression was reduced, mRNA expression levels of PTEN did not significantly change ($P > 0.05$), but the expression levels of its protein significantly increased ($P < 0.05$). In comparing the levels of PTEN protein and downstream AKT and PI3K in HCT116 cells after downregulation of miR-21 expression, the levels of AKT and PI3K protein expression significantly decreased ($P < 0.05$).

CONCLUSION: PTEN is one of the direct target genes

of miR-21. Thus, phosphatase gene and its downstream AKT and PI3K expression levels can be regulated by regulating the expression levels of miR-21, which in turn regulates the development of CRC.

Key words: MicroRNA-21; Protein kinase B; Colorectal cancer; Phosphatidylinositol 3-kinase; Phosphatase and tensin homolog

Core tip: RT-PCR and Western blot were applied to detect the expression level of microRNA-21 (miR-21) and Phosphatase gene (PTEN), including its downstream proteins protein kinase B (AKT) and phosphatidylinositol 3-kinase (PI3K) in colorectal cancer (CRC) cell lines, and to explore the regulatory mechanism of the expression of miR-21 in inhibiting CRC, respectively. Their associations were investigated to clarify whether one of the direct target genes of miR-21 is PTEN. The expression levels of miR-21, PTEN and its downstream proteins AKT and PI3K are regulated and controlled to manage the occurrence and progression of CRC.

Sheng WZ, Chen YS, Tu CT, He J, Zhang B, Gao WD. MicroRNA-21 promotes phosphatase gene and protein kinase B/phosphatidylinositol 3-kinase expression in colorectal cancer. *World J Gastroenterol* 2016; 22(24): 5532-5539 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5532.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5532

INTRODUCTION

MicroRNA (miRNA) is a kind of non-coding macromolecule RNA that includes nearly 22 nucleotides, and it is able to conjugate the 3'UTR of mRNA to facilitate target mRNA degradation and inhibition of the translation process by virtue of lowering gene expression[1-4]. The research of Calin *et al* indicated that approximately 50% of miRNA was located in tumor-related genomic regions. This work also revealed aberrant expression levels in a number of tumors, which was probably on account of the oncogenic and tumor suppressing gene functions of miRNA molecules. In addition, miRNA is involved in the occurrence and progression of human tumors[5-8]. Phosphatase gene (PTEN) is a phosphatase and tensin homologue gene derived from chromosome ten, which is associated with phosphohydrolase; its inactivation induces tumor occurrence in the human body[9-11]. In recent years, the protein kinase B/phosphatase gene/phosphatidylinositol 3-kinase (AKT/PTEN/PI3K) signaling pathway has raised increasing concern, and a number of studies found that the atypical AKT/PTEN/PI3K signaling pathway was intimately linked with numerous tumor occurrences and progression, immunity, drug resistance, metastasis, angiogenesis, *etc.*[12-16]. In addition, the AKT/PTEN/PI3K signaling pathway has been reported in pulmonary, nasopharyngeal, gastric and renal tumors, as well as in neuroglioma[17-21]. However, literature with regard to colorectal cancer (CRC) is rare. Moreover, there were reports that revealed that microRNA-21 (miR-21) could regulate the AKT/PTEN/PI3K signaling pathway to promote tumor occurrence and progression, and even tumor invasion[22-25]. Hence, quantitative real-time PCR (qRT-PCR) and Western blot were applied to detect the expression level of miR-21 and PTEN, including its downstream proteins AKT and PI3K, in CRC cell lines. Furthermore, the mechanism of miR-21 expression in inhibiting CRC and their correlations were also explored. This study will provide a theoretical and experimental basis for the early diagnosis and therapy of CRC.

MATERIALS AND METHODS

Experimental materials

Primary reagents and equipment: PTEN antibody, PI3K mouse anti-human monoclonal antibody, immunohistochemistry (IHC) kit and AKT rabbit anti-human polyclonal antibody were purchased from Beijing Zhongshan Golden Bridge Biotechnology Company. Quantitative RT-PCR kit and miR-21 primer were purchased from Takara. Fluorescent quantitative PCR detection system was purchased from ABI (United States). The PCR instrument was purchased from Bio-Rad (United States). The inverted microscope was purchased from Olympus (Japan). The refrigerated centrifuge was purchased from Thermo Scientific (United States). HCT116, HT29, Colo32 and SW480 CRC cell strains were all purchased from the Cell Bank of the Chinese Academy of Sciences.

Preparation of primary reagents: Requisite reagents: phosphate buffer solution (PBS), bovine serum albumin (BSA) solution, Tris-buffered saline and Tween-20 (TBST) buffer, BSA blocking buffer, 0.25% trypsin solution, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis buffer, transmembrane buffer, SDS-PAGE separation and stacking gel.

Experimental methods

CRC cell culture and transfection: A Dulbecco's modified Eagle's medium (DMEM) high glucose medium with 10% calf serum was used to culture HCT116, Ht29, Colo32 and SW480 CRC cell strains in 5% CO_2 at 37 ℃. Cells in the logarithmic phase were used in experiments. All cells were sorted into three groups: miR-21 inhibition group (IG), negative control (NC) and blank control (BC). HCT116 cells were inoculated in 500 μL of medium (no antibiotics) to 30%-60% degree to spare. Then, 20 pmol/L of miR-21 inhibitor was

diluted with 50 mL of DMEM medium (no serum) and incubated for five minutes at room temperature after mixing. Next, 1 μL of mixing Lipofectamine 2000 was diluted in 50 μL of DMEM (no antibiotics and serum) and incubated at room temperature for 5 min. The diluted miR-21 inhibitor was mixed with Lipofectamine 2000 and incubated at room temperature for 20 min. Then, a 100-μL transfection buffer was added, mixed and incubated for six hours in an incubator (5% CO_2 at 37 ℃). Afterward, the medium was changed to normal medium and cells were incubated for another 48-72 hours for detection.

Transwell assay: Pre-cooling non-serum DMEM was used to dilute the Matrigel matrix gel. Then, it was paved on the chamber of the polycarbonate filtering membrane followed by inoculation of diluted HCT116 cells (100 μL). Afterwards, 10% fetal calf serum was added to the lower chamber. After 24 h of culture in 5% CO_2 at 37 ℃, the chamber was washed twice, stained with 0.1% crystal violet for five minutes, and washed again. Cells were randomly counted in five views at 100 × objective and the average was calculated. This was performed in three replicates for each group.

Real-time quantitative RT-PCR to detect miR-21 and PTEN mRNA levels: Total RNA was extracted using Trizol RNA extraction methods, based on the Molecular Clone Technique Experimental Manual. One mL of Trizol reagent could be added to approximately 100-mg tissues. A mortar was used to grind the tissues to powder, which eventually reached complete decomposition. The lysate was drawn into a 1.5-mL tube and incubated for 10 min on ice. After centrifuging at 12000 rpm for 10 min at 4 ℃, the supernatant was drawn into a new 1.5-mL tube. Isopropanol in equal volume was added and mixed, plated on ice for 10 min, and centrifuged at 12000 rpm for another 10 min at 4 ℃. The supernatant was discarded, 1 mL of 75% ethanol was added, the precipitate was washed, and centrifuged for another five minutes. Afterwards, the supernatant was discarded, placed into a tube in room temperature for 10 min, waited until the residue entirely volatilized, then 100 μL of diethyl pyrocarbonate H_2O was added to dissolve the precipitate, and it was stored in liquid nitrogen for use. Extracted RNA purity and concentration was detected using a spectrophotometer. The normal value of optical density (OD) OD_{260}/OD_{280} was between 1.8 and 2.1, respectively. Then, cDNA was synthesized by reverse transcription reaction. RT-PCR was used to detect miR-21 reaction conditions: 95 ℃ for 10 s, one cycle, 95 ℃ for 5 s, 60 ℃ for 34 s, 45 cycles; computational formula: relative amount = $2^{-\Delta\Delta CT}$, where -ΔΔCT = (CTmiR-21-CTu6) tumor-(CTmiR-21-CTu6) normal tissue. RT-PCR was used to detect PTEN mRNA reaction conditions: 95 ℃ for 10 s, one cycle, 95 ℃ for 5 s, 60 ℃ for 20 s, 45 cycle; and PTEN mRNA expression level was calculated on the basis of ΔCt = Ct(PTEN) - Ct(β-actin), where Folds = $2^{-\Delta\Delta CT}$, and the average result was used with three repeats.

Western blot: After 48-72 h of transfection, the medium was discarded and 100 μL of RIPA lysate was added into each well. The lysate was mixed for 5 min, centrifuged at 12000 rpm for 15 min at 4 ℃, and the supernatant was stored for use. Samples mixed with 5 × loading were boiled for 10 min at 95 ℃ and loaded after centrifugation. Eight μL of purified and desalted antibody was loaded, and 2-3 μL of the marker was loaded. The antibody was confirmed according to the marker position. Electrophoresis: stacking gel in 80 V for 20-30 min, and separation gel in 120 V for 40 min. Transmembrane: 300 mA for 90 min. After transmembrane, the membrane was blocked with non-fat milk, washed with PBS for three times, and incubated with the primary antibody (PTEN antibody, PI3K antibody, AKT antibody and β-actin antibody; 1:500 dilution) overnight at 4 ℃. On the next day, cells were washed with TBST for seven times and incubated with the secondary antibody (horseradish peroxidase-labeled goat-anti rabbit secondary antibody, 1:2500 dilution) for one hour. Then, cells were washed with TBST for another six times. Chemiluminescence reagent A and B solutions were mixed and added on the membrane based on a 1:1 proportion, and β-actin protein was used as an internal reference.

Statistical analysis

SPSS 19.0 was used to analyze all data; measurement data were presented as mean ± SD. The *t*-test was used to compare miR-21 and PTEN expression levels, and the variance analysis method was used to compare the mean value of transfection in the IG, NC and BC groups. $P < 0.05$ was considered statistically significant.

RESULTS

miR-21 and PTEN expression in CRC cells

miR-21 had the highest protein expression in HCT116 cells, but had the lowest expression in SW480 cells (Figure 1). In contrast, PTEN protein expression was the lowest in HCT116 cells, but had the highest expression in SW480 cells (Figure 2). The protein expression level between miR-21 and PTEN was inversely related, and the difference was statistically significant ($P < 0.01$).

Invasive ability changes in cells after transfection

In counting the cells that crossed after transfection in the Transwell assay, we found that cells transfected with miR-21 in IG were reduced by 39.1% compared to BC and 36.9% compared to NC. However, the difference between BC and NC was not statistically significant (Figure 3).

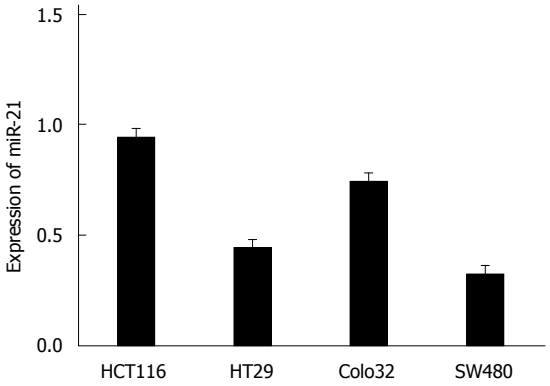

Figure 1 Expression of miR-21 in colorectal cancer cell lines (*n* = 3).

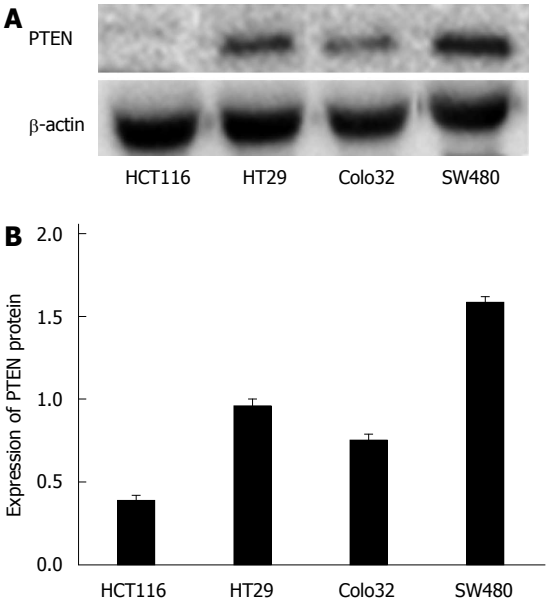

Figure 2 Expression of PTEN protein in colorectal cancer cell lines. A: Western blot assay; B: the expression of PTEN in colorectal cancer cell lines (*n* = 3).

RT-PCR detection of miR-21 expression after transfection

miR-21 expression levels after transfection in IG, NC and BC are shown in Figure 4. It can be observed that miR-21 expression levels in BC and NC were higher than in IG, and the difference was statistically significant ($P < 0.05$).

PTEN mRNA and protein expression in HCT116 cells after downregulation of miR-21

As shown in Figure 5A, after reducing the expression of miR-21, PTEN mRNA expression levels in HCT116 cells revealed no statistical significance after transfection in IG, NC and BC ($P > 0.05$). As illustrated in Figure 5B, when miR-21 was downregulated, PTEN protein expression levels were markedly elevated, and the difference was statistically significant ($P < 0.05$). The difference between BC and NC was not statistically significant ($P > 0.05$)

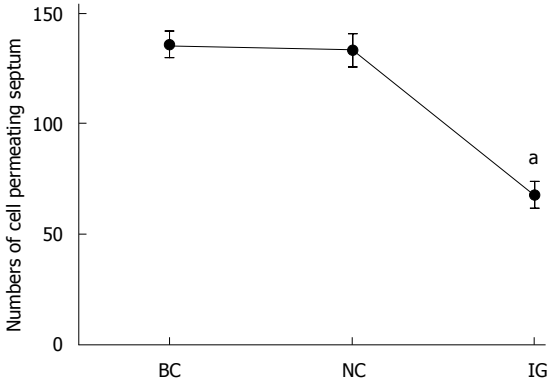

Figure 3 Changes in cell invasion ability after transfection ($^aP < 0.05$, $n = 3$). IG: Inhibition group; NC: Negative control; BC: Blank control.

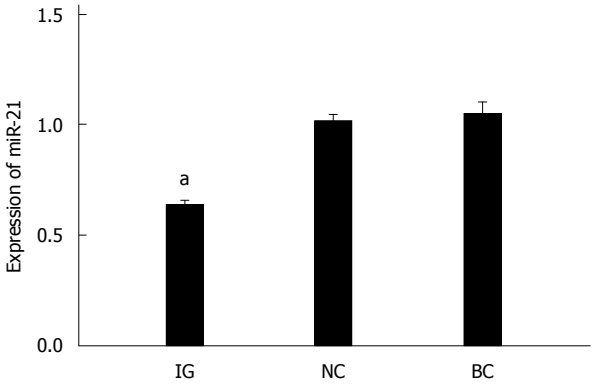

Figure 4 Effect of the miR-21 inhibitor on miR-21 expression in HCT116 cells ($^aP < 0.05$, $n = 3$). IG: Inhibition group; NC: Negative control; BC: Blank control.

PTEN and its downstream proteins AKT and PI3K expression in HCT116 cells after downregulating miR-21

As shown in Figure 6, PTEN protein expression levels in HCT116 cells were elevated in IG, and the difference was statistically significant ($P < 0.05$). However, AKT and PI3K expression levels in HCT116 cells in IG decreased; and the difference was statistically significant ($P < 0.05$).

DISCUSSION

Much research has indicated that CRC occurrence in patients is a complicated process, which consists of multiple stages and factors. One momentous feature is its oncogenic activation and tumor-suppressing gene expression dysregulation or absence[26,27]. In recent years, it has been found miRNA possesses oncogenic and tumor suppressing gene functions[22]. One representative miRNA, miR-21, possesses a crucial oncogenic function and exhibits high expression levels in a number of tumors; it is closely associated with tumor occurrence and chemotherapy sensitivity[28,29]. PTEN is derived from chromosome ten, and acts as a tumor-suppressing gene. PTEN exerts vital effects on cell growth, proliferation, migration, signal transmission,

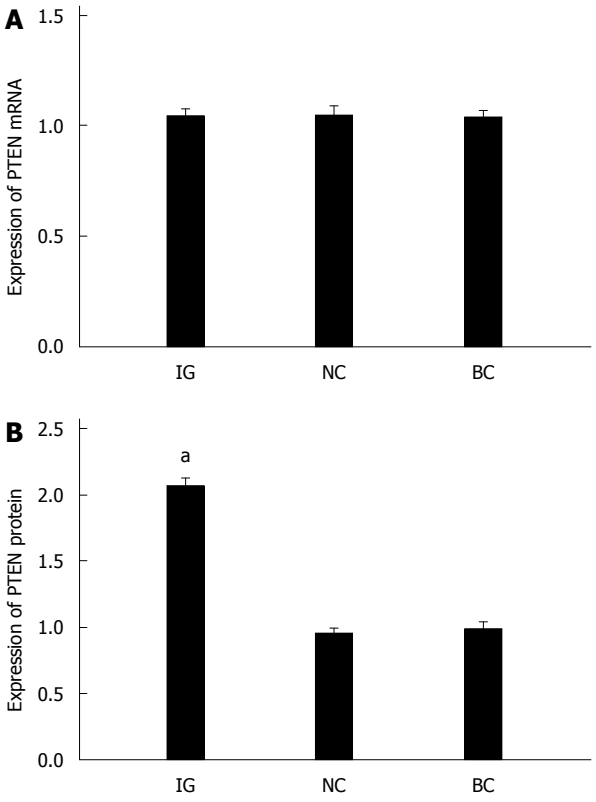

Figure 5 Effect of the miR-21 inhibitor on PTEN mRNA (A) and protein (B) expression in HCT116 cells ($^aP < 0.05$, $n = 3$). IG: Inhibition group; NC: Negative control; BC: Blank control.

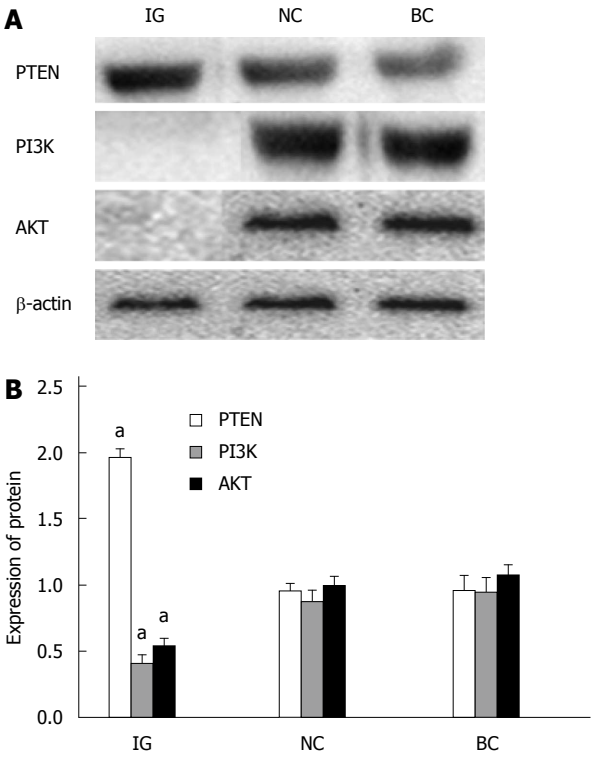

Figure 6 Protein expression of PTEN/AKT/PI3K in colorectal cancer HCT116 cell lines. A: Western blot assay; B: Protein expression in HCT116 cell lines ($^aP < 0.05$, $n = 3$). IG: Inhibition group; NC: Negative control; BC: Blank control.

invasion and apoptosis; it has an inhibitory role on tumor cell growth, invasion, proliferation, metastasis and apoptosis[30]. The AKT/PTEN/PI3K signaling pathway plays a significant part in regulating cell growth, metabolism, differentiation and apoptosis; it is closely associated with tumor occurrence and progression[31-36]. PTEN protein phosphatase activity (dephosphorylation) can inhibit AKT function, leading to cell apoptosis. Furthermore, the deletion or mutation of PTEN is able to enhance AKT activity to increase PI3K expression[37]. AKT/PTEN/PI3K signaling pathway activation is capable of inhibiting cell apoptosis to facilitate cell differentiation and proliferation. This also exerts a crucial role in tumor occurrence and progression, and participates in tumor metastasis and invasion[38-44].

miR-21 and PTEN expression in CRC cells

miR-21 and PTEN protein expression was detected in HCT116, HT29, Colo32 and SW480 CRC cell lines through RT-PCR and Western blot. Results revealed that miR-21 expression level was highest in HCT116 cells and lowest in SW480 cells. Furthermore, PTEN expression level was lowest in HCT116 cells and highest in SW480 cells. The protein expression level between miR-21 and PTEN was inversely correlated. These results indicate that miR-21 could be involved in the regulation of PTEN protein expression. In addition, due to the high expression level of miR-21 in HCT116 cells, HCT116 cells were selected for this study. Transwell invasion assay revealed that the invasive capacity of tumor cells was obviously reduced after inhibiting CRC cell miR-21 expression, which in turn indicates that miR-21 probably plays a promoting role in cell malignancy transformation.

miR-21 and PTEN mRNA and protein expression in HCT116 cells after downregulating miR-21

After downregulating the expression of miR-21, the expression level of miR-21 in HCT116 cells in IG was obviously lower compared to NC and BC, which illustrates the successful inhibition of miR-21. As a known miR-21 target gene, PTEN has been verified to be a target gene of miR-21 by Western blot. When we inhibited the expression of miR-21, PTEN mRNA expression levels did not reveal apparent change, but protein levels were obviously elevated; which indicates that miR-21 could regulate PTEN expression at the post-transcriptional level and that PTEN was a target gene for miR-21. Meng *et al*[45] recently found that miR-21 targeted tumor-suppressing gene PTEN in liver and bile duct cancer cells to promote tumor growth, invasion and migration, manifesting that PTEN was a target gene of miR-21.

PTEN and its downstream AKT and PI3K expression in HCT116 cells after reducing miR-21 expression

After inhibiting the expression of miR-21, PTEN protein

level was significantly elevated, but its downstream AKT and PI3K expression obviously decreased, indicating that miR-21 could regulate and control PTEN and the expression level of downstream AKT and PI3K in terms of its effects on tumor cell invasion and migration. Certain studies have shown that change in miR-21 expression level is able to induce PTEN downstream molecule AKT phosphorylation, metalloproteinase 2 and focal adhesion kinase expression; these are altered to restrain tumor cell invasion and migration[45-50].

In conclusion, our study revealed that PTEN is one of the direct target genes of miR-21. By altering miR-21 expression, downstream AKT and PI3K expression levels could be regulated and controlled, in order to control CRC occurrence and progression. Nevertheless, the mechanisms of miR-21 and PTEN function, as well as their effects in tumors, need to be further investigated. As more in-depth research on miRNA is being undertaken, especially regarding tumor cell regulatory mechanisms, more roles and targets of miR-21 will probably be unraveled.

COMMENTS

Background

Colorectal cancer (CRC) is complicated and combines multiple stages and factors. In recent years, we have found that miRNA possesses oncogenic and tumor-suppressing gene functions. One representative miRNA, microRNA-21 (miR-21), possesses a crucial oncogenic function that shows high expression levels in a number of tumors; this is closely associated with tumor occurrence and chemotherapy sensitivity. PTEN acts as a tumor-suppressing gene derived from chromosome ten. PTEN exerts vital effects in cell growth, proliferation, migration, signal transmission, invasion and apoptosis; it has an inhibitory role in tumor cell growth, invasion, proliferation, metastasis and apoptosis. The AKT/PTEN/PI3K signaling pathway plays a significant part in regulating cell growth, metabolism, differentiation, and apoptosis; it is closely associated with tumor occurrence and progression.

Research frontiers

In addition, the AKT/PTEN/PI3K signaling pathway has been reported in pulmonary, nasopharyngeal, gastric and renal tumors, as well as in neuroglioma; however, literature with regard to CRC is rare. Moreover, there have been reports that revealed that miR-21 could regulate the AKT/PTEN/PI3K signaling pathway, in order to promote tumor occurrence and progression, and even tumor invasion. Hence, RT-PCR and Western blot were applied to detect the expression levels of miR-21 and PTEN, including its downstream proteins AKT and PI3K in CRC cell lines, as well as the inhibition of miR-21 expression in CRC cell lines. Furthermore, their association was also explored. This study hopes to provide a theoretical and experimental basis for the early diagnosis and therapy of CRC.

Innovations and breakthroughs

RT-PCR and Western blot were applied to detect the expression level of miR-21 and PTEN including its downstream proteins AKT and PI3K, and the inhibition of miR-21 expression in CRC cell lines, respectively. This association was also investigated to demonstrate that PTEN was one of the direct target genes of miR-21. Through regulating and controlling the expression level of miR-21, PTEN and its downstream proteins AKT and PI3K expression level are regulated to control the occurrence and progression of CRC.

Applications

This study revealed that PTEN was one of the direct target genes of miR-21, and that its downstream AKT and PI3K expression levels could be regulated and controlled through altering miR-21 expression, in order to control CRC occurrence and progression. Nevertheless, the mechanisms of miR-21 and PTEN function, as well as their effects in tumors, need to be further investigated. As in-depth research on mRNA is currently being implemented, particularly regarding the regulatory mechanism of tumor cells, more roles and targets of miR-21 will probably be unraveled.

Peer-review

This is an interesting manuscript about the miR-21 promotion of PTEN and its downstream proteins AKT and PI3K in CRC. Over all, this study is well designed and the manuscript is well written.

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ORIGINAL ARTICLE

Basic Study

Effects of sphincter of Oddi motility on the formation of cholesterol gallstones

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Author contributions: Rong ZH and Zhang ZH contributed equally to this work; Zhang ZH, Rong ZH, Xian GZ, and Qin CK designed the research; Rong ZH, Chen HY, Wang XX, Wang ZY, and Ma BZ performed the research; Rong ZH and Zhang ZH analyzed the data; Rong ZH and Zhang ZH wrote the paper.

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Institutional review board statement: The study was reviewed and approved by the Provincial Hospital Affiliated to Shangdong University Institutional Review Board.

Institutional animal care and use committee statement: The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (room temperature 23 ℃, 12-h light and dark cycle, 50% humidity, ad libitum access to food and water) for two weeks prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection.

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

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at zhangzhenhai410@126.com. Participants gave informed consent for data sharing.

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Abstract

AIM: To investigate the mechanisms and effects of sphincter of Oddi (SO) motility on cholesterol gallbladder stone formation in guinea pigs.

METHODS: Thirty-four adult male Hartley guinea pigs were divided randomly into two groups, the control group (n = 10) and the cholesterol gallstone group (n = 24), which was sequentially divided into four subgroups with six guinea pigs each according to time of sacrifice. The guinea pigs in the cholesterol gallstone group were fed a cholesterol lithogenic diet and sacrificed after 3, 6, 9, and 12 wk. SO manometry and recording of myoelectric activity were obtained by a multifunctional physiograph at each stage. Cholecystokinin-A receptor (CCKAR) expression levels in SO smooth muscle were detected by quantitative real-time PCR (qRT-PCR) and serum vasoactive intestinal peptide (VIP), gastrin, and cholecystokinin octapeptide (CCK-8) were detected by enzyme-linked immunosorbent assay at each stage in the process of cholesterol gallstone formation.

RESULTS: The gallstone formation rate was 0%, 0%, 16.7%, and 83.3% in the 3, 6, 9, and 12 wk groups, respectively. The frequency of myoelectric activity in the 9 wk group, the amplitude of myoelectric activity in the 9 and 12 wk groups, and the amplitude and the frequency of SO in the 9 wk group were all significantly decreased compared to the control group. The SO basal pressure and common bile duct pressure increased markedly in the 12 wk group, and the CCKAR expression levels increased in the 6 and 12 wk groups compared to the control group. Serum VIP was elevated significantly in the 9 and 12 wk groups and gastrin decreased significantly in the 3 and 9 wk groups. There was no difference in serum CCK-8 between the groups.

CONCLUSION: A cholesterol gallstone-causing diet can induce SO dysfunction. The increasing tension of the SO along with its decreasing activity may play an important role in cholesterol gallstone formation. Expression changes of CCKAR in SO smooth muscle and serum VIP and CCK-8 may be important causes of SO dysfunction.

Key words: Cholesterol gallstone; Sphincter of Oddi; Manometry; Myoelectric activity; Cholecystokinin-A receptor

Core tip: This study investigated the role of sphincter of Oddi (SO) motility in cholesterol gallstone formation in a guinea pig model. The myoelectric activity and manometry of SO were measured at different stages of stone formation. As SO motility is controlled by neurological and hormonal factors, we detected the expression of serum vasoactive intestinal peptide (VIP), gastrin, cholecystokinin octapeptide (CCK-8), and CCK-A receptor (CCKAR) in the SO at different stages of stone formation. We found that a cholesterol gallstone-causing diet can induce SO dysfunction and expression changes of CCKAR in SO smooth muscle and serum VIP and CCK-8 may be important causes of SO dysfunction.

Rong ZH, Chen HY, Wang XX, Wang ZY, Xian GZ, Ma BZ, Qin CK, Zhang ZH. Effects of sphincter of Oddi motility on the formation of cholesterol gallstones. *World J Gastroenterol* 2016; 22(24): 5540-5547 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5540.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5540

INTRODUCTION

Gallstone disease is one of the most common digestive disorders requiring hospital admission in Western countries, with a morbidity of 10%-15% in adults[1]. In China, the incidence of cholesterol gallstones has been increasing in the past few decades due to changing lifestyles[2]. Cholesterol gallstone formation is a complicated process and is still not fully understood. Cholesterol supersaturation of bile, biliary stasis, and mucus hypersecretion are universally known to be important factors in the process of cholesterol gallstone formation[3,4]. Among them, biliary stasis is thought to be a key factor because it allows time for cholesterol nucleation and then retention of the precipitated microcrystals[4,5]. The sphincter of Oddi (SO) may play an important role in gallstone formation because it is the only way by which bile is discharged into the duodenum. There are very few reports investigating the relationship between SO motility and cholesterol gallstone formation, and the conclusions are controversial[6-8].

It is well-established that cholecystokinin (CCK) is one of the major gastrointestinal hormones responsible for gallbladder contraction and SO relaxation[9]. The biological actions of CCK in the alimentary tract are mediated by the CCK-A receptor (CCK-AR)[10]. A series of studies focused on the expression level of CCK-AR in the gallbladder in the pathogenesis of cholesterol gallstone disease[5,11]. However, no report on CCK-AR expression in the SO in animals with gallstones is available yet.

The aim of this study was to investigate the role of SO motility in cholesterol gallstone formation in a guinea pig model. The myoelectric activity and manometry of SO were measured at different stages of stone formation in this model. As SO motility is controlled by neurological and hormonal factors, we detected serum vasoactive intestinal peptide (VIP), gastrin, CCK-8, and CCK-AR expression in the SO at different stages of stone formation.

MATERIALS AND METHODS

Experimental animals

Thirty-four adult male Hartley guinea pigs, weighing between 230 g and 270 g, were purchased from Huishan Jiangnan Laboratory Animal Company (License SCXK SU: 2009-0005). The animals were housed in climate-controlled rooms under an alternating 12-h light and dark cycle and permitted continuous access to food and water. After a 2-wk equilibration, the animals were divided randomly into two groups (control and cholesterol stone groups): the control group (n = 10) was fed a normal diet, and the cholesterol stone group (n = 24) was sequentially divided into four subgroups with six guinea pigs each according to time of sacrifice, fed a cholesterol lithogenic diet[6], and sacrificed after 3, 6, 9, and 12 wk. The lithogenic diet consisted of 1% cholesterol[10] (purchased from Trophic Animal Feed High-Tech Co. Ltd., Nantong, China). Gallstones were defined as macroscopically visible sediment. The calculi were tested by infrared spectrometry to verify the sample as cholesterol gallstones. SO manometry and myoelectric activity of the guinea pigs were determined at 3, 6, 9, and 12 wk.

Table 1 Primer sequences of *CCKAR* and *GAPDH*

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
CCKAR	ACGGAGGGTAGTGAACTCCA	TCGCAGGCAGAAGTGATGTT
GAPDH	GCACCGTCAAGGCTGAGAAT	CATCACGAACATAGGGGCATC

CCKAR: Cholecystokinin-A receptor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Measurement of myoelectric activity of the SO

At the end of the feeding period, the animals were anesthetized by injecting pentobarbital sodium (45 mg/kg) into the peritoneal cavity. The guinea pigs were fixed in the supine position, and the skin of the superior abdomen was prepared and sterilized. A longitudinal incision was made and the papilla determined. Details of the myoelectric system were described previously[12]. In brief, two polar hook metal electrodes were inserted 0.2 mm into the subserosa of the SO by megaloscope (× 10 magnification). The fan-out of the two signals was connected with the two polars of the physiological recorder (BL-420 F; Chengdu Taimeng Software, Chengdu, China), and a piece of metal needle was inserted into the legs of the animals to connect with the earth pole of the recorder. The myoelectric signal was collected by the electrode, imported into the computer, and stored after processing by a physiological recorder and the software system specialized in electromyographic signals. The setup parameters were as follows: scanning speed, 500 ms/div; sensitivity, 200 μV; time parameter, 1 s; and frequency filtering, 10 Hz. Finally, the myoelectric figure was dealt with by digital filtering of 10-30 Hz.

SO manometry

Details of manometry were described previously[12]. In brief, a manometry catheter was modified from a pedo bi-lumen central venous catheter (4F and 30 cm long). The catheter was inserted into the common bile duct (CBD) and SO through the duodenal ampulla. The pressure transducer was used for receiving the dynamic pressure change from the manometric lumen. The frequency of SO phasic contraction, SO amplitude, SO basal pressure, and CBD pressure were measured and recorded. A physiological recorder and relevant manometry program were used to record and analyze the tracings.

Detection of serum VIP, gastrin, and CCK-8

Four milliliters of venous blood were obtained from the guinea pigs in the early morning before they were sacrificed and placed in a test tube. The blood was centrifuged at 1500 r/min for 15 min, and serum was isolated, placed in Eppendorf tubes and stored at -70 ℃. Serum VIP, gastrin, and CCK-8 were measured by enzyme-linked immunosorbent assay (ELISA). The ELISA testing kit was supplied by USCN Life Science Inc. (Houston, TX, United States).

Quantification of CCKAR mRNA in SO tissues

The SO of each animal was quickly removed and transferred to normal saline after animals were euthanized. The smooth muscle was removed carefully using sharp dissection. Total RNA from SO tissue samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). Every 50 mg of SO tissue sample was extracted with 1 mL of TRIzol reagent according to the manufacturer's protocol. One microgram of total RNA was used for the synthesis of cDNA using a FastQuant RT Kit and target genes were assayed using SYBR Green Real-time PCR Master Mix (*via* Roche Light Cycler, Roche, Basel, Switzerland) with their respective primers. The PCR conditions were as follows: 95 ℃ for 30 s; 40 cycles of 95 ℃ for 5 s, 57 ℃ for 10 s, and 72 ℃ for 15 s. Transcription levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal control to calculate fold induction, and the fold changes in transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences of CCKAR and GAPDH are shown in Table 1.

Statistical analysis

Statistical analysis was performed using Student's *t* test. Data were analyzed with software SPSS 17.0 (SPSS Inc. Chicago, IL, United States), and $P < 0.05$ was set as the level of significance. The results are expressed as mean ± SE.

RESULTS

Gallstone formation rate

No gallstone was found in guinea pigs in the control group. In contrast, the gallstone formation rate in the cholesterol stone group fed with a cholesterol lithogenic diet was 0%, 0%, 16.7%, and 83.3% in the 3, 6, 9, and 12 wk groups, respectively.

SO myoelectric activity analysis

Compared with the control group, the frequency of myoelectric activity decreased markedly in the 9 wk group (Figure 1A and B, Table 2). The amplitude of myoelectric activity decreased significantly in the 9 and 12 wk groups (Figure 1A-C, Table 2).

SO manometry analysis

Compared with the control group, the amplitude and frequency of SO decreased significantly in the 9 wk group (Table 3), and SO basal pressure and common

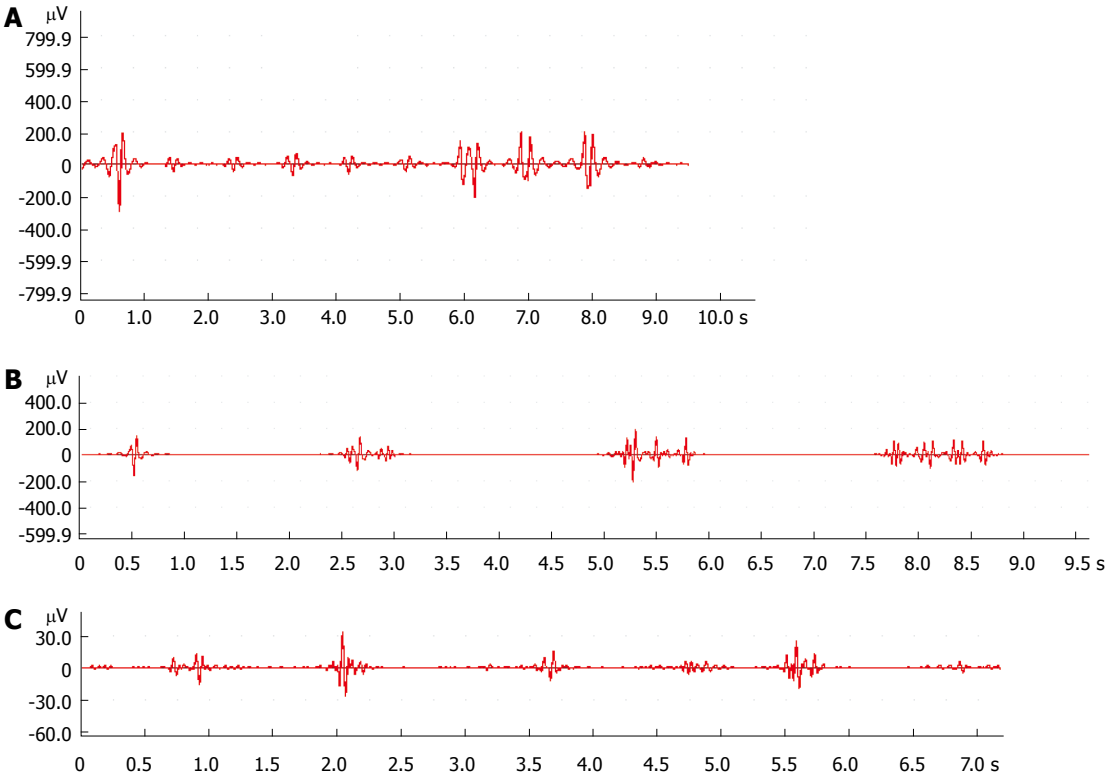

Figure 1 Sphincter of Oddi myoelectric activity analysis. A: The frequency and amplitude of myoelectric activity in the control group; B: The frequency and amplitude of myoelectric activity in the 9 wk group; C: The frequency and amplitude of myoelectric activity in the 12 wk group.

Table 2 Changes of sphincter of Oddi myoelectric activity in the process of gallstone formation

Group	Amplitude	Frequency
Control group	142.45 ± 71.25	16.56 ± 4.05
3-wk group	125.06 ± 59.76	13.70 ± 2.88
6-wk group	152.96 ± 81.05	15.10 ± 4.13
9-wk group	100.27 ± 50.42[a]	8.68 ± 2.35[a]
12-wk group	40.60 ± 15.03[c]	13.80 ± 3.79

[a]$P < 0.05$, [c]$P < 0.01$ *vs* the control group.

bile duct pressure increased significantly in the 12 wk group (Figure 2, Table 3).

Changes in serum VIP, gastrin and CCK-8

Compared with the control group, serum VIP was elevated significantly in the 9 and 12 wk groups (Table 4), and serum gastrin was decreased significantly in the 3 and 9 wk groups (Table 4). There was no difference in serum CCK-8 between the groups (Table 4).

Quantitative expression of CCK-AR mRNA

Compared with the control group, expression levels of the CCK-A receptor mRNA were increased significantly in the 6 and 12 wk groups (Figure 3).

DISCUSSION

Gallstone disease is one of the most common and most expensive digestive disorders requiring admission to the hospital[13-15], with a prevalence of 10%-15% in adults in Europe and the United States. In Western countries, cholesterol gallstones account for 80%-90% of gallstones at cholecystectomy[15], but the mechanism underlying the pathogenesis of cholesterol gallstone disease is not completely understood.

Epidemiological evidence indicates that multiple environmental factors and genetic elements are involved in cholesterol gallstone formation. Among them, biliary stasis is thought to be an important factor. A series of studies in human and animal models have shown that formation of cholesterol gallstones is causatively related to decreased gallbladder contractility[16]. Since SO is the only gate through which bile is discharged into the duodenum, bile filling and excretion of the gallbladder are closely related to the motility state of the SO[17]. However, there is limited research on the role of SO motility in the process of cholesterol gallstone formation. SO manometry (SOM) is the only method that can assess directly the motor function of the SO and is considered the gold standard for assessing SO dysfunction (SOD)[18]. However, manometry changes of the SO in cholelithiasis are controversial. Research by Pang *et al*[19] indicated that the base pressure of the SO was significantly increased in rabbits fed a cholesterol lithogenic diet. On the other hand, a study in prairie dogs showed that SO resistance remained normal throughout the period of gallstone formation. These completely different results may be species dependent. Meanwhile, the mechanical activity recorded may not represent the features of

Table 3 Sphincter of Oddi manometry in the process of gallstone formation

Group	SO basal pressure	CBD pressure	Amplitude of SO	Frequency of SO
Control group	26.59 ± 8.16	23.25 ± 8.35	8.72 ± 2.05	11.27 ± 3.74
3-wk group	21.25 ± 1.38	18.48 ± 1.94	8.33 ± 3.85	11.50 ± 1.64
6-wk group	27.57 ± 8.67	25.82 ± 8.26	8.03 ± 3.15	9.33 ± 3.27
9-wk group	34.11 ± 11.56	32.15 ± 11.64	5.89 ± 1.41[a]	7.67 ± 3.44[a]
12-wk group	52.38 ± 12.84[c]	50.11 ± 12.59[e]	6.82 ± 1.34	10.60 ± 3.51

[a]$P < 0.05$, [c]$P < 0.01$, [e]$P < 0.001$ *vs* the control group. SO: Sphincter of Oddi; CBD: Common bile duct.

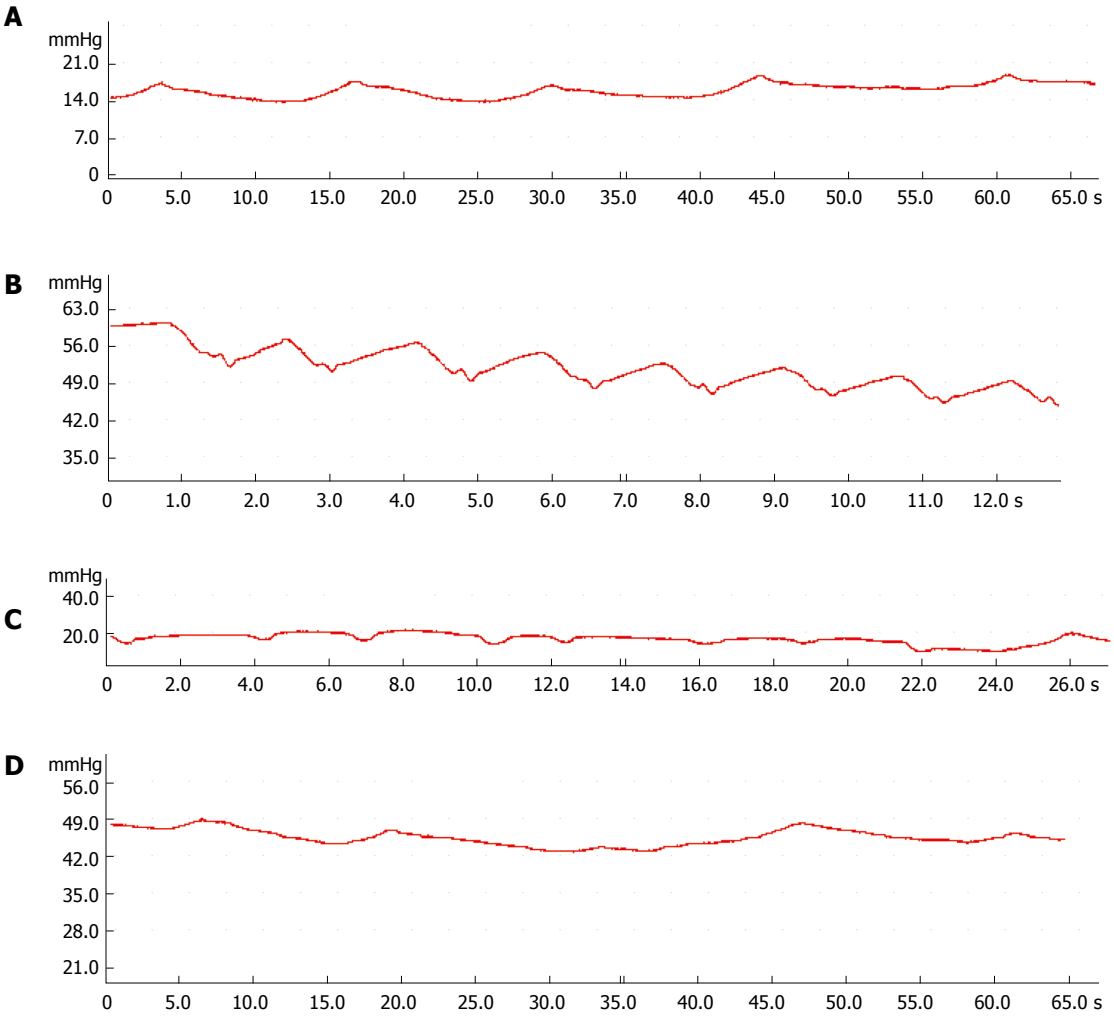

Figure 2 Sphincter of Oddi manometry analysis. A: Sphincter of Oddi (SO) basal pressure in the control group; B: SO basal pressure in the 12 wk group; C: Common bile duct pressure in the control group; D: Common bile duct pressure in the 12 wk group.

SO activity, especially in the relaxed SO. Another measurement reflecting SO function is recording the myoelectric activity of the SO[20].

In this study, we investigated whether SOD was present and what role it played in the process of cholesterol gallstone formation in guinea pigs. SOM and myoelectric activity of SO were investigated simultaneously at different stages of stone formation. We found that gallstones did not occur until 9 wk, with an incidence rate of 16.7%, increasing to 83.3% after 12 wk. SO myoelectric activity analysis indicated that the frequency and amplitude of myoelectric activity decreased significantly in the 9 wk group compared to control. Subsequent SO manometry analyses showed the same result: both SO amplitude and SO frequency decreased significantly in the 9 wk group, and the most important indications, the SO basal pressure and common bile duct pressure, increased significantly in the 12 wk group. All these findings are consistent with weakening of the myoelectric activity of SO and gradual increasing of SO tension in the process of gallstone formation. Disturbance of SO motor function impedes the flow of bile into the duodenum, and biliary stasis occurs.

Table 4 Changes in VIP, gastrin and CCK-8 in the process of cholesterol stone formation (pg/mL)

Group	3-wk	6-wk	9-wk	12-wk
VIP				
Control	9.36 ± 2.72	9.30 ± 8.91	6.91 ± 3.14	8.39 ± 0.99
Cholesterol stone	11.83 ± 3.57	7.08 ± 2.31	31.20 ± 7.78[a]	22.15 ± 2.87[c]
Gastrin				
Control	17.83 ± 2.35	18.56 ± 5.77	17.42 ± 6.39	19.41 ± 4.58
Cholesterol stone	0.08 ± 0.03[c]	4.18 ± 2.87	2.16 ± 0.44[a]	18.92 ± 8.37
CCK-8				
Control	1970.33 ± 439.82	2353.32 ± 13.18	2100.27 ± 29.70	2107.77 ± 171.70
Cholesterol stone	2214.61 ± 174.96	2044.18 ± 147.03	2034.75 ± 138.39	2042.61 ± 265.01

[a]$P < 0.05$, [c]$P < 0.01$ *vs* the control group. VIP: Vasoactive intestinal peptide; CCK-8: Cholecystokinin octapeptide.

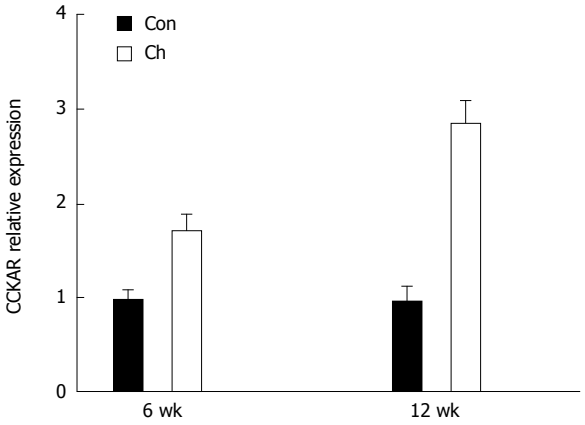

Figure 3 Cholecystokinin-A receptor mRNA expression levels in different groups. CCKAR: Cholecystokinin-A receptor.

The mechanism of cholesterol gallstone-causing diet-induced SOD has not been fully elucidated. Pang *et al*[19] considered that the potential mechanisms of hypercholesterolemia (HC)-induced SOD were the intracellular calcium overload and calcium oscillation abnormality. Szilvassy *et al*[21] suggested that the impairment of the SO relaxation function was related to the alteration of the nitric oxide signal caused by hypercholesterolemia and hypertriglyceridemia. SO motility was controlled by numerous neurotransmitters and gastrointestinal hormones[22]. The most important hormone in the regulation of SO function is CCK[23]. In normal conditions, CCK can contract the gallbladder smooth muscle and reduce tone in the SO to regulate bile flow from the liver through the bile duct into the duodenum. During fasting, hepatic bile enters the gallbladder for storage. Eating initiates gallbladder emptying by neural and hormonal (predominantly CCK) influences. We found serum CCK-8 level was not increased in the 9 and 12 wk groups, meaning that the gallbladder evacuation resistance increased during eating in these groups. Decreases in serum CCK-8 level can cause gallbladder bile stasis, increase the volume of the gallbladder, and induce SOD by increasing the tension of SO.

CCK modulates SO motility mainly *via* excitatory receptors on the smooth muscle and inhibitory receptors in the neural endings[24]. A study in cats[24] showed that the SO relaxation by CCK was abolished by complete denervation induced by tetrodotoxin. Beagle dogs after cholecystectomy[25] showed an increased CBD pressure, and SO relaxation response to CCK was weakened. These findings were thought to be due to destruction of neural pathways with the operation. In our study, we examined the expression of CCK-AR in the SO using quantitative real-time PCR for the first time. We found that the expression levels of *CCK-AR* mRNA increased in SO in the 6 and 12 wk groups. The increase in CCK-AR may result in changes in tension of SO, and, therefore, the excreting resistance of the bile may be enhanced significantly.

VIP, an alkaline intestinal peptide composed of 28 amino acids, can relax gallbladder smooth muscle and decrease gallbladder pressure. In the present study, serum VIP level in the 9 and 12 wk groups was higher than that of the control group. We restated the role of VIP in the formation of cholesterol gallstones that had been stressed in our previous study[12]. We found that the frequency of myoelectric activity decreased markedly in the 9 wk group, the amplitude of myoelectric activity decreased in the 9 and 12 wk groups, and the SO basal pressure and common bile duct pressure increased significantly in the 12 wk group. Elevation of VIP may play an important role in the mechanism of SOD.

Gastrin can increase the SO basal pressure amplitude[12]. Elevation of serum gastrin may be related to SOD with characteristics of high tension in patients with post-cholecystectomy pain[26]. However, we found that serum gastrin was decreased significantly in the 3 and 9 wk groups. This effect may have resulted from changes in diet and may not necessarily be related to the formation of gallstones.

In conclusion, we have indicated that a cholesterol gallstone-causing diet may induce SOD, the increasing tension of SO along with its decreasing activity may play an important role in cholesterol gallstone formation, and expression changes in CCK-AR in SO smooth muscle and serum CCK-8 and VIP may be important causes of SOD. The exact mechanism of cholesterol gallstone-causing diet-induced SOD needs further study. Control

of a cholesterol diet and regulation of SO motility are important in the prevention of cholesterol gallstone formation.

COMMENTS

Background

The sphincter of Oddi (SO) may play an important role in gallstone formation because it is the only way by which bile is discharged into the duodenum. However, there is limited research on how the motor function of the SO works and what mechanism by which stones are formed in the process of cholesterol gallstone formation.

Research frontiers

SO manometry is the only method to directly assess the motor function of the SO and is considered the gold standard for assessing SO dysfunction (SOD). However, manometry changes of SO in cholelithiasis are quite controversial. Another measurement reflecting SO function is recording the myoelectric activity of the SO.

Innovations and breakthroughs

This study showed that a cholesterol gallstone-causing diet can induce SOD. The increasing tension of the SO along with its decreasing activity may play an important role in cholesterol gallstone formation. Expression changes of cholecystokinin-A receptor in SO smooth muscle, serum vasoactive intestinal peptide, and cholecystokinin-octapeptide may be important causes of SOD.

Applications

The study results suggest that a cholesterol gallstone-causing diet can induce SOD, and disturbance of SO motility may play a role in gallstone formation. Control of a cholesterol diet and regulation of SO motility are important in the prevention of cholesterol gallstone formation.

Peer-review

This is an interesting paper. The authors describe the results of a basic study in a guinea pig model to investigate the role of SO motility in cholesterol gallbladder stone formation. They concluded that a cholesterol gallstone causing diet may induce increasing tension and decreasing activity of SO, and these effects could play an important role in cholesterol gallstone formation. This work represents a well-conducted basic study that contributes useful information about the role of SO motility in the process of cholesterol gallstone formation and confirms previous limited data that indicated a significant increase in the base pressure of the SO in rabbits with a cholesterol lithogenic diet.

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ORIGINAL ARTICLE

Case Control Study

Comprehensive risk assessment for early neurologic complications after liver transplantation

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Abstract

AIM: To determine risk factors for early neurologic complications (NCs) after liver transplantation from perspective of recipient, donor, and surgeon.

METHODS: In all, 295 adult recipients were enrolled consecutively between August 2001 and February 2014 from a single medical center in Taiwan. Any NC in the first 30 d post-liver transplantation, and perioperative variables from multiple perspectives were collected and analyzed. The main outcome was a 30-d NC. Generalized additive models were used to detect the non-linear effect of continuous variables on outcome, and to determine cut-off values for categorizing risk. Risk factors were identified using multiple logistic regression analysis.

RESULTS: In all, 288 recipients were included, of whom 142 (49.3%) experienced at least one NC, with encephalopathy being the most common 106 (73%). NCs prolonged hospital stay (35.15 ± 43.80 d *vs* 20.88 ± 13.58 d, $P < 0.001$). Liver recipients' age < 29 or ⩾ 60 years, body mass index < 21.6 or >

27.6 kg/m^2, Child-Pugh class C, history of preoperative hepatoencephalopathy or mental disorders, day 7 tacrolimus level > 8.9 ng/mL, and postoperative intra-abdominal infection were more likely associated with NCs. Novel risk factors for NCs were donor age < 22 or ⩾ 40 years, male-to-male gender matching, graft-recipient weight ratio 0.9%-1.9%, and sequence of transplantation between 31 and 174.

CONCLUSION: NCs post- liver transplantation occurs because of factors related to recipient, donor, and surgeon. Our results provide a basis of risk stratification for surgeon to minimize neurotoxic factors during transplantation.

Key words: Risk; Liver transplantation; Neurotoxicity syndromes; Donor; Learning curve

Core tip: The study uses generalized additive models and logistic regression in statistics to control confounders in the case-control study. We identified 11 risk factors for early neurologic complication after transplantation. From liver recipients' perspective, age < 29 or ⩾ 60 years, body mass index < 21.6 or > 27.6 kg/m^2, Child-Pugh class C, history of preoperative hepatoencephalopathy or mental disorders, day 7 tacrolimus level > 8.9 ng/mL, and postoperative intra-abdominal infection were at risk. From donors' perspective, age < 22 or ⩾ 40 years, male-to-male gender matching, graft-recipient weight ratio 0.9%-1.9% was at risk. From surgeons' perspective, sequence of transplantation between 31 and 174 were at risk.

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INTRODUCTION

Neurologic complications (NCs) are common after solid organ transplantation, especially liver transplantation[1]. While mortality rates decreased with advances in surgical technique and postoperative care in recent years, NCs continue to affect more than one-third of transplanted patients and cause significant morbidity, mortality, and prolonged hospital stay[2,3]. The liver recipient is vulnerable to NCs since many patients have preoperative hepatic encephalopathy, which is a well-known risk factor[3-6]. Moreover, the unfavorable condition of recipients including metabolic, nutritional, and electrolyte imbalances may predispose them to NCs[7,8]. Generally, NCs develop early, with more than 80% of patients developing NCs within 30 d after liver transplantation[6,9]. Encephalopathy is the most frequent etiology[3,5,9-12]. As it is multifactorial in nature, the transplant team should identify patients at risk and avoid predisposing variables during the perioperative period to minimize its incidence.

In the last decade, the risk factors and mechanism of NCs have been investigated in several studies, most of which were retrospective[3,6,9-12]. The majority of these focused on recipients' factors depending on timing of transplant, such as presence of hepatic encephalopathy, etiology of cirrhosis before transplant, hyponatremia, cerebrovascular insult during transplant, immunosuppressant toxicity and central nervous system infection after transplant. However, the nature of liver graft, donor-recipient matching, and experience of surgeon and transplant team also impact outcome, and few studies have investigated NCs after liver transplantation from these perspectives. No study showed how the accumulated experience of the transplant team influences NC development. Besides, the non-linear effect of certain continuous variables, such as age, body mass index (BMI) and graft-recipient weight ratio (GRWR), was not considered in the statistical analysis of prior studies. Our study aimed to identify new risk factors of 30-d NCs after liver transplantation from multiple perspectives. In particular, we performed statistical analyses to explore how the non-linear effect of certain continuous variables increase NC risk.

MATERIALS AND METHODS

Patients

From August 2001 to February 2014, 295 consecutive adult liver transplantation surgeries were performed in the Tri-Service General Hospital (Taipei, Taiwan). All causes of mortality were included in the study except for surgical mortality unrelated to NCs. All procedures were performed after approval of the Ethics Committee. The transplant team included 2 qualified transplant surgeons, 2 fellows and 2 senior residents. The immunosuppressant induction protocol consisted of intravenous methylprednisolone, starting with a large bolus dose during the anhepatic phase, followed by daily tapering off until postoperative day 5, and triple oral immunosuppressants (tacrolimus, mycophenolate mofetil, steroids). Serum trough levels of tacrolimus were checked daily in the first postoperative week to maintain a level of 6-10 ng/mL. If basiliximab was included, the first 20 mg were given during anhepatic phase and the second dose on postoperative day 4, along with halving the steroid dose. Diagnoses of NCs were primarily made by the transplant team based on clinical examination, and ancillary examinations such as computed tomography, magnetic resonance imaging, and electroencephalography. Neurologists were consulted for major NCs. Only new onset of

postoperative neurologic disorders was regarded as NCs. The clinical data and patients' outcomes were retrospectively reviewed from medical charts. The final follow-up was conducted until March 31, 2014.

Data collection

The medical records and transplant database were reviewed after approval of the Institutional Review Board I of Tri-Service General Hospital, National Defense Medical Center (TSGHIRB No.100-05-220). Data were collected from 4 major perspectives: recipient, donor, donor-recipient match, and surgeon. The recipient-related variables were demographic details, comorbidities other than liver diseases, primary liver disease requiring liver transplantation, preoperative Child-Pugh and Model for End-Stage Liver Disease (MELD) scores, any complications of end-stage liver disease, such as ascites, hepatic encephalopathy, variceal bleeding and hepatorenal syndrome. In addition, any mental disorders related to alcoholism or encephalopathy were recorded. The perioperative variables included intraoperative blood loss, length of the procedure, complex vascular anatomy, and splenectomy. The postoperative variables included dose and serum level of tacrolimus, inclusion of basiliximab, and blood chemistry parameters (ammonia, sodium, and magnesium) on postoperative day 7. In addition, any postoperative complications as acute rejection, intra-abdominal infection, or kidney injury requiring dialysis were recorded. The donor and donor-recipient matching-related variables were graft's type and weight, gender match, GRWR, and ABO compatibility. The surgeon variables were surgeon in charge, and the chronological sequence of transplantations.

Outcomes' evaluation

The primary outcome was occurrence of any NCs within 30 d after liver transplantation. We adapted the NC classification from Dhar *et al*[3] as follows: (1) encephalopathy: delirium, psychosis, or alteration of conscious level diagnosed after excluding specific lesions of the central nervous system; (2) seizures; (3) drug neurotoxicity: symptoms subsiding after reduction or discontinuation of immunosuppressants, with severity varying from tremors to imaging-confirmed posterior reversible encephalopathy syndrome; (4) cerebrovascular insults; (5) central nervous system infection; and (6) central pontine myelinolysis: acute para- or quadriparesis, dysphagia, dysarthria, diplopia, loss of consciousness with evident change in serum sodium levels. Patients experiencing "locked-in syndrome", that is, awake but unable to move or communicate, were categorized into "central pontine myelinolysis" if rapid changes in serum sodium level coexisted. If the definite etiology could not be determined, they were recorded separately.

Other outcome data were hospital days and complications other than NCs during the first month. In addition, the complications were classified into minor and major according to the clinical finding and its severity. Minor complications included those that improved spontaneously without sequela within 1 mo, while the others causing functional deficit, brain damage or death were considered as major complications.

Statistical analysis

Statistical analysis was performed using the R 3.1.0 software (R Foundation for Statistical Computing, Vienna, Austria). A P-value of < 0.05 was considered statistically significant.

Descriptive statistics were used to express data as mean ± SD for continuous variables, and frequency and percentage for categorical variables. Differences between the two groups, patients developing NCs and those who did not, were analyzed using Wilcoxon's rank-sum test for continuous variables, and Fisher's exact test for categorical variables.

Some continuous variables had non-linear effect, such as age and BMI. The regression model may have described poor correlation between these variables and the outcome since linearity was usually assumed during the analysis. Generalized additive models (GAMs)[13] for binary response (patients developing NCs and those who did not) were fitted to detect the potential nonlinear effects of continuous covariates; if nonlinearity existed, appropriate cut-off point(s) for discretizing the continuous covariate were selected in the GAM plots. This procedure was carried out using the vgam function (with the default values of smoothing parameters) of the VGAM package in R.

Multiple logistic regression analysis was conducted by fitting a generalized linear model to estimate the effects of predictors on the occurrence of NCs. First, all variables, donor-recipient gender match combinations, and new categorical variables obtained from previous cut-off points for discretizing continuous variables by GAM were selected. Next, a step-wise variable selection procedure went through iteration between the forward and backward steps with both the significant levels for entry and for stay set to 0.15 for being conservative. Then, the best candidate final logistic regression model was identified manually by dropping the covariates with P value > 0.05 one at a time until all regression coefficients were significantly different from 0. The final fitted logistic model was assessed with 3 goodness of fit measures: the estimated area under the receiver operating characteristic curve ($\geqslant$ 0.7 acceptable), adjusted generalized R^2 ($\geqslant$ 0.30 acceptable), and the Hosmer-Lemeshow test ($P \geqslant$ 0.05, larger means better fit). In addition, the variance inflation factor was checked for multicollinearity (no more than 10 for continuous covariates and 2.5 for categorical covariates).

RESULTS

Among the 295 patients, 7 with surgical mortality

Table 1 Baseline characteristics of the 288 consecutive liver transplantation patients

Recipient variables	
Demographics	
Age (yr)	52.3 ± 9.81
Gender (M/F)	213/75
BMI (kg/m^2)	24.4 ± 3.72
Primary diagnosis, *n* (%)	
Hepatitis B	175 (60.8)
Hepatitis C	73 (25.3)
Alcoholic liver disease	77 (26.7)
HCC	138 (47.9)
Severity of liver disease	
Child-Pugh score	10.0 ± 2.49
MELD score	15.0 ± 9.01
Complication, *n* (%)	
Hepatic encephalopathy	132 (45.8)
Ascites	189 (65.6)
Variceal bleeding	114 (39.6)
Comorbidities, *n* (%)	
Diabetes mellitus	103 (35.7)
Hypertension	52 (18.1)
Uremia	14 (4.9)
Mental disorders	99 (34.4)
Blood test before transplant	
Glucose (mg/dL)	129 ± 76.07
Albumin (g/dL)	3.08 ± 0.66
Creatinine (mg/dL)	1.22 ± 1.53
INR	1.62 ± 0.99
T. bilirubin (mg/dL)	7.78 ± 11.19
Platelet (× 10^3/μL)	85.4 ± 53.85
Ammonia (μg/dL)	138.0 ± 97.05
Perioperative variables	
Blood loss (mL)	2896 ± 3409
Operation time (min)	575 ± 122.7
Splenectomy, *n* (%)	87 (30.2)
Complex vascularity, *n* (%)	75 (26.0)
Postoperative variables	
Tacrolimus dose (mg/d)	3.4 ± 1.67
Basiliximab, *n* (%)	51 (18)
Serum level - Day 7	
Tacrolimus level (ng/mL)	7.4 ± 6.24
Ammonia (μg/dL)	69.6 ± 130.8
Sodium (mmol/L)	136 ± 4.92
Magnesium (mEq/L)	1.73 ± 0.34
Donor variables	
Age (yr)	33 ± 11.21
Male gender, *n* (%)	171 (59.4)
Graft weight (g)	872 ± 357.88
Graft type	95/47/142/4
(whole/left/right/S67), *n* (%)	(33.0/16.3/49.3/1.4)
Donor-recipient matching	
GRWR (%)	1.34 ± 0.57
ABO incompatible, *n* (%)	9 (3.1)
Gender match	128/44/86/30
(MM/MF/FM/FF), *n* (%)	(44.4/15.3/29.9/10.4)
Surgeon variables	
Surgeon A/B/C, *n* (%)	234/51/3 (81/18/1)
Sequence of transplantation	1-288

BMI: Body mass index; HCC: Hepatocellular carcinoma; MELD: Model for end-stage liver disease; GRWR: Graft-recipient weight ratio.

unrelated to NCs were excluded: 2 failed to awake due to irreversible brain damage secondary to fulminant hepatitis, and 5 experienced primary graft failure. Finally, 288 patients were enrolled in this study, with 95 receiving deceased donor liver transplantation (DDLT) and 193 receiving living donor liver transplantation (LDLT). Baseline characteristics of the study population are shown in Table 1.

Table 2 Type of complication after liver transplantation *n* (%)

	Event	Minor/major
Neurologic complications	145 (100)	97 (67)/48 (33)
Encephalopathy	106 (73)	88 (83)/18 (17)
Delirium	75 (52)	64 (85)/11 (15)
Psychosis	5 (4)	4 (80)/1 (20)
Change in consciousness	26 (18)	20 (77)/6 (23)
Seizures	10 (7)	0 (0)/10 (100)
Cerebrovascular events	5 (4)	0 (0)/5 (100)
Drug neurotoxicity	10 (7)	5 (50)/5 (50)
Locked-in syndrome	12 (8)	4 (33)/8 (67)
Central pontine myelinolysis	2 (1)	0 (0)/2 (100)
Other complications		
Acute kidney injury	1	
Intra-abdominal infection	9	
Graft failure	2	
Reoperation	31	
Acute rejection	61	
Hepatitis B recurrence	10	
Tuberculosis infection	8	
Cytomegalovirus infection	7	
Total complications	85	

The outcome data are shown in Table 2. One hundred and forty two patients experienced 145 events of NCs, with an overall incidence of 49.3%. The most frequent events were encephalopathy (n = 106, 73%), including delirium (n = 75, 52%), conscious change (n = 26, 18%) and psychosis (n = 5, 4%). The average hospital stay was significantly longer in those with postoperative NCs (35.15 ± 43.80 d *vs* 20.88 ± 13.58 d, P < 0.001).

Variables associated with NCs

The differences between the patients with NC or not are shown in Table 3. Both groups were similar in demographics of recipient and donor, liver graft type, surgeon and sequence of transplantation. For primary diagnosis, alcoholic liver cirrhosis was more common in the NC group (P = 0.001), while hepatocellular carcinoma was prevalent in the control group (P < 0.001). The NC group included patients with more severe liver disease before transplant, more preoperative mental disorders and hepatic encephalopathy, more intraoperative complex vascular anatomy, higher day 7 tacrolimus level, more postoperative complications of acute rejection, intra-abdominal infection, and kidney injury requiring dialysis.

GAMs were fitted to continuous variables with potential non-linear effect on outcome. The selected GAM plots for continuous variables on the NC group are shown in Figure 1. According to the GAM plots, the following scales of variables were associated with higher probability of NCs: age < 29 or ⩾ 60, recipient BMI < 21.6 or > 27.6 kg/m^2, Child-Pugh score >

Table 3 Comparison of variables from multiple perspectives between the neurologic complications and control groups

	NC (n = 142)	No NC (n = 146)	P value
Recipient variables			
Preoperative			
Age (yr)	51.75 ± 10.51	52.77 ± 9.09	0.567
Gender (M/F)	109/33	104/42	0.347
BMI (kg/m^2)	24.65 ± 4.25	24.26 ± 3.11	0.617
Hepatitis B	86	89	1.000
Hepatitis C	30	43	0.136
Alcoholic liver disease	50	27	0.001
HCC	51	87	< 0.0001
Child-Pugh score	10.26 ± 2.26	8.27 ± 2.32	< 0.0001
MELD score	20.3 ± 9.4	14.0 ± 7.4	< 0.0001
Hepatic encephalopathy	91	41	< 0.0001
Variceal bleeding	51	63	0.229
Ascites	106	83	0.002
Mental disorder	66	33	< 0.0001
Serum Albumin (g/dL)	2.95 ± 0.57	3.21 ± 0.72	0.01
Serum T. bilirubin (mg/dL)	10.91 ± 13.16	4.74 ± 7.79	< 0.0001
Perioperative			
Blood loss (mL)	3329 ± 3953	2474 ± 2728	0.081
Operation time (min)	558.98 ± 99.63	579.9 ± 127.0	0.160
Complex vascularity	45	30	0.033
Postoperative			
Day 7 tacrolimus level (ng/mL)	8.23 ± 7.42	6.54 ± 4.69	0.023
Acute rejection	38	23	0.030
Intra-abdominal infection	32	12	< 0.0001
Kidney injury requiring dialysis	18	7	0.021
Donor variables			
Donor age (yr)	32.5 ± 11.4	33.6 ± 11.0	0.234
Donor gender (M/F)	89/53	82/64	0.282
Graft type (whole/left/right/S67)	45/21/74/2	50/26/68/2	0.827
Donor-recipient matching			
GRWR	1.33 ± 0.56	1.35 ± 0.57	0.726
Surgeon variables			
Surgeon A/B/C	116/25/1	118/26/2	0.855
Sequence of transplantation	95.35 ± 69.66	108.12 ± 74.41	0.186

NCs: Neurologic complications; HCC: Hepatocellular carcinoma; MELD: Model for end-stage liver disease; GRWR: Graft-recipient weight ratio; BMI: Body mass index.

9, MELD score between 11 and 20, serum glucose > 8 mmol/L, serum albumin between 2.9 and 4.2 g/dL, serum creatinine between 0.6-1.6 mg/dL, the logarithm of INR > 0.3, platelet count < 70 × 10^3/μL, serum ammonia > 268 μg/dL, day 7 tacrolimus level > 9 ng/mL, day 7 serum ammonia level > 98 μg/dL, day 7 serum sodium level > 143 mmol/L, day 7 serum magnesium level > 1.8 mEq/L, donor age < 22 or ⩾ 40 years, GRWR between 0.9% and 1.9%, and sequence of transplantation between 31 and 174. New categorical variables for regression analysis were obtained after grouping these continuous variables.

After stepwise multiple logistic regression analysis, 12 independent variables were identified as significant in the final logistic regression model (Table 4). Eleven positive predictors of NCs were: recipient age < 29 or ⩾ 60 years, BMI < 21.6 or > 27.6 kg/m^2, Child-Pugh score > 9, preoperative hepatic encephalopathy, history of mental disorder, day 7 tacrolimus level > 8.9, postoperative intra-abdominal infection, donor age < 22 or ⩾ 40 years, male-to-male gender match, GRWR between 0.9% and 1.9%, and sequence of transplantation between 31 and 174. On the other hand, recipients with history of variceal bleeding were less likely to develop NCs (OR = 0.431; 95%CI: 0.221-0.821). The assessment of final logistic regression model showed fair goodness-of-fit (area under the receiver operating characteristic curve = 0.8553 > 0.7 with 95%CI: 0.8119-0.8988; adjusted R^2 = 0.471 > 0.3; Hosmer-Lemeshow goodness-of-fit test P = 0.1446 > 0.05). Furthermore, variance inflation factors for each covariate in the selected final logistic regression model were between 1.075 and 1.582, indicating no multicollinearity.

DISCUSSION

Our results confirmed the prevalence of NCs following liver transplantation, with three-quarters of patients developing encephalopathy. The risk factors were not only from recipient's perspective, but also from perspectives of donor, donor-recipient match and surgeon. Since randomization of subjects is hardly possible in critical conditions as those requiring liver transplantation, studies in this area almost have case-control design. The novelty we have in this study came from better control of confounding effects by flexible statistical tools. The GAM plots enabled more proper stratification of continuous variables on outcome, and the regression analysis controlled the confounding bias by considering multiple covariates all at a time.

The incidence of NCs in the study was relatively higher than in other reports (49.3% *vs* 20%-30%) because of more diagnoses of minor encephalopathy (88/145, 60.7% of overall NC events). In fact, the diagnostic criteria for encephalopathy are not universal among physicians, especially for minor degrees. In our institution we tend to broaden the diagnostic criteria to include any transient delirium, psychosis or consciousness' level change. This enables us to correct metabolic disorders and use immunosuppressants more properly. Our categorical system here is limited by not differentiating anoxic, septic or metabolic etiologies[14] accounting for intra-abdominal infection as an independent risk factor (OR = 5.193, 95%CI: 2.114-13.67).

Preoperative hepatic encephalopathy and mental disorders significantly increased the risk of NCs, with an OR 2.432 and 2.517, respectively. Hepatic encephalopathy, both episodic and active before the operation, is a well-known risk factor[3,4,6,12,14]. It is hypothesized that excess serum ammonia in end stage liver disease interferes with cerebral metabolism, and the condition is not immediately reversed after

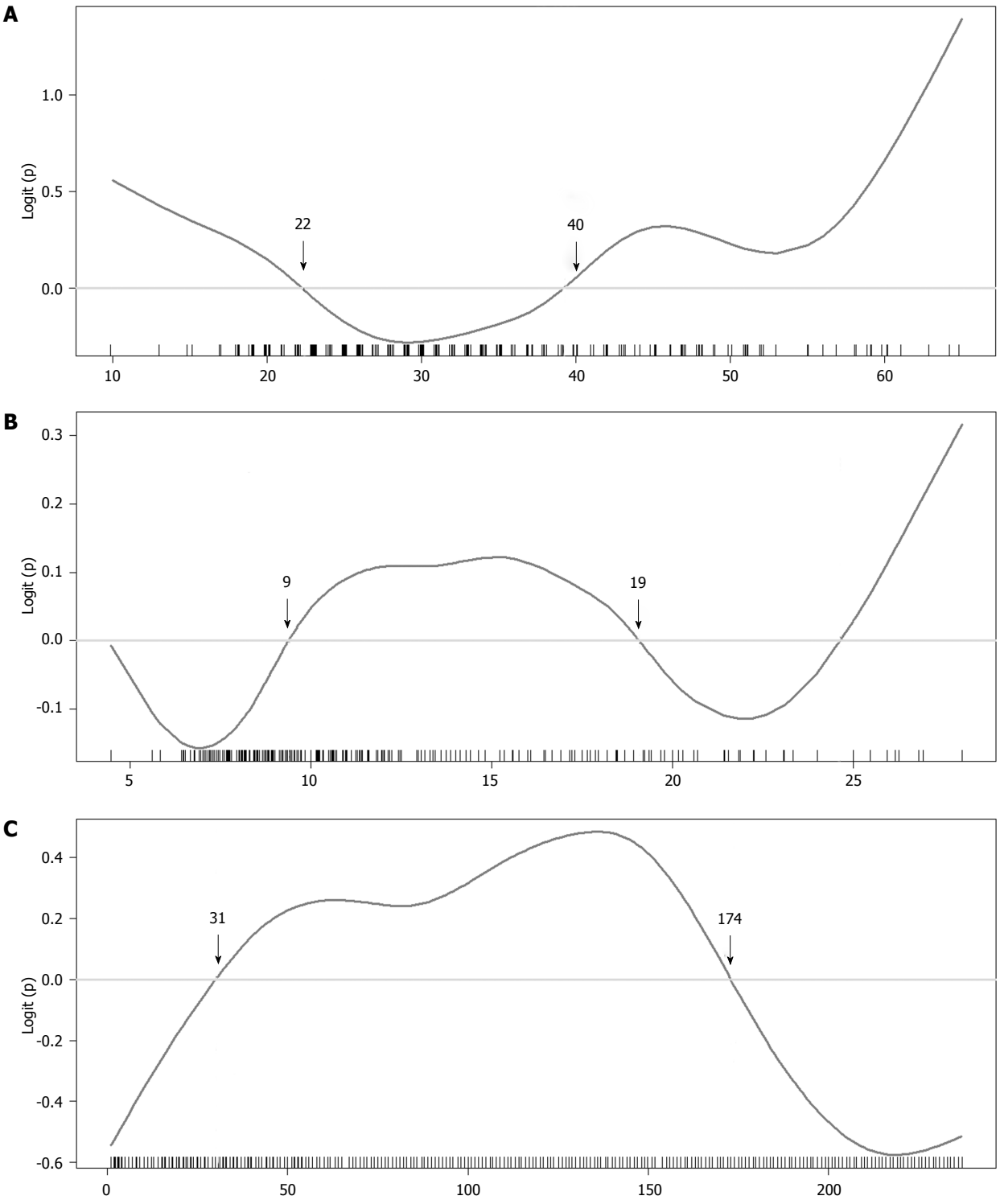

Figure 1 Influence of continuous covariates on neurologic complications resulting from fitting a generalized additive model to the data. A: Donor age; B: Graft-recipient weight ratio; C: Sequence of transplantation.

transplantation. Several investigators have emphasized the importance of comprehensive preoperative neurologic examinations including neuropsychiatric and neuromuscular assessments.

In addition to hepatic encephalopathy, chronic alcohol abuses also contribute to neurotoxicity, which impairs cognitive function especially memory. Alcoholism also renders patients at risk for thiamine deficiency. A prospective study that performed neuropsychological assessments before and after liver transplantation found that recipients with alcoholic etiology had poorer cognitive indexes in memory; in addition, a multivariate analysis determined that alcohol etiology, diabetes mellitus, and hepatic encephalopathy were predictors of poor global cognitive function after transplantation[15]. However,

Table 4 Multiple logistic regression model for neurologic complications

	OR	95%CI	*P* value
Recipient variables			
Preoperative			
Age < 29 or ⩾ 60 yr	2.071	1.024-4.272	0.045
BMI < 21.6 or > 27.6 kg/m^2	1.877	1.007-3.552	0.049
Child-Pugh score > 9 (Class C)	1.509	1.288-1.790	< 0.001
Hepatic encephalopathy	2.432	1.232-4.860	0.011
Variceal bleeding	0.431	0.221-0.821	0.012
Mental disorder	2.517	1.279-5.064	0.008
Postoperative			
Day 7 tacrolimus level > 8.9 ng/mL	1.131	1.068-1.205	< 0.001
Intra-abdominal infection	5.193	2.114-13.67	< 0.001
Donor variable			
Donor age < 22 or ⩾ 40 yr	2.245	1.207-4.271	0.012
Donor-recipient matching			
Male to male gender match	2.36	1.266-4.506	0.008
0.9% < GRWR < 1.9%	1.95	1.069-3.624	0.032
Surgeon variable			
31 ⩽ Sequence of transplantation < 174	2.773	1.479-5.363	0.002

NCs: Neurologic complications; BMI: Body mass index; MELD: Model for end-stage liver disease; GRWR: Graft-recipient weight ratio.

in line with the result of recent studies on LDLT[10,14,16], the current study, using regression analysis adjusted by other covariates, did not find alcoholic liver disease to be a predictor of NCs. The discrepancy may be related to the multifactorial nature of neurologic complications, and it is difficult to attribute to a specific cause of NC in patients with alcoholic liver disease. As a result, clinicians believe that hepatic encephalopathy, which is prevalent in cirrhotic patients, could be the most common cause of NC. A controlled study with the exact definition of alcohol abuse, complete neurologic examination before and after transplantation, standard postoperative care, and exclusion of postoperative complications confounding the result is needed to answer the question.

On the other hand, recipients with history of variceal bleeding were not likely to have NCs after controlling of potential confounders in the regression model. It was somewhat difficult to explain. One possible reason was that our recipients with variceal bleeding tended to receive living donor liver transplantation earlier than those without did, which enabled transplant proceeding under less severe liver disease. Another reason might be related to the shunt ligation procedure which we often done during transplantation blocking portosystemic encephalopathy.

Moreover, patients with NCs had more severe liver disease before transplant. Child-Pugh class C was an independent risk factor, with an odds ratio of 1.509 (95%CI: 1.288-1.790), while MELD score was not when adjusted by serum creatinine level and other factors in regression analysis. These results were consistent with data obtained in previous studies. In a retrospective study by Dhar *et al*[3] investigating factors associated with NCs, preoperative Child-Pugh Class C was a significant variable in the univariate analysis, while only active preoperative hepatic encephalopathy was significant in the multivariate analysis. Another retrospective study by Kanwal *et al*[17] used age, sex and era-matched control group to identify risk factors of post-transplant mental status change. MELD score > 15 was an independent risk factor in both univariate and multivariate analyses and was one of the four factors included in the prediction model. These results are likely to be related to encephalopathy precipitated by higher serum level of endogenous neurotoxic substances (including ammonia) in patients with severe liver disease. Besides, the frequent changes in electrolyte levels, malnutrition and metabolic disorders in decompensated cirrhosis may result in an unfavorable postoperative environment, rendering liver recipients vulnerable to postoperative neurologic disorders.

Age < 29 or ⩾ 60 years and BMI < 21.6 or ⩾ 27.6 kg/m^2 showed increased probability of NCs in the GAM plot, and both were independent predictors in multiple logistic regression model, with odds ratios of 2.071 and 1.877, respectively (Table 4). Advanced age, preoperative cognitive impairment and multiple medical comorbidities were known risk factors for postoperative delirium after various procedures in several studies[18-20]. We believe that age ⩾ 60 years had greater impact on NCs since the mean recipients' age in our study was 52.3 ± 9.81 years, without pediatric recipients.

Underweight and overweight liver recipients had a significant risk for NCs. Extreme BMI values (< 18.5 and ⩾ 40 kg/m^2) are known risk factors for mortality after liver transplantation[21]. Patients are also more likely to develop infectious complications owing to malnutrition, as well as prolonged treatment time, and NCs secondary to of vitamins' and trace elements' deficiencies[22]. Early diagnosis and prompt treatment of nutritional deficits before transplantation may ameliorate encephalopathy and optimize transplant outcome[22,23].

In the GAM plot for postoperative variables, we found that day 7 tacrolimus level > 8.9 ng/mL, serum ammonia level > 98 μg/dL, serum sodium level > 143 mmol/L, serum magnesium level > 1.8 mEq/L were associated with higher probabilities for NCs. In the final logistic regression model, only day 7 tacrolimus level was an independent variable. This is in line with prior study[3].

Neurotoxicity during induction of immunosuppressants, mostly induced by calcineurin inhibitors (CNIs), is common in the early postoperative period. Clinical manifestations may vary from tremors, headache, and visual disturbances to altered mental status. In addition, seizures after transplantation are most often caused by drug neurotoxicity. The incidence is associated with high serum levels of CNIs while the occurrence is not excluded by normal serum CNI level. Coexisting hypomagnesemia and hypocholesterolemia

are risk factors. Rarely, patients using CNIs have posterior reversible encephalopathy syndrome, a radiographic diagnosis characterized by reversible vasogenic edema of the white matter involving the posterior circulation territory on serial magnetic resonance imaging. It is likely to occur in patients with concomitant alcoholic liver disease and infection/sepsis. Management is mainly dose reduction and shift to an immunosuppressant with another mechanism of action; however, these patients may be at risk of acute rejection from the lower maintenance dose[24]. The process of optimizing immunosuppression, aiming to minimize graft rejection and avoid neurotoxicity, may need multidisciplinary teamwork and accumulated experience.

From the donor perspective, variables of graft type, donor age, donor gender, and GRWR were not significantly different between the NC and control groups. While age and GRWR had a biphasic effect on the GAM plots (Figure 1), we adjusted the cut-off values (donor age < 22 or > 40 years, and 0.9% < GRWR < 1.9%) and found that both variables were predictors in the regression analysis. In the literature, few studies investigated the influence of donor and donor-recipient match factors on NCs. Study from the SRTR database have shown an increased risk of graft failure with donor age > 40 years[25]. Prior studies on NC risk factors including donor age and GRWR showed no significant difference of these variables[5,11]. In contrast, our study indicated that risk of NC depends on either extreme donor age, or improper GRWR. In the GAM plots, we could see the probability of NCs decreasing when GRWR was above 1.9, which could suggest better detoxification capabilities of larger grafts. Besides, small size grafts are not likely to increase NC risk, suggesting non-inferiority of small graft when graft inflow is properly controlled[26].

Male-to-male gender match was another independent risk factor for NCs in this study. The association between donor-gender match and neurologic complications has not been reported previously. The impact of gender mismatch on the outcome of liver transplantation is still controversial. Several studies demonstrated that female-to-male gender match is associated with negative outcomes in kidney, heart, lung, and liver transplantations, possibly due to the effect of estrogen and the relative small-for-size of female grafts[27-30]. It is unclear whether other confounding variables specific to male gender play a role, such as prevalent alcoholic liver diseases. Further prospective, controlled studies are needed to confirm the gender match effect on NCs.

The sequence of transplantation was a notable finding. The risk of NCs decreased after 174 transplantations by the team, and the order between 31 and 174 was an independent risk factor for NC development. The lower risk in the first 30 cases could be related to strict patient selection and relatively conservative strategy of immunosuppression. Learning curve effect in liver transplantation has been described, which is affected by the volume of the center or the year of transplant[31-34]. For successful liver transplantation, multidisciplinary team work is needed. The improvement is important, not only in the surgical technique but also in donor selection, timely decision for surgery, standardized postoperative care and optimal immunosuppression. Compared to graft survival rate commonly used in studies with a learning curve, NCs are more likely to be related to non-operative factors[35,36]. It is the environment influenced by perioperative risk factors from all perspectives that results in complications. To avoid NCs, the transplant team may need risk stratification during patient selection and donor matching, correcting risk factors before the operation as much as possible, and should remain watchful during the perioperative period. Ideal immunosuppression aims at minimizing acute rejection and avoids neurotoxicity. It is crucial for the transplant surgeon to recognize this process of development and to conduct interdisciplinary learning as well as continuously improving the patient care quality of the team.

This study has limitations owing to its retrospective design. First, the complications included were those that were identified and reported by the clinician. While serious complications are rarely excluded, clinicians may have missed minor complications that resolved spontaneously without treatment. Second, NCs often manifests as one or more signs, but it is possible that only the most serious ones were documented, leading to misclassification. Third, patients in the control group might have shared features and exposures with those in the NC group. Even if we have considered as many potential important variables as we could, residual confounders are still possible. Despite these limitations, the risk factors identified in the study could be subjective for validation in further prospective, randomized studies.

In conclusion, NCs after liver transplantation occur frequently and affect almost half of liver recipients, with encephalopathy being the most common (73%). In this study, we identified 11 risk factors for neurologic complications. From the recipient's perspective, age < 29 or ⩾ 60 years along with BMI < 21.6 or > 27.6 kg/m^2 significantly increased NC risk, and Child-Pugh score > 9, hepatic encephalopathy, mental disorder, 7-d tacrolimus level > 8.9 ng/mL, intraabdominal infection were complementary to previous studies. Patients with history of variceal bleeding were less likely to develop NCs. Novel risk factors from donor's and surgeon's perspective were donor age < 22 or ⩾ 40 years, male-to-male gender match, GRWR between 0.9 and 1.9 and sequence of transplantation between 31 and 174. Our results provide a basis for risk stratification, which would enable transplant surgeons to weigh the risk during patient selection, control unfavorable factors before operation, avoid neurotoxicity during perioperative period, and perform active surveillance

after operation. The manner in which the experience of transplant team influences NCs should also be taken into account by the team leader or health care manager who would allow the conduction of interdisciplinary education as well as strategies for continuous quality improvement.

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COMMENTS

Background

Neurologic complications (NCs) are common after liver transplantation and result in significant morbidity and mortality. Encephalopathy, the most frequent etiology, is multifactorial in nature. Surgeons should identify patients at risk and avoid predisposing variables during the perioperative period to minimize their incidence. However, known risk factors have been mainly investigated from the recipient's perspective.

Research frontiers

The authors have considered various factors from recipient, donor, donor-recipient matching, and surgeon perspectives that had potential impact on early NC after liver transplantation. They had better control of confounders from the case-control study design using more flexible statistical tools, including generalized additive models for variables, which had a nonlinear effect on outcome.

Innovations and breakthroughs

In addition to risk factors from the recipient perspective that were complementary to those in the literature, novel risk factors discovered in this study were donor age (< 22 or ⩾ 40 years), male-to-male gender matching, graft-recipient weight ratio 0.9%-1.9%, and sequence of transplantation between 31 and 174.

Applications

The results provide a basis for risk stratification for surgeons to minimize neurotoxic factors during transplantation. In addition, the transplant team leader should be aware of the higher risk of neurologic complication during the team's earlier experiences to conduct interdisciplinary education and quality improvement program.

Peer-review

The manuscript is clearly written to describe the results with pretty good discussion. The method section is really well written as a clinical report and can be one of the greatest examples for physician-scientists.

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ORIGINAL ARTICLE

Case Control Study

Relationships between cell cycle pathway gene polymorphisms and risk of hepatocellular carcinoma

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Abstract

AIM: To investigate the associations between the polymorphisms of cell cycle pathway genes and the risk of hepatocellular carcinoma (HCC).

METHODS: We enrolled 1127 cases newly diagnosed with HCC from the Tumor Hospital of Guangxi Medical University and 1200 non-tumor patients from the First Affiliated Hospital of Guangxi Medical University. General demographic characteristics, behavioral information, and hematological indices were collected by unified questionnaires. Genomic DNA was isolated

from peripheral venous blood using Phenol-Chloroform. The genotyping was performed using the Sequenom MassARRAY iPLEX genotyping method. The association between genetic polymorphisms and risk of HCC was shown by *P*-value and the odd ratio (OR) with 95% confidence interval (CI) using the unconditional logistic regression after adjusting for age, sex, nationality, smoking, drinking, family history of HCC, and hepatitis B virus (HBV) infection. Moreover, stratified analysis was conducted on the basis of the status of HBV infection, smoking, and alcohol drinking.

RESULTS: The HCC risk was lower in patients with the *MCM4* rs2305952 CC (OR = 0.22, 95%CI: 0.08-0.63, *P* = 0.01) and with the *CHEK1* rs515255 TC, TT, TC/TT (OR = 0.73, 95%CI: 0.56-0.96, *P* = 0.02; OR = 0.67, 95%CI: 0.46-0.97, *P* = 0.04; OR = 0.72, 95%CI: 0.56-0.92, *P* = 0.01, respectively). Conversely, the HCC risk was higher in patients with the *KAT2B* rs17006625 GG (OR = 1.64, 95%CI: 1.01-2.64, *P* = 0.04). In addition, the risk was markedly lower for those who were carriers of *MCM4* rs2305952 CC and were also HBsAg-positive and non-drinking and non-smoking (*P* < 0.05, respectively) and for those who were carriers of *CHEK1* rs515255 TC, TT, TC/TT and were also HBsAg-negative and non-drinking (*P* < 0.05, respectively). Moreover, the risk was higher for those who were carriers of *KAT2B* rs17006625 GG and were also HBsAg-negative (*P* < 0.05).

CONCLUSION: Of 12 cell cycle pathway genes, *MCM4*, *CHEK1* and *KAT2B* polymorphisms may be associated with the risk of HCC.

Key words: Cell cycle pathway genes; Hepatocellular carcinoma; Single nucleotide polymorphism; Case-control study; Genetic susceptibility

Core tip: We analyzed the effects of polymorphisms of 12 cell cycle pathway genes on the risk of hepatocellular carcinoma (HCC) in a large population of 1019 HCC cases and 1138 controls. The results suggest that *MCM4* rs2305952 CC and *CHEK1* rs515255 TC, TT, TC/TT may be significantly associated with a decreased risk of HCC. *KAT2B* rs17006625 GG may increase the risk of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a serious threat to human health worldwide. It is the fourth most common cancer and the second leading cause of cancer death, with nearly 746000 deaths per year[1]. The incidence of this fatal disease continues to increase. HCC occurrence and development are related to environmental factors, such as infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), cigarette smoking, and alcohol consumption, as well as genetic susceptibility[2-4]. Many studies strongly support that single nucleotide polymorphisms (SNPs) of a variety of genes are associated with HCC[5-7]. However, the genetic mechanism underlying the inherited component of HCC is still not fully understood.

The cell cycle comprises the events that result in the formation of two daughter cells through division of the parent cell. Cell cycle progression, including cell division, is influenced by three different types of molecules: cyclin, cyclin-dependent kinases, and cyclin kinase inhibitors[8]. The associations between the genetic susceptibility of genes which regulate the cell cycle and the risk of cancer are well known. For instance, a polymorphism of the *p27* generates an increased risk of squamous cell carcinoma of the head and neck[9], while polymorphisms of *p27* and *p21* are associated with a significantly increased risk of HCC[10]. Other cell cycle pathway genes implicated in cancer include *cyclinD1*[11], *p53*[12], *CHEK2*[13] and *P21*[14].

During the last several decades, an increasing number of studies have shown an association between genetic variants, mainly in the form of SNPs, and the risk of cancer, including breast[15], colorectal[16], cervical, and vulvar cancers[17], and HCC[18]. Despite investigations into the association of polymorphisms in cell cycle pathway genes with cancer susceptibility[19,20], in the case of HCC this association remains unclear. Therefore, in this hospital-based study we investigated the associations between the polymorphisms of SNPs in cell cycle pathway genes and the risk of HCC.

MATERIALS AND METHODS

Study population

For this case-control study, 2327 subjects were consecutively recruited from June 2007 to December 2013. The 1127 HCC patients were from the Tumor Hospital of Guangxi Medical University and were newly diagnosed with HCC based on biochemical (α-fetoprotein > 20 μg/L) and histopathological examinations. None had undergone radiotherapy or chemotherapy before blood sampling. The 1200 controls from the First Affiliated Hospital of Guangxi Medical University consisted of non-tumor patients admitted within the same period of time. Informed

Table 1 Summarized information of selected single nucleotide polymorphisms in cell cycle pathway genes

Genes	SNPs	Chromosome (position)	Allele	MAF (hapmap-HCB)
MCM4	rs2305952	8 (47962049)	C/T	C = 0.18
YWHAB	rs2425675	20 (44906293)	A/G	A = 0.20
CDKN2A	rs3088440	9 (21968160)	A/G	A = 0.08
TGFB3	rs3917148	14 (75980178)	A/C	C = 0.10
RBL2	rs3929	16 (53490396)	C/G	C = 0.20
RAD21	rs6987652	8 (116870042)	A/G	A = 0.12
SMAD3	rs11556090	15 (67194045)	A/G	G = 0.09
	rs8025774	15 (67190938)	C/T	C = 0.45
KAT2B	rs17006625	3 (20119604)	A/G	G = 0.14
	rs4858770	3 (20152931)	C/T	T = 0.47
MCM7	rs2070215	7 (100099174)	A/G	G = 0.29
	rs2261360	7 (100095370)	A/C	A = 0.37
CDKN1A	rs3176320	6 (36679011)	A/G	G = 0.17
CDC25C	rs3734166	5 (138329634)	A/G	G = 0.38
CHEK1	rs515255	11 (125627250)	C/T	T = 0.44

MAF (minor allele frequency) was derived from HCB population in HapMap website (http://hapmap.ncbi.nlm.nih.gov/). SNPs: Single nucleotide polymorphisms.

consent was obtained from all participants, who also agreed to truthfully complete the questionnaires.

Information and sample collection

General demographic and behavioral information, hematological indices, and data on the patients' age, sex, nationality, drinking habit, smoking habit, HBV infection, and family history of HCC were obtained in face-to-face interviews by trained investigators. Peripheral venous blood was collected in a vacuum EDTA anticoagulant tube from each participant. Genomic DNA was extracted using a standard phenol-chloroform extraction method and stored at -80 ℃.

SNP selection

From the GEO database (https://www.ncbi.nlm.nih.gov/geo/), we found three sets of whole genome expression microarray data which were related to HCC (GSE14520, GSE25097, and GSE12941). A total of 3826 different genes were selected using SPSS 16.0 software (SPSS Inc., Chicago, IL, United States) ($P < 0.05$). Gene ontology classification and pathway enrichment analysis were performed by blast2GO and DAVID (https://david.ncifcrf.gov/) and 40 cell cycle pathway genes involved in the cellular process were chose. The genotype information was downloaded from Hapmap website (http://hapmap.ncbi.nlm.nih.gov/), and functional SNPs were selected using Haploview 4.2 software (Cambridge, MA o2141, United States) based on a function prediction website (http://snpinfo.niehs.nih.gov/snpfunc.htm). Referring to the existing literature on these SNPs with HCC, 15 SNPs in 12 genes (*MCM4* rs2305952, *YWHAB* rs2425675, *CDKN2A* rs3088440, *TGFB3* rs3917148, *RBL2* rs3929, *RAD21* rs6987652, *SMAD3* rs11556090, rs8025774, *KAT2B* rs17006625, rs4858770, *MCM7* rs2070215, rs2261360, *CDKN1A* rs3176320, *CDC25C* rs3734166, and *CHEK1* rs515255) were selected in this study. Information of selected SNPs is shown in Table 1.

SNP genotyping

Before genotyping, each DNA sample was quantified using a UV-Vis spectrophotometer Q5000 (Quawell Technology, Inc., United States) and diluted to a final concentration of 50 ng/μL. SNP genotyping was performed using a MassARRAY system (Sequenom, San Diego, CA, United States) and a matrix-assisted laser desorption ionization-time of flight mass spectrometry method according to the manufacturer's instructions. Primers for PCR and extension were designed using the Assay Designer software package (Sequenom). For quality control, 5% of the samples were randomly chosen and genotyped twice for each locus. Among the 1127 patient samples and 1200 control samples, genotyping was successful for all 15 SNPs in both groups, with a success rate of 92.7%. Thus, all 1019 HCC patients and 1138 controls were included in the final analysis.

Statistical analysis

Statistical analyses were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, United States). Continuous variables were evaluated using the two-sample *t*-test. Categorical variables and genotype frequencies between the HCC patients and controls were compared using the Pearson's χ^2 and Fisher's exact test. Hardy-Weinberg equilibrium (HWE) was evaluated by a goodness-of-fit χ^2 test to compare the observed genotype frequencies with the expected ones. The association between SNP genotypes and HCC risk was estimated using unconditional logistic regression analysis and an odds ratio (OR) with 95% confidence interval (CI). All statistical tests were two-sided. A P-value < 0.05 was considered to indicate statistical significance.

RESULTS

Characteristics of the participants

The 2157 unrelated Chinese subjects enrolled in this study included 881 (86.5%) males and 138 (13.5%) females with HCC. The mean age of these patients was 48.54 ± 11.44 years. The control group consisted of 982 (86.3%) males and 156 (13.7%) females, with a mean age of 48.01 ± 11.5 years. The general demographic characteristics and behavior information on the patients and controls are provided in Table 2. There were no significant differences between the HCC patients and the controls in terms of age, sex, and nationality; however, HCC patients had a significantly higher rate of a positive history of HBV infection, a family history of HCC, smoking, and drinking.

Table 2 General demographic characteristics and behavioral information among hepatocellular carcinoma patients and controls

Variable	HCC patients *n* = 1019	Controls *n* = 1138	t/χ^2	*P* value
Age	48.54 ± 11.44	48.01 ± 11.50	-1.076	0.28
Gender				
Male	881	982	0.013	0.91
Female	138	156		
Nationality				
Han	673	708	3.591	0.17
Zhuang	332	410		
Others	14	20		
Drinking				
Yes	345	145	136.527	< 0.001
No	674	993		
Smoking				
Yes	355	158	130.222	< 0.001
No	664	980		
Chronic HBV infection				
Yes	794	109	1031.687	< 0.001
No	225	1029		
Family history of HCC				
Yes	80	2	86.597	< 0.001
No	939	1136		

HCC: Hepatocellular carcinoma.

Allele frequencies and genotype distribution

In the control group, the genotype frequencies of the 15 SNPs, all but *CDKN1A* rs3176320, were in line with the HWE ($P > 0.05$), which indicated that these study participants were from a homogeneous group. The allele frequencies and genotype distribution of SNPs among the HCC patients and controls from this study are listed in Table 3.

Association analysis of genetic polymorphisms and HCC

The association between SNPs and the risk of HCC was examined using unconditional logistic regression analysis. According to the crude ORs and their 95%CIs, *SMAD3* rs11556090 AG or AG/GG and *MCM7* rs2070215 GG carried an increased risk of HCC when compared with the wild genotype *SMAD3* rs11556090 AA and *MCM7* rs2070215 AA, respectively. Individuals with *CDC25C* rs3734166 GG or GA/GG and *KAT2B* rs4858770 TT had a lower risk of HCC than those with the wild genotype *CDC25C* rs3734166 AA and *KAT2B* rs4858770 CC, respectively. However, the association disappeared after adjusting for age, sex, nationality, smoking, drinking, family history of HCC, and HBV infection. Using individuals with the wild genotype AA as the reference, individuals carrying the GG variant of *KAT2B* rs17006625 had a higher risk of HCC (adjusted OR = 1.64, 95%CI: 1.01-2.64, $P = 0.04$) after adjusting for confounding factors. In addition, compared with the wild genotypes *MCM4* rs2305952 TT and *CHEK1* rs515255 CC, individuals carrying the CC variant of *MCM4* rs2305952 or the TC, TT, TC/TT variants of *CHEK1* rs515255 had a significantly lower risk of HCC (adjusted OR = 0.22, 95%CI: 0.08-0.63, $P = 0.01$; adjusted OR = 0.73, 95%CI: 0.56-0.96, $P = 0.02$; adjusted OR = 0.67, 95%CI: 0.46-0.97, $P = 0.04$; adjusted OR = 0.72, 95%CI: 0.56-0.92, $P = 0.01$, respectively). The associations are shown in Table 4.

Table 3 Allele frequencies and genotype distribution of single nucleotide polymorphisms *n* (%)

SNP	Genotype	HCC patients *n* = 1019	Control *n* = 1138	χ^2	*P* value of HWE
rs2305952	TT	801 (78.61)	883 (77.59)		
	TC	209 (20.51)	238 (20.91)	0.04	0.83
	CC	9 (0.88)	17 (1.49)		
rs2425675	GG	632 (62.02)	724 (63.62)		
	AG	348 (34.15)	374 (32.86)	0.96	0.33
	AA	39 (3.83)	40 (3.51)		
rs3088440	GG	750 (73.60)	813 (71.44)		
	GA	249 (24.44)	300 (26.36)	0.19	0.66
	AA	20 (1.96)	25 (2.20)		
rs3917148	AA	773 (75.86)	882 (77.50)		
	CA	233 (22.87)	235 (20.65)	1.32	0.25
	CC	13 (1.28)	21 (1.85)		
rs3929	GG	619 (60.75)	688 (60.46)		
	GC	349 (34.25)	395 (34.71)	0.03	0.86
	CC	51 (5.00)	55 (4.83)		
rs6987652	GG	743 (72.91)	843 (74.08)		
	AG	251 (24.63)	270 (23.73)	0.38	0.54
	AA	25 (2.45)	25 (2.20)		
rs11556090	AA	622 (61.04)	749 (65.82)		
	AG	352 (34.54)	346 (30.40)	0.15	0.70
	GG	45 (4.42)	43 (3.78)		
rs17006625	AA	526 (51.62)	620 (54.48)		
	AG	412 (40.43)	446 (39.19)	0.48	0.49
	GG	81 (7.95)	72 (6.33)		
rs2070215	AA	465 (45.63)	554 (48.68)		
	AG	424 (41.61)	480 (42.18)	< 0.01	1.00
	GG	130 (12.76)	104 (9.14)		
rs2261360	CC	460 (45.14)	484 (42.53)		
	CA	433 (42.49)	497 (43.67)	2.61	0.11
	AA	126 (12.37)	157 (13.80)		
rs3176320	AA	579 (56.82)	687 (60.37)		
	GA	383 (37.59)	377 (33.13)	5.05	0.02
	GG	57 (5.59)	74 (6.50)		
rs3734166	AA	421 (41.32)	421 (36.99)		
	GA	481 (47.20)	539 (47.36)	0.06	0.8
	GG	117 (11.48)	178 (15.64)		
rs4858770	CC	445 (43.67)	465 (40.86)		
	CT	461 (45.24)	515 (45.25)	0.65	0.42
	TT	113 (11.09)	158 (13.88)		
rs515255	CC	408 (40.04)	411 (36.12)		
	TC	469 (46.03)	553 (48.59)	0.29	0.59
	TT	142 (13.94)	174 (15.29)		
rs8025774	CC	313 (30.72)	335 (29.44)		
	CT	514 (50.44)	547 (48.07)	1.32	0.25
	TT	192 (18.84)	256 (22.50)		

HCC: Hepatocellular carcinoma; SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium

Association between SNPs and HCC risk stratified by behavioral factors

HBV infection, alcohol intake status, and smoking status are important behavioral factors that can

Table 4 Associations between single nucleotide polymorphisms with the risk of hepatocellular carcinoma

SNP	Genotype	OR (95%CI)[1]	*P* value[1]	OR (95%CI)[2]	*P* value[2]
rs2305952	TT	Reference		Reference	
	TC	0.97 (0.79-1.19)	0.76	0.97 (0.72-1.32)	0.85
	CC	0.58 (0.26-1.32)	0.19	0.22 (0.08-0.63)	0.01[a]
	TC/CC	0.94 (0.77-1.16)	0.57	0.89 (0.66-1.19)	0.43
rs2425675	GG	Reference		Reference	
	AG	1.07 (0.89-1.28)	0.49	1.92 (0.71-1.20)	0.54
	AA	1.12 (0.71-1.76)	0.63	0.97 (0.51-1.85)	0.93
	AG/AA	1.07 (0.90-1.28)	0.44	0.93 (0.72-1.20)	0.56
rs3088440	GG	Reference		Reference	
	GA	0.90 (0.74-1.09)	0.29	1.02 (0.76-1.35)	0.92
	AA	0.87 (0.48-1.58)	0.64	1.46 (0.62-3.44)	0.38
	GA/AA	0.90 (0.74-1.09)	0.26	1.04 (0.79-1.37)	0.77
rs3917148	AA	Reference		Reference	
	CA	1.13 (0.92-1.39)	0.24	1.18 (0.88-1.59)	0.28
	CC	0.71 (0.35-1.42)	0.33	1.05 (0.41-2.68)	0.92
	CA/CC	1.10 (0.90-1.34)	0.37	1.17 (0.88-1.56)	0.29
rs3929	GG	Reference		Reference	
	GC	0.98 (0.82-1.18)	0.84	0.97 (0.75-1.26)	0.82
	CC	1.03 (0.69-1.53)	0.88	1.39 (0.80-2.42)	0.25
	GC/CC	0.99 (0.83-1.18)	0.89	1.02 (0.79-1.30)	0.90
rs6987652	GG	Reference		Reference	
	AG	1.06 (0.87-1.29)	0.60	0.92 (0.69-1.23)	0.59
	AA	1.14 (0.65-1.99)	0.66	1.26 (0.55-2.88)	0.59
	AG/AA	1.06 (0.88-1.29)	0.54	0.95 (0.72-1.25)	0.71
rs11556090	AA	Reference		Reference	
	AG	1.23 (1.02-1.47)	0.03	1.11 (0.85-1.44)	0.44
	GG	1.26 (0.82-1.94)	0.29	1.02 (0.54-1.91)	0.96
	AG/GG	1.23 (1.03-1.47)	0.02	1.10 (0.85-1.42)	0.47
rs17006625	AA	Reference		Reference	
	AG	1.09 (0.91-1.30)	0.35	1.07 (0.83-1.38)	0.61
	GG	1.33 (0.95-1.86)	0.10	1.64 (1.01-2.64)	0.04[a]
	AG/GG	1.12 (0.95-1.33)	0.18	1.14 (0.89-1.46)	0.29
rs2070215	AA	Reference		Reference	
	AG	1.05 (0.88-1.26)	0.58	0.95 (0.73-1.24)	0.71
	GG	1.49 (1.12-1.98)	0.01	1.39 (0.93-2.08)	0.11
	AG/GG	1.13 (0.95-1.34)	0.16	1.03 (0.81-1.32)	0.81
rs2261360	CC	Reference		Reference	
	CA	0.92 (0.77-1.10)	0.35	0.84 (0.64-1.09)	0.19
	AA	0.84 (0.65-1.10)	0.21	0.89 (0.60-1.31)	0.55
	CA/AA	0.90 (0.76-1.07)	0.22	0.85 (0.66-1.09)	0.19
rs3734166	AA	Reference		Reference	
	GA	0.89 (0.74-1.07)	0.22	0.92 (0.71-1.21)	0.56
	GG	0.66 (0.50-0.86)	0.002	0.86 (0.59-1.25)	0.43
	GA/GG	0.83 (0.70-0.99)	0.04	0.91 (0.71-1.17)	0.45
rs4858770	CC	Reference		Reference	
	CT	0.94 (0.78-1.12)	0.47	0.96 (0.74-1.24)	0.74
	TT	0.75 (0.57-0.98)	0.04	0.80 (0.54-1.20)	0.28
	CT/TT	0.89 (0.75-1.06)	0.19	0.92 (0.72-1.18)	0.51
rs515255	CC	Reference		Reference	
	TC	0.85 (0.71-1.03)	0.09	0.73 (0.56-0.96)	0.02[a]
	TT	0.82 (0.63-1.07)	0.14	0.67 (0.46-0.97)	0.04[a]
	TC/TT	0.85 (0.71-1.01)	0.06	0.72 (0.56-0.92)	0.01[a]
rs8025774	CC	Reference		Reference	
	CT	1.01 (0.83-1.22)	0.95	0.95 (0.72-1.27)	0.74
	TT	0.80 (0.63-1.02)	0.08	0.94 (0.66-1.32)	0.71
	CT/TT	0.94 (0.78-1.13)	0.52	0.95 (0.73-1.24)	0.69

[1]OR and 95%CI without adjusting for confounding factors; [2]OR and 95%CI after adjusting for age, sex, nationality, smoking, drinking, family history of hepatocellular carcinoma, and HBV infection. [a]$P < 0.05$ was considered statistically significant. OR: Odds ratio; CI: Confidence interval; SNPs: Single nucleotide polymorphisms.

increase the risk of HCC. To account for the role of these factors, a stratified analysis was conducted. Thus, when the patients were stratified, we found that the variant genotype CC of *MCM4* rs2305952 was associated with a significantly lower risk of HCC among HBsAg-positive individuals, non-drinkers, and non-smokers (adjusted OR = 0.25, 95%CI: 0.08-0.80, *P* = 0.02; adjusted OR = 0.19, 95%CI: 0.06-0.60, *P*

Table 5 Stratified analysis on the association between single nucleotide polymorphism genotype and hepatocellular carcinoma risk according to hepatitis B virus infection status

SNP	HBsAg-positive				HBsAg-negative			
	Case	Control	OR (95%CI)[1]	*P* value[1]	Case	Control	OR (95%CI)[1]	*P* value[1]
rs2305952								
TT	624	80	Reference		177	803	Reference	
TC	161	24	0.86 (0.53-1.42)	0.56	48	214	1.05 (0.72-1.52)	0.80
CC	9	5	0.25 (0.08-0.80)	0.02[a]	0	12	-	1.00
TC/CC	170	29	0.76 (0.48-1.21)	0.25	48	226	0.99 (0.68-1.43)	0.95
rs17006625								
AA	411	60	Reference		115	560	Reference	
AG	323	42	1.15 (0.75-1.76)	0.54	89	404	1.07 (0.77-1.48)	0.68
GG	60	7	1.36 (0.59-3.17)	0.47	21	65	1.79 (1.02-3.12)	0.04[a]
AG/GG	383	49	1.18 (0.78-1.77)	0.44	110	469	1.17 (0.86-1.59)	0.32
rs515255								
CC	301	39	Reference		107	372	Reference	
TC	377	52	0.93 (0.59-1.46)	0.75	92	501	0.64 (0.46-0.89)	0.01[a]
TT	116	18	0.81 (0.44-1.50)	0.51	26	156	0.69 (0.36-0.96)	0.03[a]
TC/TT	493	70	0.90 (0.59-1.37)	0.62	118	657	0.63 (0.46-0.86)	0.003[a]

[1]OR and 95%CI after adjusting for age, sex, nationality, smoking, drinking and family history of hepatocellular carcinoma. [a]$P < 0.05$ was considered statistically significant. OR: Odds ratio; CI: Confidence interval; SNPs: Single nucleotide polymorphisms.

Table 6 Stratified analysis on the association between single nucleotide polymorphism genotype and hepatocellular carcinoma risk according to drinking status

SNP	Drinking				Non-drinking			
	Case	Control	OR (95%CI)[1]	*P* value[1]	Case	Control	OR (95%CI)[1]	*P* value[1]
rs2305952								
TT	273	111	Reference		528	772	Reference	
TC	69	33	0.82 (0.44-1.52)	0.53	140	205	1.02 (0.72-1.44)	0.93
CC	3	1	0.51 (0.03-9.74)	0.66	6	16	0.19 (0.06-0.60)	0.004[a]
TC/CC	72	34	0.81 (0.44-1.49)	0.49	146	221	0.91 (0.65-1.27)	0.57
rs515255								
CC	145	56	Reference		263	355	Reference	
TC	154	71	0.69 (0.40-1.19)	0.18	315	482	0.73 (0.54-0.99)	0.05[a]
TT	46	18	1.10 (0.50-2.43)	0.82	96	156	0.56 (0.36-0.86)	0.01[a]
TC/TT	200	89	0.77 (0.46-1.29)	0.31	411	638	0.69 (0.52-0.92)	0.01[a]

[1]OR and 95%CI after adjusting for age, sex, nationality, smoking, family history of hepatocellular carcinoma, and hepatitis B virus infection. [a]$P < 0.05$ was considered statistically significant. OR: Odds ratio; CI: Confidence interval; SNPs: Single nucleotide polymorphisms.

= 0.004; adjusted OR = 0.17, 95%CI: 0.05-0.56, *P* = 0.004, respectively). The variant genotypes TC, TT, and TC/TT of *CHEK1* rs515255 were associated with a significantly lower risk of HCC in HBsAg-negative individuals (adjusted OR = 0.64, 95%CI: 0.46-0.89, *P* = 0.01; adjusted OR = 0.69, 95%CI: 0.36-0.96, *P* = 0.03; adjusted OR = 0.63, 95%CI: 0.46-0.86, *P* = 0.003) and in non-drinkers (adjusted OR = 0.73, 95%CI: 0.54-0.99, *P* = 0.05; adjusted OR = 0.56, 95%CI: 0.36-0.86, *P* = 0.01; adjusted OR = 0.69, 95%CI: 0.52-0.92, *P* = 0.01, respectively). Among smokers, those with the TC variant genotype of *CHEK1* rs515255 had a significantly lower risk of HCC (adjusted OR = 0.54, 95%CI: 0.32-0.93, *P* = 0.03), while among non-smokers the risk was significantly lower in those with the TT variant genotype (adjusted OR = 0.60, 95%CI: 0.39-0.94, *P* = 0.03). In addition, the variant genotype GG of *KAT2B* rs17006625 was shown to carry a significantly higher risk of HCC among HBsAg-negative individuals (adjusted OR = 1.79, 95%CI: 1.02-3.12, *P* = 0.04). These findings are summarized in Tables 5-7 (only significant SNPs are shown).

DISCUSSION

We performed this case-control study to investigate the associations between the 15 SNPs in 12 cell cycle pathway genes and the risk of HCC. The *KAT2B* rs17006625 GG was associated with an increased risk of HCC. Furthermore, this harmful effect was more marked in HBsAg-negative carriers. Conversely, the *CHEK1* rs515255 TC, TT, TC/TT and the *MCM4* rs2305952 CC were associated with a decreased risk of HCC. In addition, the risk was markedly lower for those who were carriers of *MCM4* rs2305952 CC and were also HBsAg-positive and non-drinking and non-smoking and for those who were carriers of the TC,

Table 7 Stratified analysis on the association between single nucleotide polymorphism genotype and hepatocellular carcinoma risk according to smoking status

SNP	Smoking				Non-smoking			
	Case	Control	OR (95%CI)[1]	*P* value[1]	Case	Control	OR (95%CI)[1]	*P* value[1]
rs2305952								
TT	274	124	Reference		527	759	Reference	
TC	77	32	1.05 (0.58-1.91)	0.87	132	206	0.94 (0.66-1.34)	0.75
CC	4	2	0.54 (0.06-4.97)	0.59	5	15	0.17 (0.05-0.56)	0.004[a]
TC/CC	81	34	1.01 (0.57-1.82)	0.96	137	221	0.84 (0.60-1.19)	0.33
rs515255								
CC	145	53	Reference		263	358	Reference	
TC	155	84	0.54 (0.32-0.93)	0.03[a]	314	469	0.81 (0.59-1.10)	0.17
TT	55	21	0.87 (0.41-1.85)	0.72	87	153	0.60 (0.39-0.94)	0.03[a]
TC/TT	210	105	0.61 (0.67-1.02)	0.06	401	622	0.75 (0.56-1.01)	0.06

[1]OR and 95%CI after adjusting for age, sex, nationality, drinking, family history of hepatocellular carcinoma, and HBV infection. [a]$P < 0.05$ was considered statistically significant. OR: Odds ratio; CI: Confidence interval; SNPs: Single nucleotide polymorphisms.

TT, TC/TT genotype of *CHEK1* rs515255 and were also HBsAg-negative and non-drinking. No significant associations were observed between other 12 SNPs and HCC risk.

The cell cycle pathway is one of the most important cellular signaling pathways, as it regulates both cell division and apoptosis. DNA damage readily leads to dysregulation of the cell cycle, which is an essential step in the initiation and development of human malignancies[21-23]. In the present study, we reported that three SNPs in cell cycle pathway genes (*MCM4*, *CHEK1*, and *KAT2B*) were significantly associated with the risk of HCC.

MCM4, a member of the mini-chromosome maintenance family of proteins, which interact with cell cycle checkpoints and recombinant proteins to stabilize the S phase, is essential for the initiation of eukaryotic genome replication[24,25]. Several reports have shown that MCM4 protein is overexpressed in esophageal carcinomas[26], cervical cancer[27], and cervical squamous cell carcinoma[28]. In our study, we found that the polymorphism of *MCM4* rs2305952 was associated with a lower risk of HCC. However, the mechanism of *MCM4* polymorphisms in HCC development remains unclear. Ishimi *et al*[29] found that MCM4 is one of the crucial targets of DNA replication checkpoint and the phosphorylation of MCM4, which is caused by the activation of ATR-CHK1 pathway and CDK2, results in the DNA replication through the inactivation of the MCM4/6/7 complex. It is also found that *MCM4* mutations may cause tumors by affecting the formation of the MCM4/6/7 complex[30,31].

CHEK1 is a mediator of cell cycle arrest in response to DNA damage. In addition to controlling cell cycle progression[32], it regulates DNA repair[33] and coordinates cell survival and death[34,35]. It is reported that CHEK1 plays an important role in the checkpoint of DNA damage and DNA replication through the ATR-CHK1 pathway[36-38]. Lin *et al*[39] performed a meta-analysis to explore the association of *CHEK1* SNPs with breast cancer in patients registered in the database of the Utah Breast Cancer Study. They found that *CHEK1* polymorphisms are significantly associated with the risk of breast cancer. However, in that study common alleles of *CHEK1* are not implicated in breast cancer risk or in the survival of breast cancer patients after meta-analysis. Our results showed an association between the *CHEK1* rs515255 genetic variant and a decreased risk of HCC, after adjusting for age, sex, nationality, smoking, drinking, family history of HCC, and HBV infection. The conflicting results may reflect the different cancers evaluated and/or differences in the study population. This remains to be clarified in further investigations.

KAT2B, also known as *PCAF*, encodes the cofactor PCAF (P300/CBP associated factor) of activated nucleoprotein that is important in cell cycle regulation. KAT2B induces cell cycle arrest and/or apoptosis by regulating p53 and affects the acetylation and stability of E2F1 in the presence of DNA damage[40,41]. Overexpression of PCAF was reported in samples of both central nervous system tumors and Wilm's tumors[42]. In addition, an association between *KAT2B* gene polymorphisms and several human diseases and behaviors has been reported. For example, the *KAT2B* SNP rs9829896 is associated with drug abuse in African Americans[43]. We also found that the risk of HCC was higher in individuals with the *KAT2B* rs17006625 GG genotype than with the AA genotype, after adjusting for age, sex, nationality, smoking, drinking, family history of HCC, and HBV infection.

HBV infection status, drinking status, and smoking status are well known to influence the occurrence and development of HCC[44-47]. Moreover, some genotypes have no effect on HCC risk when considered within a population as a whole, but the subgroup analysis may show an effect on HCC risk among alcohol drinkers and/or smokers[48,49]. Therefore, in our study, we evaluated the role of risk factors such as drinking status and smoking status in a stratified analysis and

found that these environmental factors may interact with the analyzed SNPs.

Our study had several limitations. First, the research population was drawn only from the Guangxi Zhuang Autonomous Region. Whether the results apply to the Chinese population as a whole or to other ethnic groups remains to be seen. Second, because our study used a case-control format, recall bias was difficult to avoid. However, we sought to minimize recall bias by choosing patients newly diagnosed with HCC. Finally, the functional influence of the examined SNPs and the potential mechanisms need to be determined in functional validation tests.

In conclusion, *MCM4* rs2305952 CC and *CHEK1* rs515255 TC, TT, TC/TT may decrease the risk of HCC and *KAT2B* rs17006625 GG may increase the risk of HCC. In addition, we observed an increased risk associated with *KAT2B* rs17006625 GG in HBsAg-negative patients. Furthermore, we also observed a decreased risk associated with *MCM4* rs2305952 CC in HBsAg-positive patients and in also non-drinking patients and non-smoking patients, and with *CHEK1* rs515255 TC, TT, TC/TT in HBsAg-negative patients and in also non-drinking patients. Our results suggest that the genetic variants in the cell cycle pathway genes affect the risk of HCC, however, further studies are needed to confirm the findings.

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COMMENTS

Background

The uncontrollable proliferation of cancer cells is a crucial mechanism in cancer development and progression. Previous studies have shown that polymorphisms of cell cycle pathway genes are associated with cancer. However, their relationship with hepatocellular carcinoma (HCC) is unclear.

Research frontiers

Despite reports of an association between polymorphisms in cell cycle pathway genes and cancer risk, little is known about the relationship between these polymorphisms and HCC risk.

Innovations and breakthroughs

This study enrolled 1127 cases newly diagnosed with HCC and 1200 non-tumor patients. It comprehensively investigated the relationship between 15 SNPs in 12 cell cycle pathway genes and HCC risk.

Applications

Since individuals with the *KAT2B* rs17006625 GG genotype may have an increased risk of HCC, they should be carefully monitored to reduce the occurrence and development of HCC.

Terminology

A single nucleotide polymorphism (SNP) is a variation in the genomic DNA sequence. SNPs in some genes may cause an increased or decreased risk of HCC.

Peer-review

The manuscript is interesting and provides relevant information. The study is a descriptive paper analyzing the polymorphism in HCC in a wide number of patients. The analyses are consistent with the results and the conclusions asserted in the manuscript.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Hepatitis E in Israel: A nation-wide retrospective study

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Abstract

AIM: To investigate the epidemiology, risk factors and clinical course of acute hepatitis E virus (HEV) infection in Israel, an industrialized country.

METHODS: A retrospective analysis of acute HEV cases diagnosed in Israel from 1993 to 2013. Acute HEV was defined by ALT/AST elevation and a positive HEV PCR test or positive anti-HEV-IgM serology. HEV RNA was tested by quantitative reverse transcription PCR. Antibodies to HEV were tested retrospectively using an ELISA assay. HEV-RNA was sequenced using RT-PCR of ORF1 and ORF2 regions to diagnose genotype of the virus. Epidemiologic and clinical data were collected by reviewing the clinical files and through a telephone interview according to a structured questionnaire.

RESULTS: Acute HEV was diagnosed in 68 patients. Among the 59 patients who gave an informed consent and were interviewed, 41% of infections were autochthonous (acquired in Israel), 44% travel-related and 15% imported by foreign workers. Autochthonous patients were mainly females (62.5%), more than half of them pregnant, 26% recalled consuming food or water in areas with poor sanitation, 44% ate non-kosher meat. Fulminant hepatitis developed in 3 patients (5%), all of them were females, two of them with post-partum infection, all acquired the disease in Israel (autochthonous). Israeli travelers with imported infection were predominantly males (73%), acquired the disease in the Indian subcontinent (81%), with 100% reporting having consumed fresh vegetables and drinks with ice cubes abroad. Six patients' sera were tested for genotype and revealed HEV genotype 1 (all cases acquired in the Indian subcontinent).

CONCLUSION: This is the first report which highlights the existence of hepatitis E as an autochthonous infection in Israel. Imported HEV originates mostly from the Indian subcontinent.

Key words: Hepatitis E; Autochthonous; Travel; Foreign workers; Pregnancy; Post-partum; India; Nepal; Indian subcontinent; Israel

Core tip: This is the first epidemiologic report on hepatitis E virus (HEV) in Israel. This report demonstrates the significant presence of autochthonous acute HEV in Israel, serving as an example of occurrence in an industrialized country. Suspected risk factors in Israel include consumption of water and food in areas with poor sanitation, exposure to animals and eating a non-Kosher meat. The high risk group for fulminant hepatitis was pregnant women in their final trimester. Additionally, imported HEV, originating mainly from the Indian subcontinent, is also seen in Israel. Awareness of this disease is important both among physicians in Israel as well as those in other industrialized countries.

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INTRODUCTION

Hepatitis E virus (HEV) infection is currently one of the leading causes of viral hepatitis worldwide[1]. The first epidemic of HEV infection was recognized in India, by a retrospective epidemiologic and serologic survey performed in India and the United States in the early 1980s. This led to the recognition of HEV as a water-borne associated hepatitis. Similar epidemics were subsequently identified in Central and Southeast Asia, the Middle East and North Africa[2-4]. The viral genome was later cloned and sequenced using samples of bile obtained from experimentally infected macaques, and the virus was named Hepatitis E[5-8].

HEV is a single strand RNA virus classified in the genus Hepevirus, family Hepeviridae[9]. Identification of four HEV genotypes has subsequently been used to study the molecular epidemiology of HEV infection worldwide. Genotypes 1 and 2, restricted to humans, are mostly seen in developing countries causing large waterborne outbreaks of hepatitis. Genotype 1 is mostly associated with outbreaks in Asia and Africa, whereas genotype 2 has been detected in Mexico and some African countries[10]. In recent years there has been increasing evidence of an autochthonous HEV infection in industrialized countries, contrasting with previous reports of HEV as only an imported infection from endemic, developing countries. In these cases, genotypes 3 and 4 have been identified, and found to be responsible for sporadic cases of autochthonous hepatitis E in industrialized countries[11-15]. In contrast to genotypes 1 and 2, genotypes 3 and 4 infect not only humans but also other mammalian species such as pigs, boars, and deer[11].

HEV is transmitted predominantly *via* the fecal-oral route, causing a self-limiting disease which resolves spontaneously within 4-6 wk[6]. Occasionally, in immune-suppressed patients and in pregnant women, a fulminant form of hepatitis develops[16]. Chronic infection has been identified almost exclusively among immunocompromised persons, including organ-transplant recipients, patients receiving cancer chemotherapy, and HIV-infected persons[17].

Israel is an industrialized country located amid HEV endemic countries and home to immigrants and refugees from African countries (such as Egypt, Sudan and Ethiopia, all endemic for genotype 1 of the virus). Furthermore, since a portion of Israel's population eats only kosher food (*i.e.,* avoiding pork, game meat or seafood), the index of suspicion for autochthonous cases has been low. Data obtained through old and non-validated immune-assays regarding sero-epidemiology of hepatitis E in Israel revealed a seroprevalence of 2.81% and 1.81% in the Jewish and Arab population, respectively[18]. However, data regarding acute HEV revealed only one case report of acute HEV infection acquired in Israel[19] while the remaining published cases were travel-related[20,21].

The aim of this study was to identify whether there is a change in the epidemiology of acute HEV in Israel, with cases acquired in Israel (autochthonous cases) and to characterize the epidemiology, risk factors and clinical presentation of all documented acute HEV infections in patients diagnosed in Israel.

MATERIALS AND METHODS

Study design

A descriptive, retrospective, nation-wide study.

Patient population

The study included all patients diagnosed with acute HEV infection in Israel from October 1993 to 2013 at the laboratory of the Liver Unit at the Hadassah Medical Center in Jerusalem. During the study period, this laboratory was the only reference laboratory in Israel for HEV detection. Epidemiologic and clinical data were collected by reviewing the clinical files and through telephone interviews in accordance with a structured questionnaire. The study was approved by the Sheba-Medical Centers' institutional review board.

Case definitions

"*Definite acute HEV*" was defined as acute hepatitis manifested by ALT/AST elevation and a positive HEV PCR (polymerase chain reaction) test or positive anti-HEV-IgM serology. "*Probable acute HEV*" was defined as acute non-A, non-B, non-C hepatitis with negative HEV PCR and negative anti-HEV-IgM serology but positive for anti-HEV-IgG serology, where serum samples were taken later in the course of the disease, and with a clinical course that fit HEV infection, and no other proven etiology. "*Fulminant hepatitis*" was defined as a rapid development of acute liver injury with evidence of coagulation abnormality, an international normalized ratio (INR) > 1.5, and any degree of hepatic encephalopathy in a patient without pre-existing liver disease; and after exclusion of the conventional etiologies for acute liver failure[22].

Laboratory tests

Serologic detection of anti-HEV antibodies: Antibodies to HEV (IgG and IgM) were tested using an ELISA micro-titer plates assay [DS-EIA-anti-HEV-G, DS-EIA-anti-HEV-M, DSI S.R.L. Serronno (VA), Milan, Italy] according to the manufacturer's instructions. Micro-titer plates were coated with HEV peptides able to detect all four genotypes of HEV. Ten μL serum samples were diluted 1:10 with 90 μL diluent and incubated for 30 min at 37 ℃ and rinsed three times, followed by a second incubation with 100 μL conjugated antibodies for another 30 min at 37 ℃. After rinsing three times, 100 μL substrate was incubated for 30 min at room temperature. Finally, 100 μL stop solution was added followed by reading the plates at a 450 nm wave length. Sample reading > 0.2 OD were considered as positive.

A pangenotypic evaluation by CDC of 6 serologic assays for IgM against HEV identified the assay manufactured by diagnostic systems, which was used in this study, as having the best performance characteristics. Its diagnostic sensitivity and specificity were 98% and 95.2%, respectively[23].

We used an assay for the detection of IgG against HEV from the same manufacturer, with a sensitivity of 100% and specificity of 97.5%[24].

Detection of HEV-RNA by Taqman real time PCR: Detection of HEV RNA was performed by quantitative reverse transcription PCR (qRT-PCR)[25]. RNA was extracted from 200 μL serum with TRI reagent (Bio Lab), then diluted in 10 μL DEPC water. A 10 μL RNA aliquot was used for one step RT-PCR assay in a final volume of 20 μL. The region used for the real time assay is a highly conserved region, junction of ORF's 2/3 of HEV: HEV Forward primer GGTGGTTTCTGGGGTGAC, HEV Reverse primer AGGGGTTGGTTGGATGAA, HEV Probe FAM-TGATTCTCAGCCCTTCGC-BHQ.

HEV sequence: To define HEV genotype in the sera samples, we ran reverse transcription polymerase chain reaction (RT-PCR) of two regions of the virus: ORF1 and ORF2. PCR products were sent for cleaning and sequencing in a service laboratory (HY-lab). We used the programs CHROMAS and CLUSTAL in order to analyze the sequences.

Statistical analysis

Quantitative variables are presented as mean ± SD or as medians and range. Qualitative variables are presented as frequencies and ratios (percent). The χ^2 and the Fisher's exact tests were applied to assess associations between two qualitative variables. The comparison of quantitative variables between two independent groups was carried out using the two-sample *t*-test or the non-parametric Mann-Whitney test. All tests applied were two-tailed, and a *P*-value of 5% or less was considered statistically significant.

RESULTS

During the years 1993 to 2013, 651 patients with presumed acute non-A, non-B, non-C hepatitis were tested for HEV in Israel. Acute HEV was diagnosed in 68/651 patients (10.45%). Among them, 61 patients (90%) were classified as having "definite acute HEV" confirmed by a positive HEV-RNA PCR result (n = 50) or positive anti-HEV-IgM serology (n = 10). One patient had a positive PCR result from a stool sample taken abroad. Probable acute HEV was diagnosed in 7 patients.

Altogether the cohort of acute HEV infection included 68 patients, 58.8% male, with a mean age of 39.4 years. The greatest number of patients were between the ages of 17-40 years (63.5%). Comparing acute HEV positive patients with non-A-non-B-non-C-non-E acute hepatitis patients revealed no significant differences in gender or age distribution (Table 1).

Among this cohort of 68 patients with a history of acute HEV, 59 patients gave an informed consent and

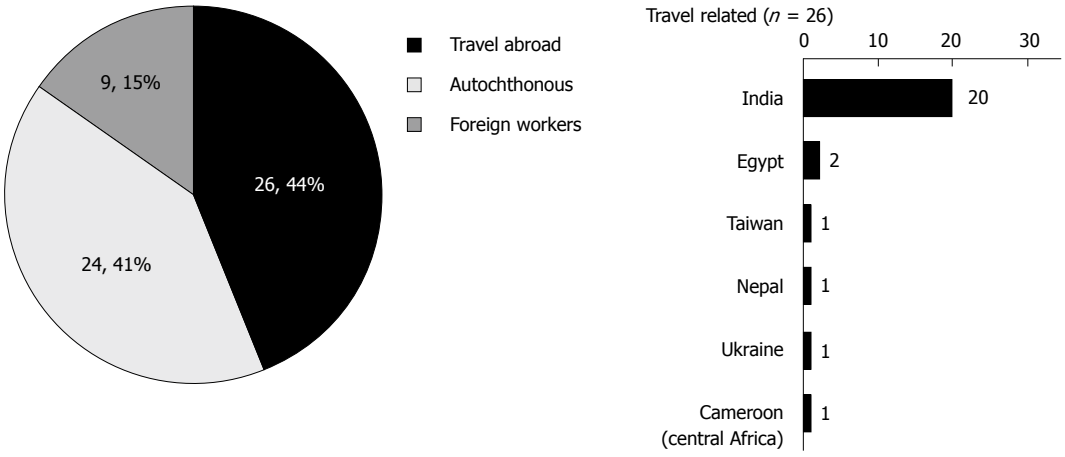

Figure 1 Hepatitis E infection in Israel according to place of acquisition (*n* = 59). Foreign workers origin: Nepal (*n* = 5), China (*n* = 1), 3 unknown.

Table 1 Demographic characteristics of patients with acute hepatitis E *vs* non A-B-C-E acute hepatitis

	Acute hepatitis	
	Non A-B-C-E hepatitis[1] *n* = 583	Hepatitis E[1] *n* = 68
Gender		
M	51.0%	58.8%
F	49.0%	41.1%
Age (yr)		
0-16	29 (7.0)	1 (1.6)
17-40	206 (49.8)	40 (63.5)
41-60	132 (31.9)	13 (20.6)
> 60	47 (11.4)	9 (14.3)
Mean	38.96	39.38
Range	0-86	16-87

[1]Comparing the groups above in parameters of gender and age reveled non-significant *P*-value.

were further evaluated. Thirty five patients (35/59, 59%) had "imported" HEV; among them 26 cases (26/59, 44%) were travel-related HEV infections in Israeli patients and 9 cases (9/59, 15%) were diagnosed in foreign workers from HEV endemic countries (Figure 1). The majority (80%) of travel-related HEV cases were acquired in the Indian subcontinent. Finally, 41% of the patients (24/59) did not travel abroad and had no contact with people from endemic areas, and are therefore defined as "autochthonous HEV".

There was a trend of an increasing number of cases diagnosed with acute hepatitis E throughout the study years in both the travel-related and autochthonous groups (Figure 2).

Autochthonous HE

This group consisted of 24 patients, predominantly female (15/24, 62.5%), with a mean age of 42 years old (SD-15, range: 15-69 years old) and without any contact with a foreign worker in Israel. There were, however, 26% (5/19) who recalled consuming food or water from rural settlements and areas of low sanitation (the West Bank, Bedouin villages) during the 6 wk before the onset of symptoms. Other probable risk factors for HEV infection are summarized in Table 2; 44%, (8/18) ate non-kosher meat (14% ate raw meat, 10% consumed sea food); 40% (8/20) reported contact with animals (cats, dogs, chicken, parrots, geese, fish, guinea pigs, horses or a monkey). Five out of the 24 with autochthonous infections (21%) had chronic liver disease before acquiring HEV (chronic HCV, HBsAg carrier, cystic fibrosis of liver or autoimmune hepatitis). Four of them were diagnosed by positive molecular test (PCR), and one by positive anti-IgM serology for HEV. Eight percent (2/24) received immune suppressing medications (Corticosteroids, Azathioprine, Mycophenolate Moftil and Tacrolimus). Among the female patients, 53% (8/15) were pregnant or post-partum at the time of clinical presentation.

Fifteen of the patients with autochthonous HEV infection (15/24, 62.5%) were diagnosed by detection of HEV-RNA in their serum. Unfortunately, we were unable to retrospectively test for the genotype in this group of autochthonous patients due to a breakdown in refrigeration.

Travel related HEV

This group consisted 26 patients, predominantly male (19/26, 73%), with a mean age of 37.38 years, SD-16.7. Among the females, 1 out of 7 was pregnant (14.3%). Sixty four percent (16/25) developed symptoms after returning to Israel, with a mean time elapsing before symptoms of 16 d (range: 2-28 d). Duration of travel was on average 62.5 d (range: 3-240 d). Thirty six percent (9/25) were symptomatic before flying back to Israel, and among those patients, the average duration of travel was 6.5 mo (range: 1-24 mo).

Behavior during travel, possibly contributing to risk of infection is summarized in Table 3. As described, the vast majority had contact with suspected contaminated water and raw vegetables. The entire group consisted

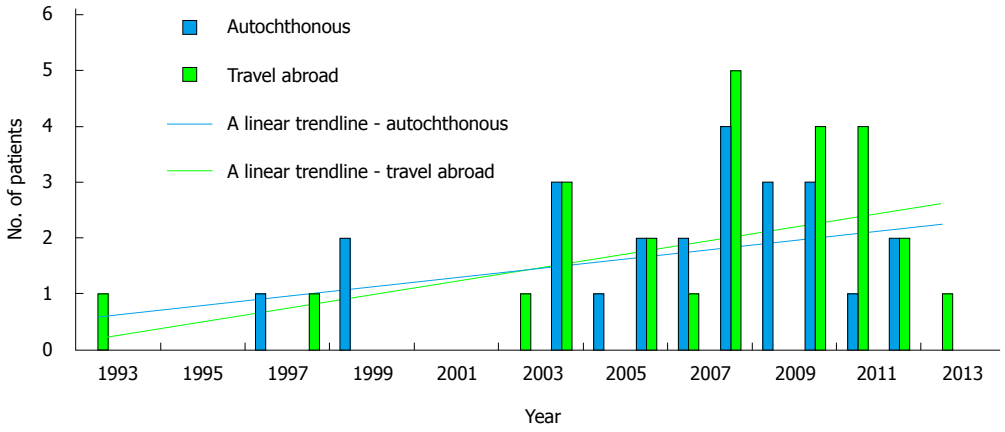

Figure 2 Number of cases of acute hepatitis E virus infection in travel related and autochthonous cases.

Table 2 Clinical characteristics and potential risk factors for hepatitis E virus in patients with autochthonous and travel-related hepatitis E virus infection[1]

Character		Autochthonous infection (n = 24)	Travel related (n = 26)	P value[2]
Demography	Gender: M	9 (37.5)	19 (73.1)	0.011
	Age: mean	41.58	37.38	0.358
	Range	15-69	20-74	
Potential risk factors: food related	Eating non-kosher meat	8 (44.4)	22 (81.8)	0.014
	Eating raw meat	3 (14.3)	3 (13.0)	1.000
	Eating sea-food	2 (10.0)	6 (28.6)	0.238
	Consuming food/water from areas with poor sanitation	5 (26.3)	2 (12.5)	0.415
	Contact with animals[3]	8 (40.0)	8 (34.8)	0.724
Potential risk factors: others	Pregnancy	8 (53.3)	1 (14.3)	0.165
	Immunosuppression[4]	2 (8.3)	0 (0.0)	0.225
	Chronic liver disease	5 (20.8)	1 (3.8)	0.064
Clinical data	Time from onset of symptoms to diagnosis (d)	59.13 (n = 15)	25.21 (n = 19)	0.009
	Duration of symptoms (average weeks)	5.94 (n = 18)	4.08 (n = 20)	0.149
	Hospitalization (percent of patients)	68.2% (15/22)	20 (80.0)	0.345
	Duration of hospitalization (d)	22.15 (n = 13)	11.11 (n = 19)	0.195
Laboratory tests (average)	Bilirubin mg/dL (STD)	10.95 (10.84)	9.24 (5.93)	0.813
	GPT (ALT) U/L (STD)	1169.3 (1279.4)	2446.4 (1604.3)	0.043
	GOT U/L(STD)	1311.7 (2114.6)	1540.4 (1412.7)	0.436
	ALKP (STD)	566.5 (986.1)	205.6 (54.2)	0.673
	GGT U/L (STD)	470.0 (625.1)	232.2 (243.3)	0.730
	LDH U/L (STD)	2613 (6400.5)	1503 (1511.4)	0.440
	ALB g/dL (STD)	3.3 (0.94)	3.9 (0.42)	0.241
	INR (STD)	1.42 (0.8)	1.21 (0.2)	0.791
Outcome	Self-limited	20 (86.9)	26 (100)	0.085
	Fulminant hepatitis	3 (13)	0 (0)	
	Chronic hepatitis	0 (0)	0 (0)	

[1]Excluding foreign workers with acute HEV; [2]Mann-Whitney *U* Test, the χ^2 and the Fisher's exact tests were applied as detailed in the text (Methods); [3]Contact with animals *i.e.,*: cats, dogs, chicken, parrots, goose, fish, guinea pigs, horses, and monkeys; [4]Immune-suppressed patients: (1) Forty five years old woman treated with immune-suppressed therapy (Imuran) for autoimmune hepatitis; and (2) 19 years old woman treated with immune suppressed therapy after liver and kidney transplantation due to cystic fibrosis.

of healthy travelers, none took immunosuppressive drugs or had a known systemic disease associated with immunosuppression, with the exception of one patient who had chronic hepatitis B, diagnosed in the past. Foreign workers from endemic countries (n = 9) were not included in the travel-related HEV group.

Twenty of the patients with travel-related HEV (20/26, 77%) were diagnosed by detection of HEV-RNA in their serum. Among them, we were able to sequence the HEV genome in six of the patients with available sera. All cases revealed genotype 1.

Comparing patients with travel-related infection and autochthonous infection revealed that the autochthonous patients were predominantly female (62.5% *vs* 27%, $P < 0.05$), had lower levels of alanine aminotransferase on presentation (mean 1169 U/L *vs* 2446 U/L, $P < 0.05$) and the time from onset of symptoms to diagnosis was more than twice as long (59 d *vs* 25 d; $P < 0.01$) (Table 2). Although there was no significant statistical difference in outcome between the two

Table 3 Potential risk factors for travel-related hepatitis E virus infection

Risk factor	No. of patients (total: n = 26)	Incidence
Gender - M	19/26	73.07%
Pregnancy	1/7	14.28%
Chronic liver disease[1]	1/23	4.30%
Immunosuppression	0/23	0.00%
Eating non-kosher meat	18/22	81.81%
Eating raw meat	3/23	13.04%
Eating sea-food	6/21	28.57%
Drinking tap water abroad	8/23	34.78%
Consuming drinks with ice cubes	23/23	100.00%
Brushing teeth with tap water	20/23	86.95%
Eating fresh vegetables abroad	23/23	100.00%
Bath in fresh water	15/23	65.21%
Contact with animals[2]	8/23	34.78%
Contact with travelers having similar symptoms	2/25	8.00%

[1]Chronic hepatitis B virus; [2]Contact with animals *i.e.*,: cat, dog, chicken, parrot, goose, fish, guinea pig, horse and monkey.

groups (due to small sample size), the results may indicate that patients with autochthonous HEV are more prone to fulminant hepatitis than patients with travel-related HEV infection (13% *vs* 0% respectively). There were no significant differences in age, hospitalization rate and symptom duration between the groups.

Pregnant women

In this study, there were nine cases of HEV in pregnant women (9/28 women in the study, 32%). Only one of the women was a returning traveler from India, while the rest were diagnosed with autochthonous infection (8/9, 88.9%). Fulminant hepatitis occurred in two of the pregnant women (2/9, 22%), without fatality or vertical transmission. A detailed description of this cohort, together with other cases of acute HEV in pregnant women from Western countries, was published recently[26].

In the entire cohort of the Israeli patients, the most common signs and symptoms were fatigue and non-obstructive-jaundice in 84% (26/31) and 78% (40/51) of patients respectively; 61% (11/18) of patients developed abdominal pain and 41% (19/46) had fever (> 37.5 ℃). Other complaints were nausea (40%, 19/48), diarrhea (21%, 10/48) and headache (24%, 11/45). Nine percent (4/43), developed neurologic abnormalities including epilepsy, encephalopathy or loss of consciousness. Admission to hospital in Israel was reported by 78% of patients, for a mean duration of 15.6 d (range: 1-84 d, SD-19.4). One Israeli traveler was admitted to an Indian hospital while abroad where HEV-RNA was identified in a stool sample by PCR.

Results of conventional liver tests and INR at presentation or later were retrieved in only 22 patients (Table 2). The rate of bilirubin level varied between 0.39 to 27.9 mg/dL. No clinically significant bleeding disorders were reported although coagulopathy was recorded in 41% of the patients (7/17), who presented with prolonged INR up to 3.49. Hepatocellular injury manifested in ALT elevation was significantly higher in patients with travel-related hepatitis compared to autochthonous infection (mean 2246.4 U/dL *vs* 1169.3 U/dL respectively, $P < 0.05$).

Ninety four percent (46/49) of the patients with HEV infection had self-limiting disease (Table 2). Among the travelers, none of the patients had a chronic or fulminant course of infection. This included a pregnant woman with HEV infection in her third trimester, who had a self-limiting infection, without any pregnancy or fetal related complications. In contrast, among the patients with autochthonous infection, three patients (3/23, 13%) developed fulminant hepatitis. All three were females in their reproductive years, two of them post-partum and the third had cystic fibrosis.

DISCUSSION

The current study was designed to review retrospectively the epidemiology and clinical outcome of acute HEV cases diagnosed in Israel in the past 20 years. During this period, 68 patients were diagnosed with acute HEV infection, and from those patients, 59 gave their consent for evaluation. In this cohort, 44% were travel related, 15% affected foreign workers and surprisingly 41% were autochthonous infection. This is the first study which recognizes the existence of a relatively large cohort of autochthonous HEV infections in Israel, while previous reports suggest that almost all cases of hepatitis E were travel related as summarized in Table 4.

Recently, reports on autochthonous HE in industrialized countries have become more frequent. The distribution of autochthonous *vs* travel-related cases varies widely between different countries[13,14,27-30], as can be seen in Table 5. In Israel about half of the cases are autochthonous.

As shown in this report (Figure 2), the number of HEV cases increased during the study years in both travelers and the autochthonous groups. This may reflect an increase in infection rates, but may also be the consequence of improved diagnostic tools and growing awareness among physicians in Israel of HEV infection.

Previous studies in industrialized countries linked sporadic cases of HEV to consumption of undercooked pork[1,12,31], raw game meat, shellfish[32] and to blood transfusions[33,34]. In autochthonous HEV in Israel, the most frequent risk factors included eating non-kosher meat and being exposed to animals. Furthermore, about a quarter of the patients reported that during the 6 wk that preceded the symptoms, they consumed food and/or water from the West Bank or other rural areas in Israel known to have relatively lower hygienic standards as compared to the rest of the country.

Table 4 Summary of the published case reports of acute hepatitis E virus in Israeli patients

Ref.	Year of follow-up	No. of patients	Travel related/autochthonous	Diagnosis
Schwartz *et al*[20]	1992-1998	5	Travel related (all cases acquired in the Indian subcontinent)	Serology tests (Abbott Laboratories, Abbott Park, IL, United States)
Lachish *et al*[21]	1997-2012	19	Travel related (84% acquired in the Indian subcontinent)	Molecular or Serology (IgM/IgG) tests (EIA, Abbott Laboratories, Abbott Park, IL, United States)
Mechnik *et al*[19]	2001	1	Autochthonous	Molecular test (HEV-RNA pos. in serum sample)

HEV: Hepatitis E virus.

Table 5 Hepatitis E virus in industrialized countries[1]

Ref.	No. of patients	Travel related	Autochthonous
Norder *et al*[27]	248	88.3%	11.7%
Romanò *et al*[14]	134	81.3%	18.7%
Ramalingam *et al*[28]	16	62.5%	37.5%
Israel (current paper)	68	44.0%[2]	41.0%
Drobeniuc *et al*[29]	26	42.0%	58.0%
Chalupa *et al*[13]	49	3.90%	96.1%
Mansuy *et al*[30]	62	3.20%	96.8%

[1]A Literature review of the prevalence of HEV infection in industrialized countries[13,14,27-30]; [2]the rest 15% were foreign workers. HEV: Hepatitis E virus.

The other major risk group susceptible to HEV was the Israeli travelers (44% of acute HEV in Israel). In a prospective observational study from Israel, 1% of ill-returning Israeli travelers were diagnosed with acute hepatitis (1997-2012), while HEV has become the most common hepatitis, with a prevalence of 39%, of all hepatitis cases. In the aforementioned study, 84% of the travel-related HEV cases were "imported" from the Indian subcontinent[21]. In this study, 80.7% of travel associated cases were acquired in the Indian subcontinent. In travel-related HEV, in contrast to autochthonous HEV, the majority of patients were young adult males (73% male, average age of 37 years). Only a minority of patients had chronic liver disease (3.8%). Regarding risk behaviors during travel in this population, although the majority of patients followed pre-travel instructions and did not drink tap water abroad, all patients ate raw vegetables, used ice cubes, brushed their teeth with tap water and bathed in fresh water (Table 3). A control group of travelers without HEV was not available for this study.

The comparison of characteristics of autochthonous and travel-related acute HEV infection, revealed significant differences between the two groups (Table 2); the majority of infected travelers were male, while in the autochthonous group, the majority were female. Time to diagnose was significantly longer in the autochthonous group (on average, more than twice longer), suggesting a delay in diagnosis. ALT levels were significantly higher in infected travelers than in patients with autochthonous infection.

HEV is generally a self-limiting disease, although on rare occasions it can be fulminant[6,16]. In the present study, most of the patients with acute HEV infection had a self-limited disease. Patients who had fulminant hepatitis reported one of the following risk factors: pregnancy (including post-partum women), immune-suppression or chronic liver disease.

There is scarce evidence about the mortality of pregnant women with acute HEV infection in industrialized countries[35-38]. Our previous report indicated that in an industrialized country such as Israel, pregnant women had a high risk of fulminant hepatitis during their final trimester (2/9, 22.2%), though with no mortality or vertical transmission[26].

Patients with underlying chronic liver disease who develop hepatitis E have a poor prognosis, as they frequently develop acute or sub-acute liver failure[17]. In a study of a large cohort of patients in India with chronic liver disease, patients who developed HEV associated liver failure had a significantly worse prognosis than patients who decompensated due to other causes. In this cited cohort, the 12-mo mortality with HEV infection reached 70%[39]. In industrialized countries, smaller studies have also shown a poor prognosis for HEV infected patients with underlying chronic liver disease[40,41]. In our current study, evaluation of the entire group of 69 patients with acute HEV infection, revealed 7 patients with underlying chronic liver disease (chronic HCV, chronic HBV, cystic fibrotic liver, autoimmune hepatitis, idiopathic cirrhosis). Six of the patients survived and were interviewed and only one patient with chronic HBV infection and cirrhosis died 4 years after the diagnosis of chronic hepatitis B and 3 years after the diagnosis of acute HEV infection.

The differential diagnosis of autochthonous HEV infection includes drug induced liver injury (DILI). Two reports from the United Kingdom and the United States revealed that 21.4% and 3%, respectively, of the patients with an initial diagnosis of DILI, had autochthonous HEV. Such misdiagnosis is particularly common in elderly populations with autochthonous HEV taking various potentially hepato-toxic medications and herbs[42,43]. In the present cohort, two patients treated with Isoniazide and Ketoconazole who presented with acute hepatitis, were initially misdiagnosed as DILI and subsequently were found positive for HEV RNA by PCR.

In the industrialized world, HEV genotypes 3 and 4 are responsible for sporadic cases of autochthonous HEV infection, where the disease is zoonotic. Imported,

travel related, cases are usually genotypes 1 and 2. In this study, genotyping of 6 travel-related cases with available serum revealed genotype 1 in all cases, a genotype which is prevalent in India where all cases were acquired. One case of hepatitis E in a foreign worker from Nepal who spent one month in Israel, revealed genotype 1 as well.

Israel is located amid countries endemic for HEV, with evidence for genotype 1 in most of the cases reported from those countries. For example, isolation of HEV circulating in Egypt, a country with HEV seroprevalence among the highest in the world, revealed HEV genotype 1 in acute HEV cases, both from rural and urban areas[44-46]. Studies of North African countries revealed autochthonous infection with genotype 1 as well[2]. Data on the HEV-genotype circulating in Lebanon, Syria, and Jordan is lacking.

Unfortunately, we were unable to retrospectively test for the genotype in the autochthonous group of patients. The cause included the retrospective design of this study which did not enable storage of large volumes of suspected sera and the physical state of some of the stored sera which were thawed and frozen several times prior to an attempt of sequencing.

However, a recent Israeli study confirmed the presence of HEV in 8.2% of the 169 sewage samples tested throughout the country during 2013-2015[47]. Sequencing revealed genotype 3 in all sewage samples positive for HEV. These data along with the evidence for acute autochthonous HEV in Israel shown in the present study, suggest autochthonous infection with HEV genotype 3, as seen in Europe and in contrast to the circulating HEV genotype in Israel bordering countries.

The present study has several limitations. The information of exposure related to different risk factors, was based on patients' recollections of events that happened years before the survey, and consequently might be biased. And as mentioned, we were unable to retrospectively test for the HEV genotypes in the autochthonous group of patients. Thus phylogenetic analysis of HEV confirmed autochthonous cases in Israel remains an undertaking for the future.

This report, along with previous reports from Western countries mentioned above, provides evidence for the presence of sporadic autochthonous or travel-related acute HEV cases in the industrialized world. At present, treatment of active HEV infection with viremia with an effective anti-viral agent remains an elusive goal[48].

To date, two types of recombinant HEV vaccine have been developed[49-51], but neither of them is commercially available in the Western countries. Additionally both vaccines are genotype 1-based, and although they would be very useful in pregnant women and travelers to endemic regions, their efficacy in preventing HEV infection in non-endemic areas (where other genotypes predominate) needs to be investigated.

COMMENTS

Background

Hepatitis E virus (HEV), one of the leading causes of viral hepatitis worldwide, is transmitted predominantly *via* the fecal-oral route and responsible for epidemics of acute hepatitis in developing countries. However studies have shown that HEV is an emerging infection in industrialized countries as well, with increasing evidence of autochthonous cases (contracted locally) in addition to imported infection among immigrants and travelers from endemic countries. Israel is an industrialized country located amid endemic countries and absorbs immigrants from African countries, however a significant portion of Israel's population eats only kosher food (*i.e.*, do not eat pork, game meat or seafood), therefore the existence of autochthonous cases is not obvious.

Research frontiers

This study is a nation-wide epidemiological study of acute HEV cases diagnosed in Israel between the years 1993-2013. The aim of this manuscript was to identify whether there is any evidence of acute autochthonous HEV in Israel and to characterize the epidemiology, risk factors and clinical presentation of all documented acute HEV infection in Israel.

Innovations and breakthroughs

This report demonstrates, for the first time, the significant presence of autochthonous acute HEV in Israel (41% of all cases were autochthonous). Additionally, the imported HEV cases were from travelers and foreign workers acquiring the disease mainly in the Indian subcontinent.

Applications

The presence of autochthonous cases in Israel highlights the need for medical practitioners to be acquainted with the disease, even in non-endemic countries, and accurate diagnostic means should be easily accessible.

Terminology

"Autochthonous infection" is an infection contracted in the area where reported. Autochthonous HEV infection in Israel relates to patients with acute hepatitis E, with no history of travel during the last six months before disease onset, and who are not immigrants. In contrast, imported infection is one contracted in a country endemic for HEV, and occurs among travelers and immigrants.

Peer-review

The manuscript represents an interesting study of the etiology of acute hepatitis HEV in Israel reporting well documented data of interest to the scientific community.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Comprehensive mutation screening for 10 genes in Chinese patients suffering very early onset inflammatory bowel disease

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Author contributions: Xiao Y and Wang XQ contributed equally to this work; Xiao Y and Xu CD designed this study; Xiao Y and Wang XQ performed the next generation sequencing; Yu Y, Guo Y, Xu X and Gong L collected the patients and recorded the data; Zhou T and Li XQ analyzed the data.

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Institutional review board statement: The study was reviewed and approved by the ethics committee of the Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

Informed consent statement: All the participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All the authors listed declare no conflicts of interest.

Data sharing statement: Technical appendix, statistical code, and dataset is available from the corresponding author at chundixu55@163.com.

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Abstract

AIM: To perform sequencing analysis in patients with very early-onset inflammatory bowel disease (VEO-IBD) to determine the genetic basis for VEO-IBD in Chinese pediatric patients.

METHODS: A total of 13 Chinese pediatric patients with VEO-IBD were diagnosed from May 2012 and August 2014. The relevant clinical characteristics of these patients were analyzed. Then DNA in the peripheral blood from patients was extracted. Next generation sequencing (NGS) based on an Illumina-Miseq platform was used to analyze the exons in the coding regions of 10 candidate genes: *IL-10*, *IL-10RA*, *IL-10RB*, *NOD2*, *FUT2*, *IL23R*, *GPR35*, *GPR65*, *TNFSF15*, and *ADAM30*. The Sanger sequencing was used to verify the variations detected in NGS.

RESULTS: Out of the 13 pediatric patients, ten were diagnosed with Crohn's disease, and three diagnosed with ulcerative colitis. Mutations in *IL-10RA* and *IL-10RB* were detected in five patients. There were four patients who had single nucleotide polymorphisms associated with IBD. Two patients had *IL-10RA* and

FUT2 polymorphisms, and two patients had *IL-10RB* and *FUT2* polymorphisms. Gene variations were not found in the rest four patients. Children with mutations had lower percentile body weight (1.0% *vs* 27.5%, P = 0.002) and hemoglobin (87.4 g/L *vs* 108.5 g/L, P = 0.040) when compared with children without mutations. Although the age of onset was earlier, height was shorter, and the response to treatment was poorer in the mutation group, there was no significant difference in these factors between groups.

CONCLUSION: *IL-10RA* and *IL-10RB* mutations are common in Chinese children with VEO-IBD. Patients with mutations have an earlier disease onset, lower body weight and hemoglobin, and poorer prognosis.

Key words: Pediatric inflammatory bowel disease; Very early-onset inflammatory bowel disease; Interleukin 10 receptor; *NOD2* gene; *FUT2* gene

Core tip: In this small-sample size study, we performed next generation sequencing for 10 candidate genes in Chinese pediatric patients with very early onset inflammatory bowel disease. We found that *IL-10RA* and *IL-10RB* mutations were common. There were five patients harbouring mutations in these two genes and accounted for 38.5% of all samples. Besides, there were four patients who had single nucleotide polymorphisms associated with inflammatory bowel disease. Pediatric patients with mutations had an earlier disease onset, lower body weight, markedly lower hemoglobin, and poorer prognosis.

Xiao Y, Wang XQ, Yu Y, Guo Y, Xu X, Gong L, Zhou T, Li XQ, Xu CD. Comprehensive mutation screening for 10 genes in Chinese patients suffering very early onset inflammatory bowel disease. *World J Gastroenterol* 2016; 22(24): 5578-5588 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5578.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5578

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic and recurrent gastrointestinal inflammatory disease in children. Based on clinical characteristics, laboratory tests, and endoscopic and pathological presentations, IBD can be subdivided into Crohn's disease (CD), ulcerative colitis (UC), and IBD-unclassified (IBD-U)[1]. Our previous study showed that the annual incidence of IBD in the 0- to 14-year age group of Shanghai residents steadily increased from 2000 to 2010[2]. Although pediatric IBD mainly occurs in adolescence[2], approximately 15% of IBD pediatric patients have very early-onset IBD (VEO-IBD) that begins before 6 years of age, and 1% of children develop this disease before reaching 1 year of age[3,4]. The majority of VEO-IBD cases have clinical characteristics that are distinct from those of classic IBD with adult and adolescent onset. VEO-IBD has more severe clinical symptoms, resistance to a variety of immunosuppressive therapies, and a poor prognosis after conventional treatments. Some scholars even consider VEO-IBD to be a completely different disease from classic IBD[5].

Previous studies suggested that persistent intestinal immune dysfunction in a genetically susceptible individual exposed to adverse environmental factors is an important mechanism for IBD development. Genome-wide association studies (GWAS) have discovered a total of 163 loci associated with the risk for IBD development[6]. However, disease onset at an early stage of life suggests a leading role for rare gene variations in VEO-IBD patients, especially in children with a disease onset before the age of 1 year. These low frequency mutations are difficult to detect using GWAS. Next generation sequencing technology allows for the high-throughput sequencing of exons in a series of genes concurrently; therefore, rare gene variations can be discovered[7]. Since Glocker *et al*[8] first discovered in 2009 that mutations in genes encoding the α subunit (IL-10R1, encoding gene *IL-10RA*) and the β subunit (IL-10R2, encoding gene *IL-10RB*) of the interleukin-10 (IL-10) receptor could induce VEO-IBD development, a few studies have continuously discovered mutations in genes encoding IL-10R1, IL-10R2, and IL-10[5,9-12]. However, current reports are limited, and the majority of studies are small-size case studies. Reports on the Han Chinese population are scarcer[13,14].

This study used the Illumina-Miseq platform to sequence candidate genes in Han Chinese children diagnosed with VEO-IBD. The candidate genes included genes involved in the IL-10 signaling pathway, such as *IL-10*, *IL-10RA*, and *IL-10RB*, and genes highly associated with the development of CD in previous studies, including *NOD2, FUT2, IL-23R, GPR35, GPR65, TNFSF15,* and *ADAM30*. This study furthers our understanding of the genetic factors associated with VEO-IBD development in Han Chinese children.

MATERIALS AND METHODS

Patient consent and ethic committee approval

Verbal and written consent was obtained from the parents of all of children included this study. Ethic committee approval for the study was granted by Institutional Review Boards of Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

Study subjects

A total of 13 pediatric patients with repeated diarrhea, mucus and bloody stool, or abdominal pain who were diagnosed by laboratory tests and digestive endoscopy with VEO-IBD in the Pediatric Department of Ruijin

Hospital of Shanghai Jiao Tong University School of Medicine between May 2012 and August 2014 were included in this study. All of the patients were Han Chinese. VEO-IBD was defined as IBD onset before the age of 6 years, and a disease onset before 2 years of age was called infantile-onset IBD[15,16]. The clinical characteristics of these pediatric patients, including gender, age of disease onset, body height, body weight, family history, clinical symptoms, complications, major laboratory examinations, endoscopic presentations, and therapeutic effects, were retrospectively analyzed.

Laboratory and digestive endoscopic examinations

Relevant laboratory examinations, including complete blood count (CBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), tumor necrosis factor α (TNF-α) level, immunoglobulins G, A, M, and E, vitamin D, human Immunodeficiency virus (HIV) and human cytomegalovirus (CMV) antibody detection in serum, T lymphocyte flow cytometry sorting, stool parasite tests, stool culture, and stool *Clostridium difficile* toxin detection, were performed when the patients were admitted to the hospital. Common infectious diseases and primary immunodeficiency diseases were excluded.

All patients received a colonoscopy under general anesthesia. A biopsy of the colonic mucosa under endoscopy was performed for pathological examination.

Illumina-Miseq platform sequencing

Genomic DNA extraction: After obtaining verbal and written informed consent from the patients' parents, genomic DNA in the peripheral blood from 13 pediatric patients was extracted using a FlexiGene DNA Kit (Qiagen Inc., Germany). Another 100 copies of DNA extracted from patients suffering from idiopathic short stature (ISS) in previous research were used to test frequency of mutant sites which were newly detected in our study.

Multiplex PCR primer design: Based on the stability of the Illumina-Miseq experiment and the operability of subsequent steps, the length requirement of target fragments for sequencing was < 400 bp. If the length of an exon was longer than 400 bp, an additional pair of primers was designed with overlapping bases of adjacent fragments. To avoid a high number of non-target fragment products, primers were grouped and suspended in a primer mix before the multiplex PCR was performed. The concentration of each primer in the primer mix was 10 mmol/L. The basic requirement for grouping was the lack of matching sequences between two of the amplified products. Oligo 7 software was used to design primers for exons of the encoding region of the 10 candidate genes: *IL-10, IL-10RA, IL-10RB, NOD2, FUT2, IL23R, GPR35, GPR65, TNFSF15,* and *ADAM30*. A total of 86 pairs of primers were designed. The sequences are shown in supplementary Table 1.

Multiplex PCR amplification of candidate genes: A Qiagen Multiple PCR Kit was used in this study. The PCR amplification reaction system had a total volume of 21 μL, including 4 μL of ddH_2O, 2 μL of Q-solution (5 ×), 4 μL of 10 mmol/L primer mix, 10 μL of buffer mix, and 1 μL of the DNA template (20 ng/μL). The reaction procedure consisted of pre-denaturation at 94 ℃ for 15 min, denaturation at 94 ℃ for 40 s, annealing at 63 ℃ for 1 min, and extension at 72 ℃ for 40 s. After each cycle, the annealing temperature was reduced by 0.5 ℃ for 10 cycles until the annealing temperature reached 58 ℃. Next, the amplification was continued for 30 cycles with a constant annealing temperature of 58 ℃. The final extension at 72 ℃ lasted for 10 min. The PCR products were stained with 100 × GelRed and subjected to 1% agarose electrophoresis (120 V for 60 min).

The purified multiplex PCR products were sent to Shanghai South Gene Technology Co., Ltd. for sequencing analysis with the Illumina-Miseq platform. After sequencing, the nucleotide sequence information was compared with the standard gene sequences available in GenBank. The obtained gene mutation sites were compared with information in the dbSNP, HGMD, and OMIM databases to determine if the mutations had been previously reported.

To confirm the accuracy of the results, the corresponding gene sequences for the mutations discovered using the Illumina-Miseq platform were sequenced again using the Sanger sequencing method.

The newly discovered gene variation sites were analyzed to predict their influence on protein functions using two online databases: SIFT (http:// http://sift.jcvi.org/) and PolyPhen 2 (http://genetics.bwh.harvard.edu/pph2/).

Statistical analysis

According to the sequencing results, the 13 pediatric patients were divided into two groups. The patients who harboured pathogenic mutations were in group 1. Those without pathogenic mutations (including presence of polymorphisms only or wild type) were in group 2. The differences in diagnosis, age of disease onset, growth indicators (percentiles of body weight and height were calculated according to WHO standards), complications (perianal diseases and recurrent infection), and therapeutic effects among all groups were compared. Because the sample size was small, quantitative and ranked ordinal data were subjected to nonparametric statistics. The Mann-Whitney Test was performed, and the difference was statistically analyzed using exact probability. SPSS13.0 for Windows software was used for the statistical analysis. $P < 0.05$ indicated a significant difference.

Table 1 Genotypes of 13 patients diagnosed with very early-onset inflammatory bowel disease

Patient	Gene	Variation	Homo/Heterozygote	Function defect
1	*IL-10RA*	p.R101W	Homozygote	Yes
2	*IL-10RA*	p.R101W	Compound	Yes
		p.V100G (novel mutation)	heterozygote	Pathogenic supporting by Polyphen 2 and SIFT
3	*IL-10RA*	p.R101W	Compound	Yes
		p.Y64C (novel mutation)	heterozygote	Pathogenic supporting by Polyphen 2 and SIFT
4	*IL-10RA*	p.R117H (rs199989396)	Heterozygote	Yes
	NOD2	p.R703C (rs5743277)	Heterozygote	Susceptibility to CD recorded in HGMD
	FUT2	p.I140F (rs1047781)	Heterozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]
5	*IL-10RB*	p.K47E (rs2834167)	Homozygote	SNP in a VEO-UC child reported by Galatola *et al*[29]
		p.E141K (rs387907326)	Heterozygote	Pathogenic supporting by Polyphen 2 and SIFT
	IL-10RA	p.P115P (rs22280554)	Homozygote	Susceptibility to VEO-IBD reported by Moran *et al*[30]
		p.I224V (rs22280555)	Homozygote	
	FUT2	p.I140F (rs1047781)	Heterozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]
6	*IL-10RA*	p.P115P (rs22280554)	Homozygote	Susceptibility to VEO-IBD reported by Moran *et al*[30]
		p.I224V (rs22280555)	Homozygote	
	FUT2	p.I140F (rs1047781)	Homozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]
7	*IL-10RA*	p.P115P (rs22280554)	Homozygote	Susceptibility to VEO-IBD reported by Moran *et al*[30]
		p.I224V (rs22280555)	Homozygote	
	FUT2	p.I140F (rs1047781)	Heterozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]
8	*IL-10RB*	p.K47E (rs2834167)	Homozygote	SNP in a VEO-UC child reported by Galatola *et al*[29]
	FUT2	p.I140F (rs1047781)	Heterozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]
9	*IL-10RB*	p.K47E (rs2834167)	Heterozygote	SNP in a VEO-UC child reported by Galatola *et al*[29]
	FUT2	p.I140F (rs1047781)	Heterozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]

Patients 10, 11, 12 and 13 were wild types in all these genes. CD: Crohn's disease; UC: Ulcerative colitis; VEO-IBD: Very early-onset inflammatory bowel disease; VEO-UC: Very early-onset ulcerative colitis; SNP: Single nucleotide polymorphism; HGMD: The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php).

RESULTS

Genotyping in VEO-IBD patients

_IL-10RA_, _IL-10RB_, and _IL-10_ mutations: *IL-10RA* mutations were detected in four patients, an *IL-10RB* mutation was detected in one patient, and an *IL-10* mutation was not detected in any of the 13 patients.

The detected *IL-10RA* mutations were all in exon 3: c.A191G (p.Y64C), c.T299G (p.V100G), c.C301T (p.R101W), and c.G350A (rs199989396) (p.R117H). The p.R101W mutation was the most common and was detected in three patients (patients 1-3). The other mutations were detected in only one patient. Patient 1 had a homozygous mutation, patients 2 and 3 had compound mutations, and patient 4 had a heterozygous mutation (Table 1 and Figure 1).

Among detected *IL-10RA* mutations, p.Y64C and p.V100G were new mutations that were predicted to be deleterious by SIFT and Polyphen 2. These novel mutant sites were not found in 100 ISS children. The other two mutations had been confirmed to be deleterious in several studies[5,12,14,17].

An *IL-10RB* heterozygous mutation was detected in one patient (patient 5) (Table 1 and Figure 1). This c.G421A (p.E141K) (rs387907326) mutation was located in exon 4 and was also predicted as a deleterious mutation by SIFT and Polyphen 2. A nonsense mutation in the same site was detected in previous studies[11,18].

Candidate gene polymorphisms

After the sequence analysis of the coding regions of 10 candidate genes, we found that six patients (patient 4, 5, 6, 7, 8 and 9) had many IBD-associated single nucleotide polymorphisms (SNPs) in *IL-10RA*, *IL-10RB*, *NOD2*, and *FUT2*. The SNP loci in *IL-10RA* were rs22280554: c.G525A, p. P175P and rs22280555: c.A670G, p.I224V; the SNP locus in *IL-10RB* was rs2834167: c.A139G, p.K47E; the SNP locus in *NOD2* was rs5743277: c.C2107T, p.R703C; and the SNP locus in *FUT2* was rs1047781: c.A418T, p.I140F (Table 1).

In addition to the detected p.R117H heterozygous mutation in *IL-10RA*, patient 4 also had heterozygous SNPs in *NOD2* and *FUT2*.

Patient 5 had a heterozygous p.E141K mutation (rs387907326) in *IL-10RB* and SNPs in *IL-10RA, IL-10RB,* and *FUT2*. The SNP loci in *IL-10RA* were rs22280554 and rs22280555. The homozygous SNP loci for *IL-10RB* were rs2834167. The SNP in *FUT2* was heterozygous.

Patients 6 and 7 had SNPs in *IL-10RA* and *FUT2*. Patients 8 and 9 had SNPs in *IL-10RB* and *FUT2*.

Four patients did not show any IBD-associated variations in the coding regions of the 10 candidate genes.

There was no IBD-associated variation discovered in the coding regions of six genes: *IL-10, IL-23R*, *GPR35*, *GPR65*, *TNFSF15*, and *ADAM30*.

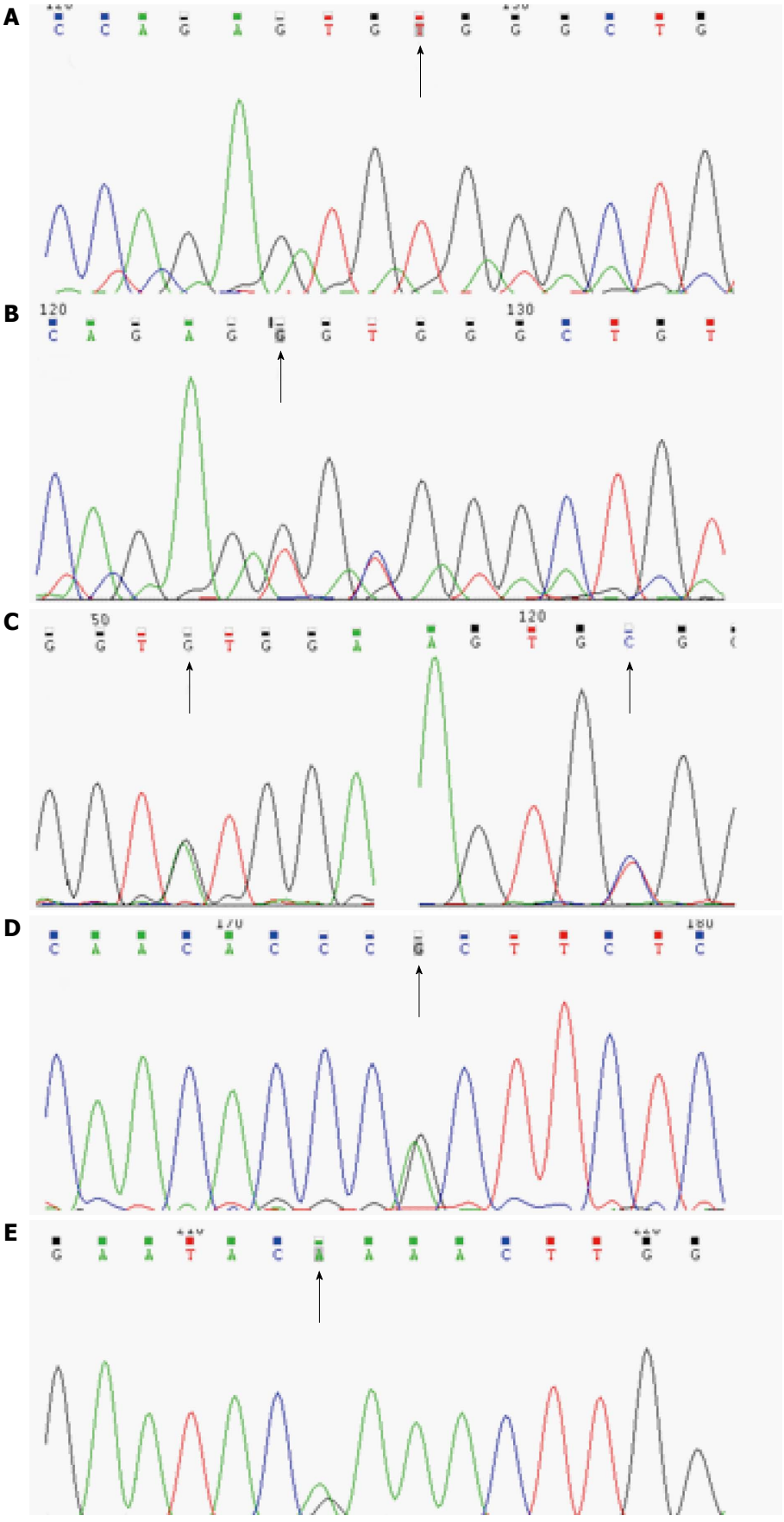

Figure 1 Causative mutations in *IL-10RA* (A-D) or *IL-10RB* (E). A: Patient 1, c.C301T, p.R101W, homozygote; B: Patient 2, c.T299G, p.V100G and c. C301T, p.R101W, compound heterozygote; C: Patient 3, c.A191G, p.Y64C and c. C301T, p.R101W, compound heterozygote; D: Patient 4, c.G35A, p.R117H (rs199989396), heterozygote; E: Patient 5, c.G421A, p.E141K (rs387907326), heterozygote.

Clinical characteristics of VEO-IBD pediatric patients

Out of the 13 VEO-IBD pediatric patients in this study, ten were diagnosed with CD (M:F = 9:1) and three had UC (M:F = 1:2). The mean age of disease onset was 5.8 ± 9.7 mo (range: birth to 3 years of age). None of the parents of the patients had a consanguineous marriage. Patient 8 had a brother that died as a neonate because of repeated diarrhea after birth. There was no clear diagnosis made at that time. The clinical symptoms of the pediatric patients included repeated abdominal pain (13/13), diarrhea (11/13), mucus and bloody stool (11/13), failure to thrive (8/13), recurrent infection (7/13), and perianal fistulas and abscesses (5/13). The colonoscopic presentation of patients with causative mutations showed pancolitis, cobblestone-like changes in mucosa, and deep and large ulcers (Figure 2). All patients received immunosuppressive treatment with glucocorticoids, 6-mercaptopurine and/or infliximab, and thalidomide; however, varying therapeutic effects were observed. Two patient died from severe sepsis or intestinal failure, 2 patients showed no change, 4 patients showed a partial alleviation of symptoms, and 5 patients showed complete clinical remission (Table 2).

Clinical characteristics of different genotypes

Based on the presence of causative mutations in *IL-10RA* and *IL-10RB*, 13 patients were divided into two groups for analysis (group 1: causative mutations in *IL-10RA* or *IL-10RB*; group 2: polymorphisms and no causative mutations). The five patients in group 1 were all diagnosed with CD (100%). Four of these patients had recurrent infections (80%), and three patients had perianal diseases (60%). In group 2 (eight patients), five patients were diagnosed with CD (62.5%), and the other three patients were diagnosed with UC (37.5%). There were only three (37.5%) and two (25%) patients that had recurrent infections and perianal diseases, respectively. Patients in group 1 had lower body weight percentile (1.0% *vs* 27.5%, $P = 0.002$) and hemoglobin concentrations (87.4 g/L *vs* 108.5 g/L, $P = 0.040$) when compared with group 2. Although patients in group 1 had a younger age of disease onset (2.7 mo), lower body height percentile (5.0%), and higher CRP (60.7 mg/L), there were no significant differences when compared with group 2 (Table 3).

DISCUSSION

The currently recognized pathogenetic mechanism of IBD is the involvement of many environmental triggers and genetic susceptibility that causes intestinal immune dysfunction. However, the influence of genes are likely more important than environmental factors for VEO-IBD patients with a disease onset prior to 6 years of age, especially for patients with an infantile onset prior to 1 year of age[19]. GWAS studies suggested that SNPs of *IL-10* and *STAT3* were associated with IBD[20-23]. Previous studies confirmed that IL-10 or IL-10 receptor gene knockout mice had severe chronic inflammation of the intestinal tract[24]. IL-10 forms a complex with two molecules of IL-10R1 and two molecules of IL-10R2 to activate Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2). This activation results in the phosphorylation of signal transducer and activator of transcription 3 (STAT3), which regulates the transcription of specific genes. Studies suggested that IL-10-mediated signals effectively reduced the number of Th17 cells and relieved intestinal inflammation in CD[25]. These data indicated that the anti-inflammatory IL-10 signaling pathway plays a critical role in the regulation of intestinal immune homeostasis.

Since Glocker *et al*[8] first reported in 2009 that gene mutations in *IL-10RA* and *IL-10RB* caused infantile onset IBD[8], studies have continuously reported mutations in *IL-10*, *IL-10RA*, and *IL-10RB* in patients with infantile onset IBD[9-12,18,26]. In these limited data, the majority of patients were Arabian or Caucasian and the offspring of a consanguineous marriage. There are few reports on the Han Chinese population, which included only three pediatric patients to date[13,14].

In this study, we used high-throughput next generation sequencing technology to sequence 10 IBD-associated genes, *IL-10*, *IL-10RA*, *IL-10RB*, *NOD2*, *FUT2*, *IL-23R*, *GPR35*, *GPR65*, *TNFSF15*, and *ADAM30*, in 13 Han Chinese children diagnosed with VEO-IBD. A total of four mutations were discovered in *IL-10RA*, including two novel mutations. There was one mutation in *IL-10RB*. These pathogenic mutations were found in five patients, which accounted for 38.5% of all VEO-IBD cases. Among these patients, one had an *IL-10RA* homozygous mutation, two had *IL-10RA* compound heterozygous mutations, one had an *IL-10RA* heterozygous mutation, and one had an *IL-10RB* heterozygous mutation. All *IL-10RA* mutations were in exon 3, and c.C301T (p.R101W) showed the highest frequency. The c.C301T (p.R101W) and c.G350A (p.R117H) mutations in *IL-10RA* were previously reported in similar pediatric patients. These mutations may disrupt signal transduction after activation of the IL-10 receptor; therefore, STAT3 is not phosphorylated and intractable inflammatory reactions in the intestinal tract of pediatric patients develop[5,12]. The two novel mutations in *IL-10RA* discovered in this study were c.A191G (p.Y64C) and c.T299G (p.V100G). Because of condition limitations, we did not perform functional studies on these mutations. However, the SIFT prediction results for these two mutations were deleterious (scores of 0 and 0.002, respectively), and the Polyphen 2 prediction results were probably damaging (both scores were 1.000). These predictions suggest that these two mutations are pathogenic. According to the recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology[27], these two

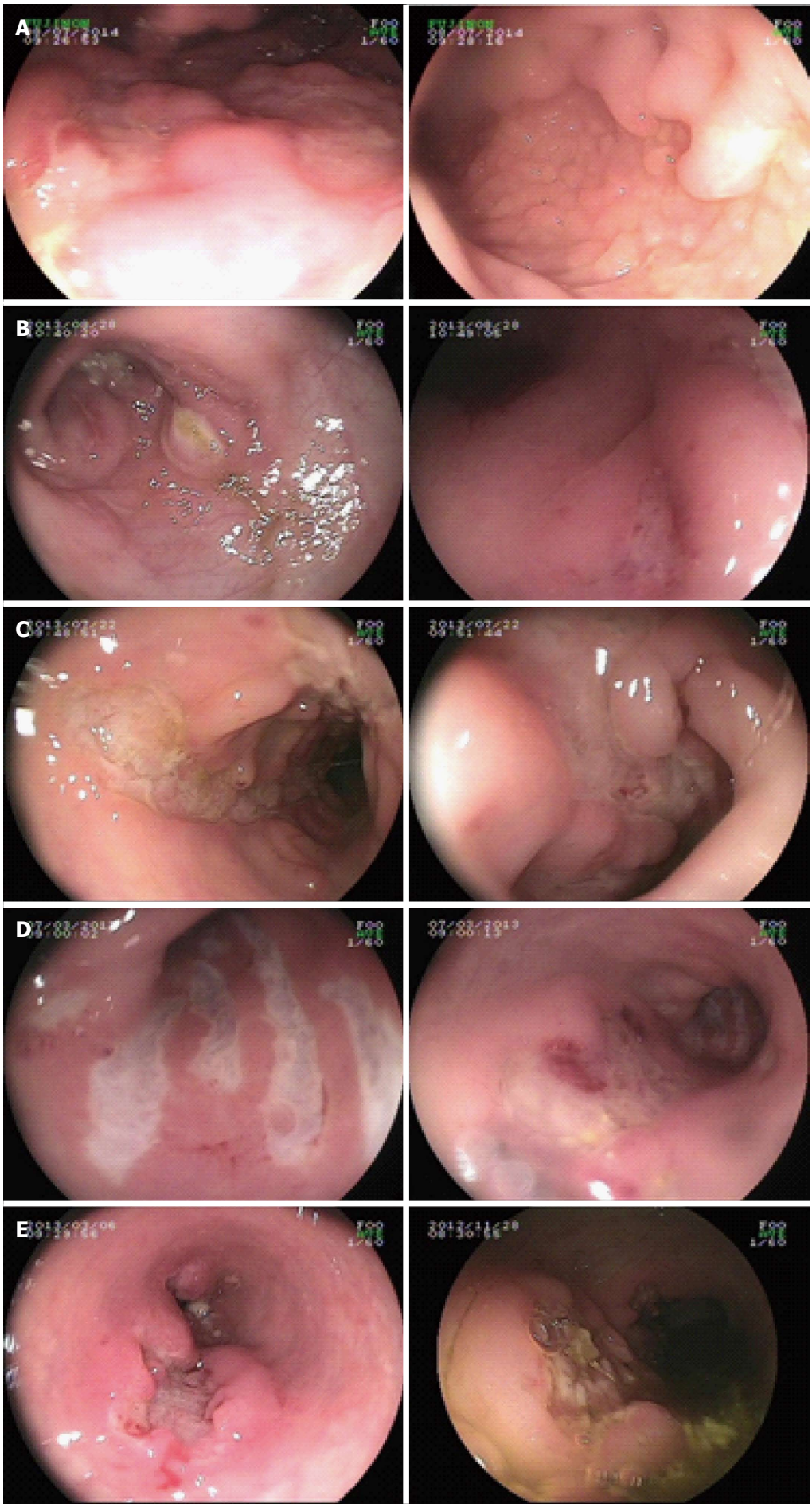

Figure 2 Colonoscopic presentation of patients with causative mutations showed pancolitis, cobblestone-like changes in mucosa, and deep and large ulcers. A to E presents patient 1 to patient 5, respectively.

Table 2 Clinical manifestations of very early-onset inflammatory bowel disease

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13
Gender	F	M	M	M	M	M	M	M	M	M	M	F	F
Age of onset (mo)	8	1	0.3	0.3	4	0.2	9	2	0.5	3	0.7	10	36
Height percentile	19%	1%	3%	1%	1%	1%	52%	1%	15%	1%	19%	16%	20%
Weight percentile	1%	1%	1%	1%	1%	20%	55%	13%	8%	15%	16%	60%	33%
Diarrhea (times/d)	> 10	7-8	> 10	10	5-10	5-6	7-8	2-4	7-8	No diarrhea	7-8	No diarrhea	2-3
Bloody stool	+	+	+	+	+	-	+	+	+	-	+	+	+
Infection	Sepsis	Pneumonia	No	Pneumonia, *Clostridium difficile* infection	Sepsis, oral candidiasis, fungemia, *Clostridium difficile* infection	Recurrent respiratory infection	No	No	No	Repeated fever of unknow origin	Oral candidiasis, gingivitis	No	No
Perianal lesion	Fistulae	No	No	Excrescence	Fistulae, abscess, excrescence	Fistulae, ulcer	No	No	No	No	Fistulae, abscess, excrescence	No	No
Clinical diagnosis	CD	CD	CD	CD	CD	CD	CD	CD	UC	CD	CD	UC	UC
Medication	GC, 6-MP	GC, IFX, THD	GC, THD	GC, IFX[1], THD	GC, IFX, THD	GC, IFX[1]	GC, IFX, MES	GC, IFX[1], THD, 6-MP	GC, MES	GC, 6-MP, THD	GC, IFX, THD, 6-MP	MES	GC, MES
Clinical status	NR	PR	Died at 2 yr because of severe sepsis	PR	Died at 3 yr because of intestinal failure	NR	CR	PR	CR	CR	PR	CR	CR

[1]Allergic to IFX. CD: Crohn's disease; UC: Ulcerative colitis; GC: Glucocorticoid; 6-MP: 6-mercaptopurine; IFX: Infliximab; THD: Thalidomide; MES: Mesalazine; NR: Non-remission; PR: Partial remission; CR: Complete remission.

mutations were defined as pathological supporting. Therefore, we speculate that these two novel mutations individually formed compound heterozygotes with the c.C301T (p.R101W) mutation to cause the disease symptoms observed in patients 2 and 3.

Previous analyses showed that the colitis caused by gene mutations in IL-10 and its receptor exhibited an autosomal recessive inheritance pattern. In the current study, patients 4 and 5 were carriers of heterozygous mutations in *IL-10RA* and *IL-10RB*, respectively. The c.G350A (p.R117H) mutation in *IL-10RA* carried by patient 4 was a pathogenic mutation[5,12,17]. The c.G421A (p.E141K) mutation in *IL-10RB* carried by patient 5 may affect protein function as predicted by SIFT (score = 0.026) and Polyphen 2 (score = 0.946). However, the clinical presentation of these two patients was similar to the symptoms of patients with other IL-10 receptor mutations: disease onset within 1 year of age, the presence of perianal diseases and recurrent infection, and resistance to conventional medication treatment. Based on currently available knowledge, there are at least 50 single-gene genetic conditions that induce IBD-like diseases, and the majority of conditions are related to immunodeficiency[4,6]. Therefore, the two patients that did not conform to a Mendelian genetic pattern might also carry abnormal sites on other genes that cause the disease symptoms. In addition to carrying a pathogenic mutation in *IL-10RA*, patient 4 also had a non-synonymous SNP (nsSNP): rs5743277 in *NOD2*. SIFT prediction results suggest that the nsSNP is deleterious (score = 0), and the Polyphen 2 prediction results suggest the nsSNP is probably damaging (score = 0.999). This polymorphism was already present in the HGMD database and has been considered to cause susceptibility to CD[28]. Patient 5 had a similar condition. In addition to carrying an *IL-10RB* mutation, patient 5 also had multiple polymorphisms: rs22280554 (homozygous) and rs22280555 (homozygous) in *IL-10RA*,

Table 3 Comparison of features between patients with mutations and polymorphisms

	Group 1	Group 2
Size of sample	5	8
Age of onset (mo)	2.7	7.7
Height percentile	5.0%	15.6%
Weight percentile[a]	1.0%	27.5%
WBC ($\times 10^{9}$)	15.2	16.3
Hemoglobin (g/L)[a]	87.4	108.5
Platelets ($\times 10^{9}$)	538.4	424.0
C reactive protein (mg/L)	60.7	35.9
ESR (mm/H)	32.2	16.6
TNFα (pg/mL)	44.5	51.6
Diagnosis of CD	100.0%	62.5%
Recurrent infection	80.0%	25.0%
Perianal disease	60.0%	25.0%

[a]$P < 0.05$. All measurement data are expressed as mean. Group 1: Mutations in *IL-10RA* or *IL-10RB*; Group 2: Polymorphisms. Height and weight percentile was calculated according to WHO charts. WBC: White blood cell; ESR: Erythrocyte sedimentation rate; TNFα: Tumor necrosis factor alpha; CD: Crohn's disease.

rs2834167 (homozygous) in *IL-10RB*, and rs1047781 (heterozygous) in *FUT2*. There are previous reports on the pathogenicity of these SNPs. For example, Galatola *et al*[29] reported that the heterozygous rs2834167 in *IL-10RB* and the heterozygous mutation in the promoter region of *IL-10RA* caused the development of UC in an 18-month-old patient. Although rs22280554 did not cause a change in the amino acid sequence of IL-10R1, a study by Moran *et al*[30] showed that rs22280554 and rs2228055 in *IL-10RA* may increase the risk for VEO-IBD, especially VEO-UC. Furthermore, in the Han Chinese population, the rs1047781 polymorphism in *FUT2* may increase the risk for CD development[31]. The above SNPs were also detected in four patients in this study. Therefore, their disease development may be due to "trans-heterzygous": the collective effects of a variety of detected mutations. Another possible cause is that the pathogenic genes were not detected in this study.

When genotypes and phenotypes were combined for analyses, the results showed that the disease phenotype in patients with mutations were more severe. The age of disease onset was earlier, the patients were more likely to have combined recurrent infections and perianal diseases, their body weight and height were low, anemia was more severe, inflammatory indicators were high, and the prognosis was much poorer. These results are in accordance with previous studies[5,8-14,18,32]. However, the sample size of this study was small, and significant differences were only found in body weight and hemoglobin parameters. Because of the influence of cultural ideas, family members find difficulty in accepting an ileostomy as a disease treatment. Past literature reported that pediatric patients with *IL-10RA* and *IL-10RB* mutations could be cured through hematopoietic stem cell transplantation[4,6,8,17]; therefore, some patients are waiting for a donor match.

In this study, we found that mutations in *IL-10RA* and *IL-10RB* were more common in Han Chinese VEO-IBD patients and accounted for 38.5% of all VEO-IBD cases. The high percentage is probably due to the small number of patients in the cohort as most of our patients who were referred by other clinical IBD centers were very ill. There was a selection bias. Because VEO-IBD is relatively rare, multi-center studies on the relationship between genotypes and phenotypes in VEO-IBD patients in China are necessary. The implementation of hematopoietic stem cell transplantation therapy is the focus in research agenda.

COMMENTS

Background

Very early-onset inflammatory bowel disease (VEO-IBD) may have stronger genetic contribution. Recently, a few studies on genetic defects in the IL-10 signaling pathway have provided new insights into IBD, especially in VEO-IBD. Furthermore, a lot of genes associated with IBD were identified, such as *NOD2*, *FUT2*, *IL-23R*, *GPR35*, *GPR65*, *TNFSF15*, and *ADAM30*. Because of different genetic background, this study was set to disclose whether mutations in these genes contributed to VEO-IBD in Chinese children.

Research frontiers

In addition to the polygenic variants associated with IBD, there are rare monogenic disorders, including many immunodeficiencies that can present with IBD-like intestinal inflammation, especially in early life.

Innovations and breakthroughs

To our knowledge, this is the first cohort study to apply NGS in 13 Chinese pediatric patients with VEO-IBD to discover gene variations in these children. The result revealed that IL-10RA and IL-10RB mutations were common in Chinese VEO-IBD, especially in infantile IBD. These monogenic IBD patients had more severe clinical features.

Applications

According to the results of this study and previous studies of VEO-IBD, the authors suggest that screening for gene mutations in IL-10 signaling pathway is necessary.

Peer-review

The clinical study is focused on gene mutation analysis in VEO-IBD by NGS. The authors conclude that mutations in the IL-10 pathway are common in VEO-IBD.

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ORIGINAL ARTICLE

Retrospective Cohort Study

miR-422a is an independent prognostic factor and functions as a potential tumor suppressor in colorectal cancer

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Abstract

AIM: To determine the expression of miR-422a in colorectal cancer (CRC) tissues and to further explore the prognostic value and function of miR-422a in CRC carcinogenesis.

METHODS: miR-422a expression was analyzed in 102 CRC tissues and paired normal mucosa adjacent to carcinoma by quantitative real-time PCR. The relationship of miR-422a expression with clinicopathological parameters was also analyzed. Kaplan-Meier analysis and Cox multivariate analysis were performed to estimate the potential role of miR-422a. Cell proliferation, migration, and invasion were used for *in vitro* functional analysis of miR-422a.

RESULTS: The levels of miR-422a were dramatically reduced in CRC tissues compared with normal mucosa ($P < 0.05$), and significantly correlated with local invasion ($P = 0.004$) and lymph node metastasis ($P < 0.001$). Kaplan-Meier survival and Cox regression multivariate analyses revealed that miR-422a expression (HR = 0.568, $P = 0.015$) and clinical TNM stage (HR = 2.942, $P = 0.003$) were independent prognostic factors

for overall survival in CRC patients. Furthermore, *in vitro* experiments showed that overexpression of miR-422a inhibited the proliferation, migration, and invasion of SW480 and HT-29 cells.

CONCLUSION: Down-regulation of miR-422a may serve as an independent prognosis factor in CRC. MiR-422a functions as a tumor suppressor and regulates progression of CRC.

Key words: Colorectal cancer; MicroRNA; miR-422a; Prognosis; *In vitro* function

Core tip: In the present study, we found that miR-422a was dramatically reduced in colorectal cancer (CRC) tissues, and significantly correlated with local invasion and lymph node metastasis. miR-422a expression and clinical TNM stage were independent prognostic factors for overall survival in CRC patients. Furthermore, *in vitro* experiments showed that overexpression of miR-422a inhibited the proliferation, migration, and invasion of SW480 and HT-29 cells. These results indicated that down-regulation of miR-422a might serve as an independent prognosis factor in CRC, and miR-422a functions as a tumor suppressor and regulates progression of CRC.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors with increasing incidence and mortality over the past several decades[1]. Despite the significant progress made in diagnostic methods and improved treatment strategies, the prognosis of CRC patients remains poor, especially in those with advanced CRC[2,3]. CRC carcinogenesis is associated with multiple alterations in oncogenes and tumor suppressor genes. A growing number of studies have revealed that microRNAs (miRNAs) might regulate up to 30% of human genes and play a pivotal role in various cellular processes including proliferation, differentiation, apoptosis, migration, and invasion[4-8]. While the functional mechanisms of miRNAs remain largely unknown in the pathogenesis of CRC, dysregulated expression of miRNAs can serve as potential biomarkers for the diagnosis and prognosis of cancer[9-11].

Several studies have determined the importance of miR-422a in human diseases such as cancer, multiple sclerosis, and postmenopausal osteoporosis. Gougelet *et al*[12] reported that miR-422a could inhibit signaling pathways regulating tumor cell proliferation in osteosarcoma. A study by Mao *et al*[13] demonstrated that miR-422a targeted key mismatch repair protein (MutLα) by suppressing MLH1 expression, resulting in genome instability and tumorigenesis. Faltejskova *et al*[14] reported that dysregulation of miR-378, miR-375, miR-422a, miR-215 and miR-135b in CRC patients played an important role in CRC pathogenesis. However, further study is needed to confirm whether miR-422a is an independent predictive factor in patients with CRC. Moreover, the functional mechanism by which miR-422a regulates CRC progression and whether it can serve as a prognostic biomarker in CRC remain largely unknown.

Previously, we have identified a serum 4-miRNA panel that included miR-19a-3p, miR-92a-3p, miR-223-3p, and miR-422a. This miRNA panel served as biomarkers for early diagnosis of colorectal adenocarcinoma[15,16]. In this study, we showed that the expression of miR-422a is dysregulated in CRC tissues. The correlation between miR-422a and certain clinical characteristics, as well as its potential as a prognostic marker for CRC, was also investigated. The effects of miR-422a on CRC cell proliferation, invasion, and migration were also assessed.

MATERIALS AND METHODS

Study population and sample collection

All written informed consent was obtained from every participant for use of tissue samples. This project was approved by the Clinical Research Ethics Committee of Qilu Hospital of Shandong University. All CRC patients were recruited from the Department of General Surgery, Qilu Hospital of Shandong University between November 2004 and December 2013. The diagnosis of CRC was confirmed by histopathology or histobiopsy. A total of 102 CRC tissues were collected and the adjacent normal mucosa tissues were used as controls since CRC is a malignant epithelial tumor and originates from glandular epithelium of the colorectal mucosa. All tissues were immediately frozen in liquid nitrogen and stored at -80 ℃ until RNA extraction. The median follow-up period for patients enrolled in this study was 63 mo (range, 14-78 mo).

Cell culture

SW480 and HT29 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The two cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, United States) at 37 ℃ in an incubator containing 5% CO_2.

Table 1 Relationship between miR-422a and clinical parameters in colorectal cancer tissues

Clinical parameter	Number of cases (n = 102)	miR-422a expression, median (min-max)	P value
Gender			0.704
Male	63	1.446 (0.074-130.774)	
Female	39	1.617 (0.167-33.543)	
Age (yr)			0.328
≤ median	49	1.637 (0.088-130.774)	
> median	53	1.715 (0.074-21.871)	
Tumor location			0.092
Colon	58	1.798 (0.088-130.774)	
Rectum	44	1.887 (0.074-33.543)	
Differentiation			0.150
Well	12	1.798 (0.074-130.744)	
Moderate	67	1.922 (0.166-21.871)	
Poor	23	2.088 (0.088-20.406)	
Tumor size			0.527
≤ 5 cm	64	2.127 (0.074-130.744)	
> 5 cm	38	2.353 (0.167-21.871)	
Local invasion			0.004
T1–T2	55	3.257 (0.206-130.744)	
T3–T4	47	1.617 (0.074-20.406)	
Lymph node metastasis			0.002
No	70	2.863 (0.359-130.744)	
Yes	32	1.445 (0.074-20.406)	
TNM stage			< 0.001
Ⅰ	23	3.877 (0.265-15.999)	
Ⅱ	42	3.253 (0.074-13.566)	
Ⅲ	37	1.445 (0.329-130.744)	

Values are median (IQR: Inter-quartile range). TNM: Tumor node metastasis.

Cell transfection

CRC cells were seeded at 2 × 10^5 cells/well in 6-well plates until 30%-50% confluency and then were transfected with miR-422a mimics or negative control mimics (RiboBio, Guangzhou, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's introduction. The transfected cells were normalized and used in subsequent assays. The transfection efficiency was determined by RT-qPCR to verify the success of transfection.

RNA preparation, cDNA synthesis and RT-qPCR

Total RNA from cell lines, CRC tissues and normal mucosa was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, United States). The cDNA was reverse-transcribed at 65 ℃ for 5 min in a 12 μL reaction system including 1 μg RNA, 1 μL reverse transcription primer (RiboBio, Guangzhou, China), 1 μL U6 reverse transcription primer and 1 μL dNTP. Then, 4 μL buffer, 2 μL DTT and 1 μL RNase inhibitor were added and incubated at 37 ℃ for 2 min followed by adding 1 μL MMLV and incubating at 37 ℃ for 50 min and 70 ℃ for 5 min. The PCR reaction was performed as follows: 95 ℃ for 1 min, and 45 cycles of 95 ℃ for 15 s, 60 ℃ for 30 s, and 72 ℃ for 45 s. U6 was used as a reference gene and each test was performed in triplicate in this study. RT-qPCR reactions were carried out on ABI Prism 7500 System (Applied Biosystems, Foster City, CA, United States).

Cell proliferation assay

miR-422a mimics and control mimics were transfected into SW480 and HT29 cells, then cultured for another 24, 48, 72, or 96 h. Cell proliferation rates was measured with CCK-8 reagent (Beyotime, Hangzhou, China) following the manufacturer's protocol. A microplate photometer (Multiskan FC, Thermo scientific, Shanghai, China) was used to determine the optical density at 450 nm.

Transwell migration and invasion assays

Transwell migration assay was performed using transwell chambers (Corning Costar, United States). After transfection for 24 h, cells were transferred into the upper chamber. RPMI 1640 medium containing 10% FBS functioning as a chemoattractant was added to matched lower chamber. SW480 and HT29 cells unable to migrate were removed from the upper surface of the transwell membrane after incubation for 48 h. Cells that were able to invade on the lower membrane surface were fixed in methanol, stained with 0.1% crystal violet, and counted using an inverted microscope (Olympus, Tokyo, Japan). For invasion assay, the inserts were pre-coated with matrix gel (BD Biosciences, Franklin Lakes, NJ, United States).

Statistical analysis

Data were analyzed using SPSS 17.0 software (IBM Corporation, Armonk, NY, United States). The expression of miR-422a between groups was compared using Mann-Whitney U test. Kaplan-Meier method was used for overall survival analysis. The Cox regression model was used for univariate and multivariate analyses to estimate the prognostic factors.

RESULTS

Expression of miR-422a in CRC tissues and correlative analysis with clinicopathological parameters

We detected the expression of miR-422a in 102 pairs of CRC tissues and normal mucosa adjacent to carcinoma using RT-qPCR. The results indicated that miR-422a levels were significantly lower in CRC tissues compared with normal mucosa (P < 0.05) (Figure 1A). Moreover, 65.7% (67 of 102) of CRC tissues had at least 2-fold down-regulated expression of miR-422a compared with normal mucosa (Figure 1B). The levels of miR-422a in stages Ⅰ and Ⅱ samples were significantly higher than those in stage Ⅲ samples (P < 0.05). There was no significant difference in miR-422a expression between stages Ⅰ and Ⅱ samples (P

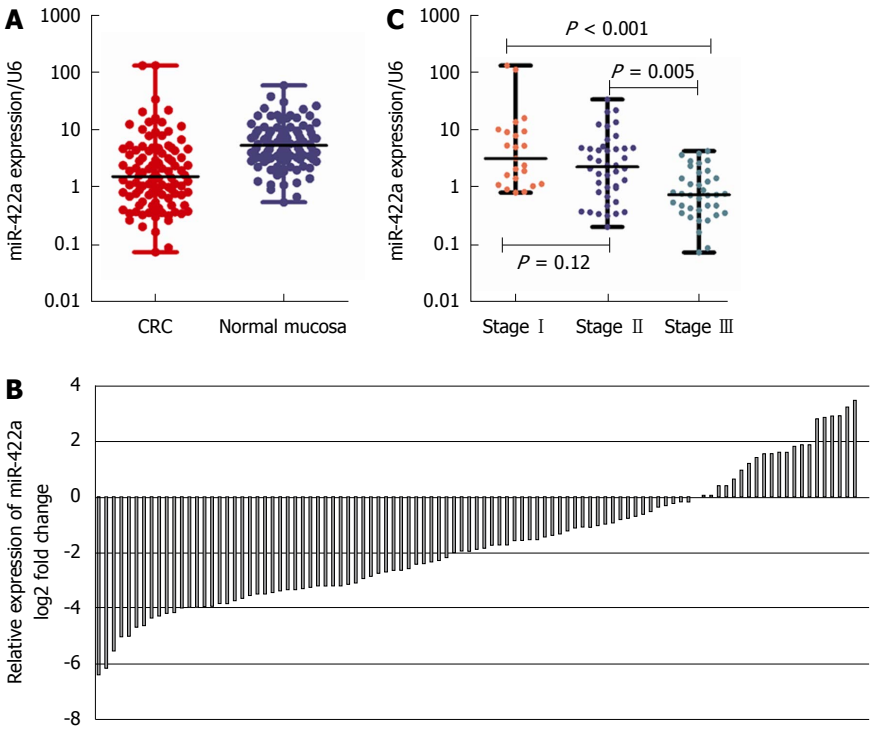

Figure 1 Expression of miR-422a in colorectal cancer and its association with TNM stage. A: Levels of miR-422a were determined in 102 colorectal cancer (CRC) tissues and their corresponding normal mucosa (NC); B: The fold changes of relative miR-422a level (CRC/NC) in each matched samples, with "< -1" defined as under-expression, "-1-1" as unchanged, and "> 1" as overexpression; C: Levels of miR-422a in stages Ⅰ/Ⅱ/Ⅲ CRC.

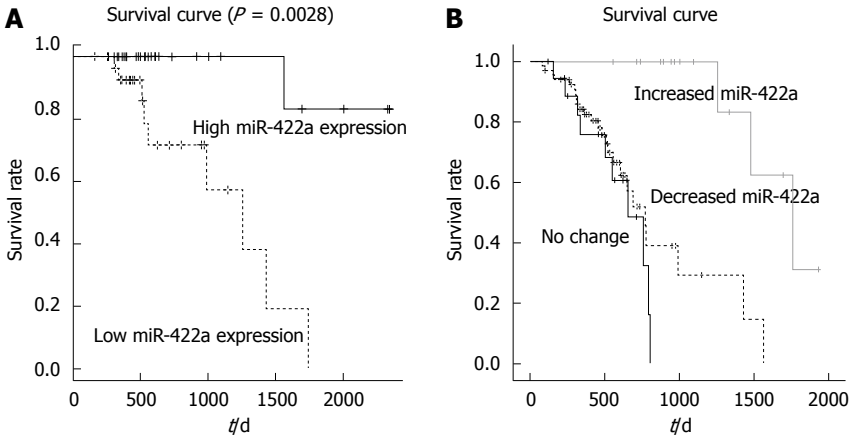

Figure 2 Kaplan-Meier curves for overall survival in 102 colorectal cancer patients based on miR-422a expression and the change of miR-422a expression compared to adjacent normal mucosa, respectively. Results showed that patients with low miR-422a expression had a significantly poorer prognosis than those with high miR-422a expression (A) (P = 0.0028), and patients with decreased miR-422a expression compared to adjacent normal mucosa had a significantly poorer prognosis than those with increased miR-422a expression (B) (P < 0.001).

> 0.05) (Figure 1C).

The association between miR-422a level and clinicopathological parameters of CRC patients is showed in Table 1. Our data showed that the level of miR-422a significantly correlated with local invasion (P = 0.004), lymph node metastasis (P = 0.002), and TNM stage (P < 0.001). However, there were no significant correlations between miR-422a level and gender, age, tumor location, differentiation or tumor size (P > 0.05).

miR-422a is an independent prognostic factor for overall survival in CRC patients

Our results demonstrated that 42 of 102 CRC patients died during the follow-up period. The 5-year overall survival rate was 58.8%. Patients were divided into high miR-422a expression and low miR-422a expression groups based on the median level. The prognosis was analyzed by Kaplan-Meier survival analysis, which revealed that patients with low miR-422a expression had a significantly poorer prognosis

Table 2 Univariate and multivariate analyses of overall survival in colorectal cancer patients

Variable	Category	Univariate analysis			Multivariate analysis		
		HR	95%CI	*P* value	HR	95%CI	*P* value
Gender	Male *vs* Female	0.943	0.493-1.872	0.810			
Age	≤ Median *vs* > Median	1.436	0.528-3.157	0.148			
Tumor location	Colon *vs* Rectum	0.862	0.484-1.706	0.603			
Tumor size	≤ 5 cm *vs* > 5 cm	1.093	0.568-2.194	0.873			
Differentiation	Well and moderate *vs* Poor	1.018	0.462-2.180	0.160	0.986	0.486-2.006	0.074
Local invasion	T1-T2 *vs* T3-T4	1.682	1.020-2.628	0.01[1]	1.460	0.746-2.632	0.482
Lymph node metastasis	Yes *vs* No	1.182	0.634-2.530	< 0.001[1]	1.262	0.584-2.680	0.306
TNM stage	Ⅰ and Ⅱ *vs* Ⅲ	2.738	1.509-4.265	< 0.001[1]	2.942	1.426-4.378	0.003[1]
MiR-422a level	Low *vs* High	0.306	0.108-0.763	0.001[1]	0.568	0.245-1.082	0.015[1]

[1]There was a significant difference between the two groups ($P < 0.05$).

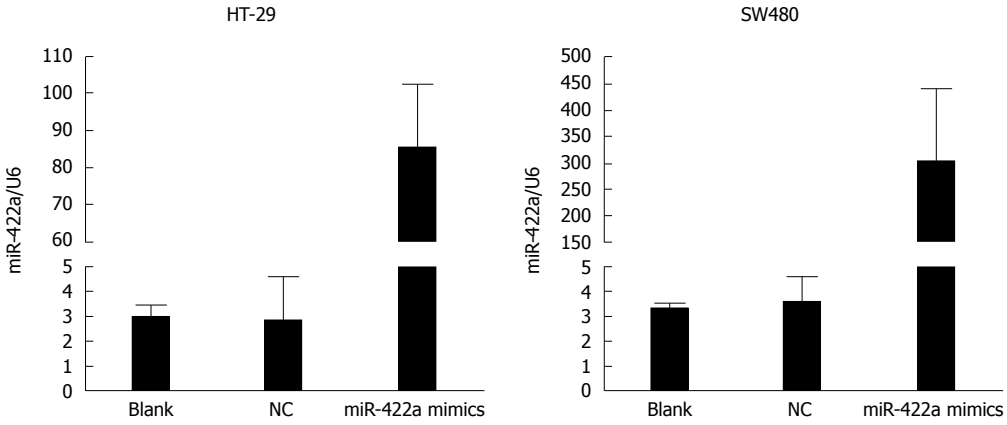

Figure 3 Transfection efficiency assays. The expression of miR-422a in HT-29 and SW480 cells was significantly increased after transfection with the mimics. All experiments were performed in triplicate.

than those with high miR-422a ($P = 0.0028$; Figure 2A). Furthermore, patients were divided into three groups (decreased, no change, and increased) based on the change of miR-422a expression compared to adjacent normal mucosa. Kaplan-Meier survival analysis showed that patients with decreased miR-422a expression had a significantly poorer prognosis than those with increased miR-422a expression ($P < 0.001$; Figure 2B). There was no significant difference between the decreased miR-422a group and no change group. In addition, Cox regression multivariate analysis was performed to determine whether miR-422a was an independent factor of overall survival in CRC patients. The analysis revealed that miR-422a expression (HR = 0.568, 95%CI: 0.245-1.082; $P = 0.015$) and clinical TNM stage (HR = 2.942, 95%CI: 1.426-4.378; $P = 0.003$) were independent prognostic factors for overall survival in CRC patients (Table 2).

Overexpression of miR-422a inhibits CRC cell proliferation

In our preliminary experiments, we detected the expression levels of miR-422a endogenously in several CRC cell lines, including HT-29, SW480, SW620 and HCT-116. The results showed that the levels of miR-422a were quite low in these CRC cells, and there were no significant differences in the expression level of miR-422a among these cell lines (data not shown). To measure the biological properties of miR-422a in CRC cells, miR-422a mimics were transiently transfected into HT-29 and SW480 cells, respectively. Subsequently, real-time RT-qPCR was performed, which showed high transfection efficiencies in both cell lines (Figure 3). The results of MTT assay showed that transfection of miR-422a mimics could significantly decrease cell number in SW480 and HT29 CRC cells ($P < 0.05$; Figure 4). It means that overexpression of miR-422a can inhibit the proliferation potential of SW480 and HT29 CRC cells.

Effect of miR-422a overexpression on cell migration and invasion

The transwell assays with and without Matrigel were performed to determine the effects of miR-422a on the migration and invasiveness of CRC cells. Overexpression of miR-422a significantly inhibited the migration of SW480 (P = 0.017; Figure 5A and B) and HT-29 cells ($P = 0.007$; Figure 5C and D). Furthermore, transwell assay with Matrigel showed that the invasiveness of these cells was significantly suppressed in cells transfected with miR-422a compared with miR control (Figure 6A-D).

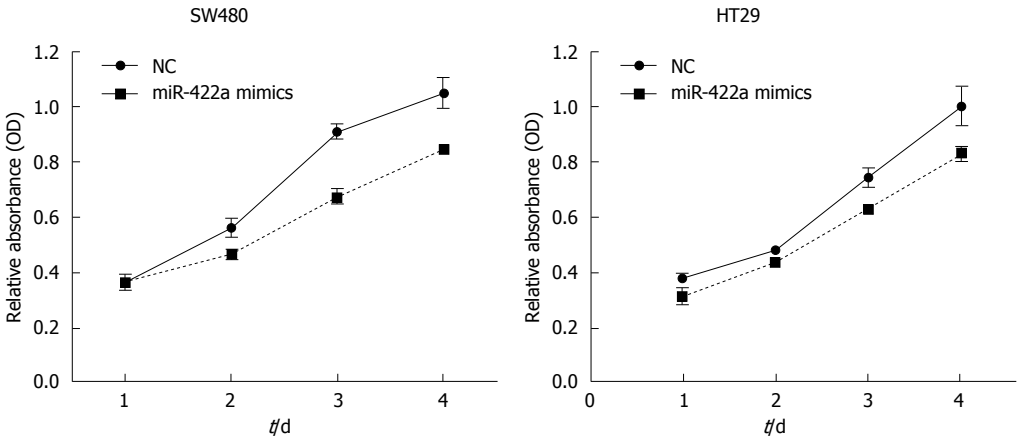

Figure 4 Overexpression of miR-422a inhibits colorectal cancer cell proliferation. Growth curves of miR-422a mimics and negative control mimics-transfected CRC cells were plotted after CCK-8 assay in SW480 and HT29 cells. All experiments were performed in triplicate. NC: Negative control.

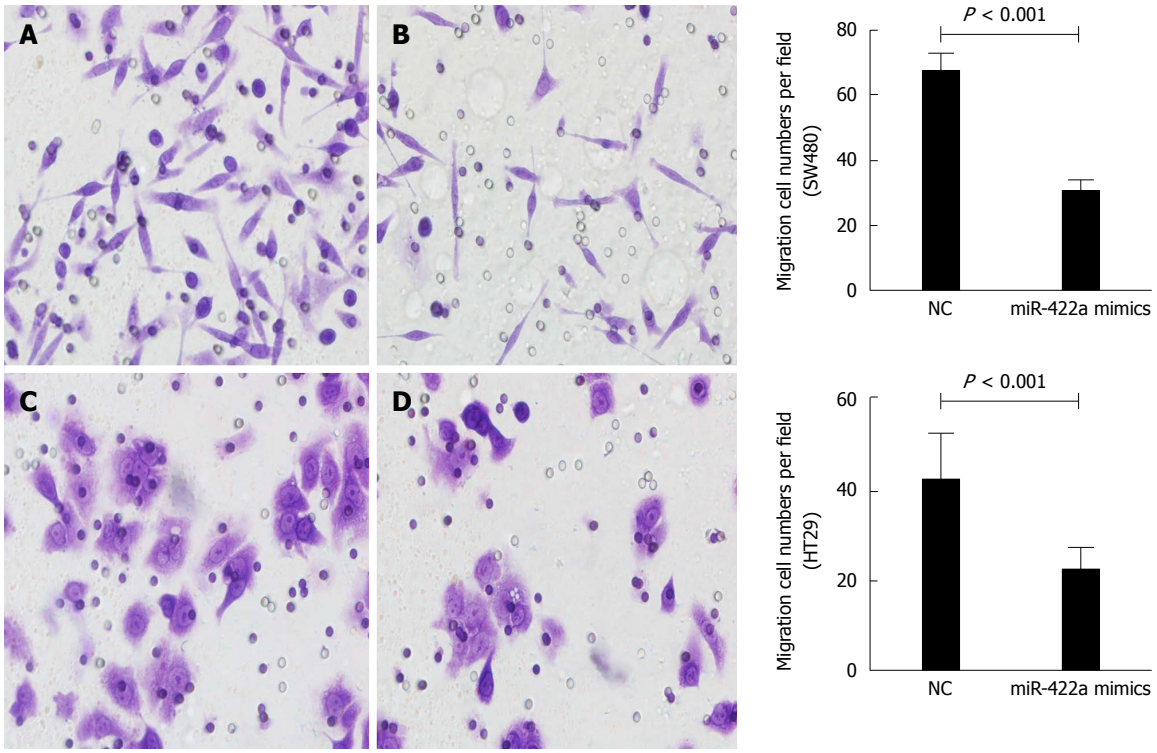

Figure 5 Effect of miR-422a overexpression on cell migration. Overexpression of miR-422a significantly inhibited the migration of SW480 (A and B) and HT29 (C and D) cells after transient transfection with miR-422a mimics. Stained cells were counted in five randomly selected fields under a light microscope. Representative photographs (left) and quantification (right) are shown. Magnification: × 400. All assays were performed in triplicate to derive the confidence intervals.

DISCUSSION

Tumor progression in CRC is a multi-step process involving a large number of genetic and epigenetic alterations[17-19]. Numerous studies have showed that miRNA target genes are involved in CRC tumorigenesis[6-8,20]. Currently, there is an ongoing effort to elucidate new deregulated miRNAs and their roles in CRC progression.

miR-422a is encoded by gene MIR422A on 15q22.31 (64, 163, 129-64, 163, 218bp). Recently, several studies have studied the functional role of miR-422a in osteosarcoma, osteoporosis, HIV infection, and other tumor types[21-25]. Our previous study demonstrated that serum miR-422a was significantly downregulated in CRC patients and is a potential biomarker with a high diagnostic accuracy[15]. However, the expression and role of miR-422a in CRC tissues, as well as its clinicopathological and prognostic value in CRC tumorigenesis remain undefined. In this study, our results demonstrated that miR-422a was down-regulated in CRC tissues compared with normal mucosa, which is consistent with a previous study[14]. We also found that the expression of miR-422a was significantly associated with lymph node metastasis and clinical stage in CRC

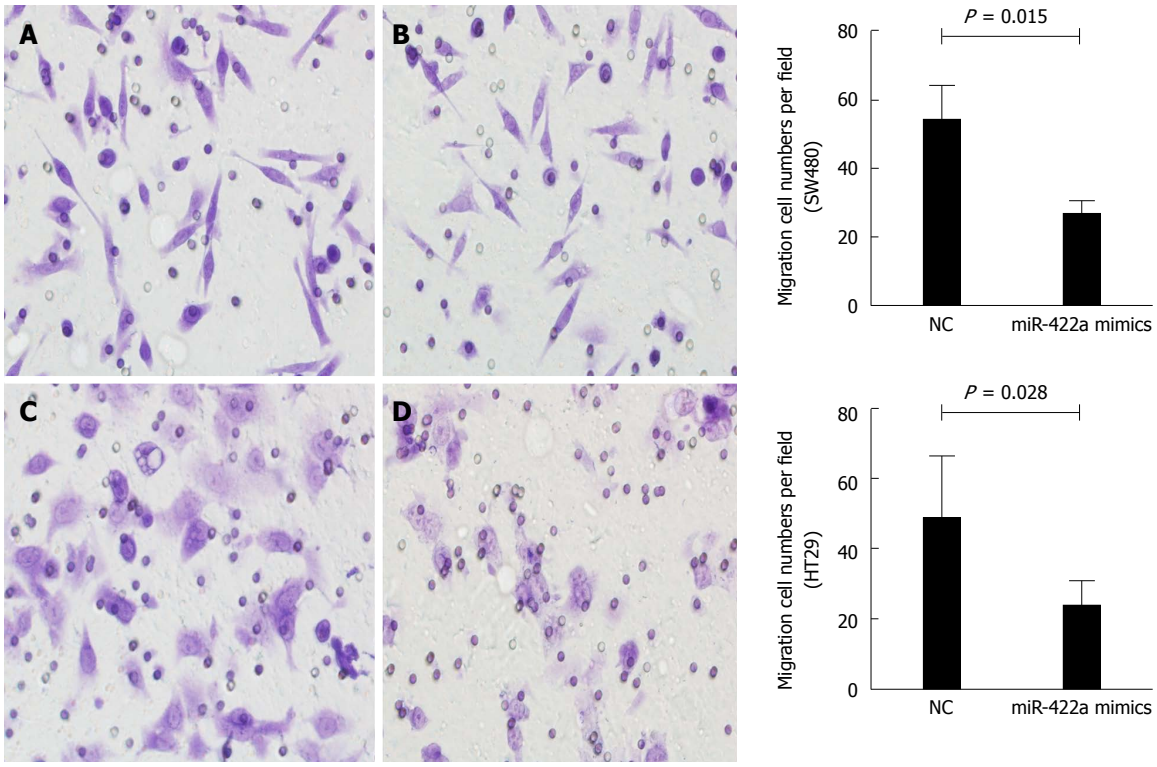

Figure 6 Effect of miR-422a overexpression on cell invasion. Overexpression of miR-422a significantly inhibited the invasiveness of in SW480 (A and B) and HT29 (C and D) cells after transient transfection with miR-422A mimics. Stained cells were counted in five randomly selected fields under a light microscope. Representative photographs (left) and quantification (right) are shown. Magnification: × 400. All assays were performed in triplicate to derive the confidence intervals.

patients, suggesting that down-regulation of miR-422a might participate in CRC progression. To further explore the value of miR-422a as a prognostic factor, we investigated the correlation between miR-422a and overall survival in CRC patients. Our results showed that patients with low expression of miR-422a and decreased miR-422a expression compared with normal mucosa had poorer survival, suggesting that miR-422a could serve as an independent prognostic factor in CRC patients. Overall, these results suggest that miR-422a plays a protective role against CRC and could be used to evaluate prognosis in CRC patients.

Until now, few studies have described the function of miR-422a[13,22,26]. A study has demonstrated that miR-422a and the mismatch repair protein Mutlα (MLH1) are involved in a feedback loop, leading to genome instability and tumorigenesis[13]. Another study showed that miR-422a significantly modulated the efficacy of IFN-α/RBV treatment *in vivo*. Exogenous IFN-α treatment led to decreased miR-422a and likely contributed to the IFN-mediated suppression of HIV-1, suggesting that restoring miR-422a treatment could be a potential therapeutic strategy for HIV-1 infection[26]. Moreover, overexpression of human telomerase reverse transcriptase (hTERT) has been associated with the invasion and metastasis of CRC cells. miR-138-5p and miR-422a were found to be hTERT-targeting miRNAs, potentially inhibiting hTERT expression[26]. Invasion and migration are required for tumor cells to spread from the primary site to lymph or blood vessels. In the present study, we found that miR-422a expression was associated with lymph node metastasis and overexpression of miR-422a inhibited CRC cell proliferation, migration and invasion. These results suggest that multiple signaling pathways regulating different aspects of tumorigenesis may be regulated by miR-422a expression, suggesting that miR-422a may be a new therapeutic target to repress cancer progression.

COMMENTS

Background

A growing number of studies have shown that deregulated miRNAs play critical roles in tumorigenesis and progression of colorectal cancer (CRC). Nevertheless, an ongoing effort to elucidate new deregulated miRNAs and their roles in CRC is still urgently needed. Several studies have demonstrated the importance of miR-422a in different types of human diseases. In our previous study, miR-422a was found to be down-regulated in serum and could be used as a potential biomarker for CRC. Therefore, it is necessary to further determine the expression of miR-422a in CRC tissues and to explore its clinicopathological, prognostic value and role in CRC carcinogenesis.

Research frontiers

Several studies have determined the importance of miR-422a in human diseases such as cancer, multiple sclerosis, and postmenopausal osteoporosis. miR-422a could inhibit signaling pathways regulating tumor cell proliferation in osteosarcoma. It was also shown that miR-422a targeted key mismatch repair protein (MutLα) by suppressing MLH1 expression, resulting in genome instability and tumorigenesis. Previously, we identified a serum 4-miRNA panel that included miR-19a-3p, miR-92a-3p, miR-223-3p, and miR-

422a. This miRNA panel served as biomarkers for early diagnosis of colorectal adenocarcinoma.

Innovations and breakthroughs

The present study indicated that down-regulation of miR-422a might serve as an independent prognosis factor in CRC. miR-422a functions as a tumor suppressor and regulates progression of CRC.

Applications

By understanding the differential expression of miR-422a in CRC patients and its relationship with clinicopathological characteristics, prognosis and *in vitro* function, the present study may provide a new prognostic factor of CRC and further reveal the mechanism of miR-422a participating in CRC carcinogenesis.

Terminology

MicroRNAs are a class of short non-coding RNAs that regulate up to 30% of human genes and play a pivotal role in various cellular processes. A growing number of miRNAs have been implicated in the initiation and progression of tumors including miR-422a. miR-422a is encoded by gene *MIR422A* on 15q22.31 (64,163,129-64,163,218bp), which has been found to be important in human diseases such as cancer, multiple sclerosis, and postmenopausal osteoporosis.

Peer-review

The authors presented a study analyzing the prognostic value and function of miR-422a in CRC carcinogenesis. The results demonstrated that miR-422a was dramatically reduced in CRC tissues and significantly correlated with local invasion and lymph node metastasis. miR-422a expression was an independent prognostic factor for overall survival. *In vitro* experiments showed that overexpression of miR-422a inhibited the proliferation, migration, and invasion of SW480 and HT-29 cells. These data indicated that miR-422a might serve as an independent prognosis factor and regulate progression of CRC by functioning as a tumor suppressor.

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ORIGINAL ARTICLE

Retrospective Study

Colostomy is a simple and effective procedure for severe chronic radiation proctitis

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Author contributions: Yuan ZX and Ma TH contributed equally to this work; Yuan ZX, Ma TH and Wang L conceived and designed the study; Yuan ZX, Zhong QH, Yu XH, and Qin QY and Wang L conducted the experiments; Yuan ZX, Ma TH, Wang HM and Wang JP analyzed and interpreted the data; Yuan ZX, Ma TH and Wang L wrote and revised the manuscript; all authors approved the final version to be published.

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Institutional review board statement: The study was approved by the Ethical Committee of the Sixth Affiliated Hospital of Sun Yat-Sen University and fulfilled the guidelines of the local responsible governmental agency.

Informed consent statement: Informed consent was waived due to the retrospective nature of this study.

Conflict-of-interest statement: The authors declare no conflicts of interests related to the publication of this study.

Data sharing statement: No additional data was available.

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Abstract

AIM: To assess the efficacy and safety of diverting colostomy in treating severe hemorrhagic chronic radiation proctitis (CRP).

METHODS: Patients with severe hemorrhagic CRP who were admitted from 2008 to 2014 were enrolled into this study. All CRP patients were diagnosed by a combination of pelvic radiation history, clinical rectal bleeding, and endoscopic findings. Inclusion criteria were CRP patients with refractory bleeding with moderate to severe anemia with a hemoglobin level < 90 g/L. The study group included patients who were treated by diverting colostomy, while the control group included patients who received conservative treatment. The remission of bleeding was defined as complete cessation or only occasional bleeding that needed no further treatment. The primary outcome was bleeding remission at 6 mo after treatment. Quality of life before

treatment and at follow-up was evaluated according to EORTC QLQ C30. Severe CRP complications were recorded during follow-up.

RESULTS: Forty-seven consecutive patients were enrolled, including 22 in the colostomy group and 27 in the conservative treatment group. When compared to conservative treatment, colostomy obtained a higher rate of bleeding remission (94% *vs* 12%), especially in control of transfusion-dependent bleeding (100% *vs* 0%), and offered a better control of refractory perianal pain (100% *vs* 0%), and a lower score of bleeding ($P < 0.001$) at 6 mo after treatment. At 1 year after treatment, colostomy achieved better remission of both moderate bleeding (100% *vs* 21.5%, $P = 0.002$) and severe bleeding (100% *vs* 0%, $P < 0.001$), obtained a lower score of bleeding (0.8 *vs* 2.0, $P < 0.001$), and achieved obvious elevated hemoglobin levels ($P = 0.003$), when compared to the conservative treatment group. The quality of life dramatically improved after colostomy, which included global health, function, and symptoms, but it was not improved in the control group. Pathological evaluation after colostomy found diffused chronic inflammation cells, and massive fibrosis collagen depositions under the rectal wall, which revealed potential fibrosis formation.

CONCLUSION: Diverting colostomy is a simple, effective and safe procedure for severe hemorrhagic CRP. Colostomy can improve quality of life and reduce serious complications secondary to radiotherapy.

Key words: Chronic radiation proctitis; Rectal bleeding; Diverting colostomy; Quality of life; Serious complication

Core tip: The study describes the efficacy and safety of diverting colostomy in treating severe hemorrhagic chronic radiation proctitis. The procedure focuses on improving the severe refractory bleeding and reducing severe complications. The advantages of diverting colostomy are as follows: it acts effectively and rapidly in controlling severe bleeding that does not respond to conservative treatment; it is a simple procedure that can be conducted in many medical centers; and it can improve quality of life dramatically and reduce serious complications that occur secondary to radiotherapy.

Yuan ZX, Ma TH, Wang HM, Zhong QH, Yu XH, Qin QY, Wang JP, Wang L. Colostomy is a simple and effective procedure for severe chronic radiation proctitis. *World J Gastroenterol* 2016; 22(24): 5598-5608 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5598.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5598

INTRODUCTION

Chronic radiation proctitis (CRP) is a common complication after radiotherapy of pelvic malignancies, accounting for 5%-20% of cases[1]. The onset of RP can be delayed for several months to years after radiotherapy. CRP develops as a result of ischemic lesions due to obliterative endarteritis and progressive fibrosis[2,3]. Rectal bleeding is the most common symptom, which accounts for > 80% of CRP patients[4]. Acute and mild CRP is usually self-limiting and easy to manage, but moderate to severe CRP is difficult to treat; especially those cases requiring blood transfusions and that are life threatening[1,5].

Various treatment modalities have been tried. Medical agents include topical sucralfate, steroids[6], sulfasalazine[7], metronidazole[8], rebamipide[9] and short-chain fatty acid[10]. Other treatment options include topical formalin[11,12], endoscopic argon plasma coagulation (APC)[13], laser therapy[14], and hyperbaric oxygen therapy[15]. However, most of these treatments are only useful for mild to moderate bleeding, and severe and refractory bleeding is still problematic[16]. Furthermore, endoscopic treatments can bring severe side effects and multiple treatment sessions are needed for severe CRP[17]. In addition, accompanying symptoms such as intractable perianal pain, urgency and tenesmus in CRP are usually hard to manage.

Diverting colostomy has been reported previously, mainly for severe CRP complications[18,19]. Colostomy can reduce the irritation injury of fecal stream to the irradiated tissues and thus decrease rectal bleeding. However, unlike formalin or APC, colostomy is now not widely used. The issue of colostomy is not well studied to date. To the best of our knowledge, no study has compared diverting colostomy to conservative treatment. The aim of this study was to assess the efficacy and safety of diverting colostomy in severe CRP. The indications, quality of life, severe CRP complications, and stoma reversals after colostomy were also investigated, when compared to conservative treatment.

MATERIALS AND METHODS

Patients and ethical statements

Hemorrhagic CRP patients who were treated at the Sixth Affiliated Hospital of Sun Yat-Sen University (SYSU) from March 2008 to October 2014 were retrospectively enrolled in this study. Electronic files and medical records were both carefully collected to extract clinicopathological data. This study was approved by the Ethical Committee of the Sixth Affiliated Hospital of SYSU and the study was conducted in accordance with the provisions of the World Medical Association's Declaration of Helsinki in 1995 (revised in Tokyo, 2004). Due to the nature of

Table 1 Modified subjective objective management analysis system to assess the severity of bleeding in radiation proctitis

Grade	Bleeding	Severity	Anemia (Hb, g/L)
1	Mild bleeding	Occasionally or occult	Mild anemia (Hb: ≥ 90 g/L)
2	Moderate bleeding	Persistent	Moderate anemia (Hb: 70-90 g/L)
3	Severe bleeding	Gross	Severe anemia, transfusion needed (Hb: < 70 g/L)

Hb: Hemoglobin.

the retrospective study, informed consent was waived.

Inclusion and exclusion criteria

Inclusion criteria were CRP patients with refractory bleeding with moderate to severe anemia with a hemoglobin level < 90 g/L. Refractory bleeding was defined as no response to conservative treatment. Patients who were treated with diverting stomas were enrolled in the study group, while those who continued to non-surgical treatment were enrolled in the control group. Patients with tumor relapses, loss to follow-up, or who underwent rectal resection with preventive colostomy were excluded.

Diagnosis, scores and definitions

All patients were diagnosed by combination of pelvic radiation history, clinical rectal bleeding, and endoscopic findings of injured rectal mucosa. Flexible colonoscopy was performed in all patients to rule out other causes of bleeding, such as recurrent tumors, inflammatory bowel disease and anal benign hemorrhagic diseases. The Vienna Rectoscopy Score[20] system was used to assess endoscopic severity.

Current scores to evaluate the severity of bleeding included Common Terminology Criteria for Adverse Events[21], Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer (EORTC) score[22]. However, most of them are based on subjective complaints of patients, instead of accurate laboratory tests. Because the severity of bleeding was mainly reflected by a decrease in hemoglobin level, we designed a modified Subjective Objective Management Analysis (SOMA) system reported in a previous study[23], to assess the severity of bleeding, included both subjective complaints of bleeding and objective hemoglobin level. All patients were scored by the system (Table 1). The remission of bleeding was defined as complete cessation of bleeding or only occasional bleeding that needed no further treatment. Failure in the conservative treatment group was defined as no improvement or even worse bleeding and decreased hemoglobin level 6 mo after treatment.

Indications of diverting colostomy

All patients, except those with fistulas, were initially treated with medical agent enemas including almagate (one mucosa-protector like sucralfate), corticosteroids, and metronidazole. Topical formalin (details listed in our previous study)[24] or endoscopic APC were suggested when they experienced recurrent bleeding. As for refractory and transfusion-dependent CRP after these conservative measures, physicians suggested a diverting stoma. If patients refused a colostomy and demanded to continue conservative treatment, they were enrolled in the control group. Other indications of diverting colostomy were as follow: (1) fistula, perforation or stricture; and (2) deep ulcer with refractory perianal pain.

Diverting loop colostomies were conducted under general anesthesia in the operating room. The transverse colon was pulled out through a small incision, then a soft catheter of a stent was inserted to prevent stoma retraction, and a double-cavity stoma of the transverse colon was then created. The catheter was removed postoperatively. Classical images of a double-cavity colostomy and a "gunsight" of stoma closure were shown (Figure 1). This technique of stoma closure can simplify wound care, decrease surgical site infection, and give a neat cosmetic result[25,26].

Follow-up

Follow-up was scheduled through outpatient visits or telephone questionnaires at 6 mo and 1 year after treatment. The quality of life before treatment and at follow-up was evaluated according to EORTC QLQ C30[27]. The primary outcome was the remission rate of bleeding at 6 mo after treatment. The secondary outcomes included hemoglobin level, remission rate of bleeding at 1 year after treatment, quality of life, stoma-related complications, severe CRP complications, and stoma reversal rate.

Statistical analysis

Comparisons of characteristics were made by Student's *t* test analysis for continuous variables. For categorical variables, the χ^2 test was used. Fisher's exact test was adopted when appropriate. For non-parameter variables, the Wilcoxon rank sum test was used. All statistical analyses were performed by SPSS version 17.0 (Chicago, IL, United States). $P < 0.05$ (two-tails) was considered to be statistically significant.

RESULTS

Demographics and characteristics

A total of 47 patients were analyzed. Twenty-two (46.8%) were treated by diverting colostomy, and 27 (53.2%) were managed by conservative treatment (Figure 2). Forty-three (91.5%) were women, and 40 (85.1%) primary malignancies were cervical cancer. Cumulative radiation dosage of one patient was about 80 Gy, which included the radiation for both sites

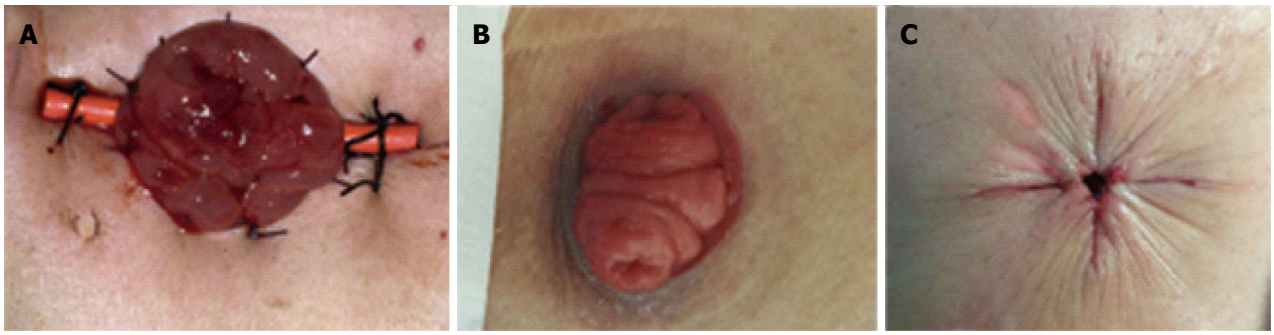

Figure 1 Classic representative images of a double-cavity colostomy. A: A double-cavity colostomy with a soft catheter as a stent; B: A colostomy after during follow-up; C: A "gunsight" skin incision and closure for stoma reversal.

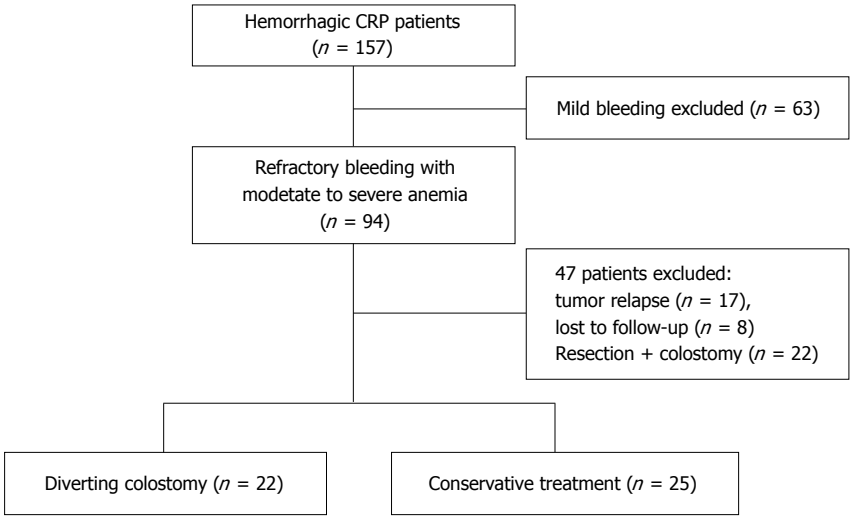

Figure 2 Flow chart of patient selection.

of primary malignancy and invasive lymph nodes. The detailed radiotherapy for those patients with gynecological cancers, especially cervical cancer, was 25 rounds (2 Gy/round) of external beam radiation and five or six episodes (6 Gy/episode) of intra-cavity brachytherapy. Patients with prostate or rectal cancers received only external beam radiation. When comparing demographics prior to treatment between the two groups, there were no significant differences in age, gender, type of primary malignancy, cumulative radiation dosage, latency period, duration from treatment to end of radiotherapy, duration of bleeding, albumin level, body mass index (BMI), concomitant radiation uropathy, radiation enteritis, and associated risk factors of CRP such as previous history of abdominal surgery, diabetes mellitus and hypertension (Table 2). Thus, these above characteristics were comparable between the two groups. However, the colostomy group had a higher score of bleeding (2.7 *vs* 2.0, $P < 0.001$) and a lower hemoglobin level (60.8 g/L *vs* 88.2 g/L, $P < 0.001$), when compared to the conservative treatment group, respectively. These results indicated that the colostomy group had more serious bleeding before treatment (Table 2).

Treatment

The indications for colostomy were as follows: (1) severe bleeding in eight (36.4%) cases; (2) fistulas in 11 (50%), including nine (40.9%) rectovaginal fistulas and two (9.1%) sigmoid-vesical-vaginal fistulas; (3) deep ulcer + refractory perianal pain in two (9.1%) cases; and (4) severe bleeding + deep ulcer + anal stricture in one (4.5%) case. Among these 11 patients with fistulas, five also had concomitant severe bleeding. Among these nine recto-vaginal fistulas, one had concomitant recto-urethral fistula, and another had concomitant recto-vesical fistula and small bowel fistula.

In the conservative treatment group, seven patients received topical formalin irrigation and one received APC treatment after enrollment. All eight (32%) cases transiently obtained bleeding remission, but only two (25%) obtained long-term remission of bleeding. The other six patients experienced recurrent bleeding and developed severe anemia. Repeat topical formalin achieved only limited efficacy in these patients with severe anemia (average 2 sessions of formalin at 2-4-wk intervals). The remaining 17 patients refused formalin treatment, and thus continued retention

Table 2 Comparisons of patient demographics between the colostomy group and conservative group before treatment *n* (%)

Characteristics	Diverting colostomy (*n* = 22)	Conservative treatment (*n* = 25)	*P* value
Age (mean ± SD)	60.1 ± 2.2	60.2 ± 2.4	0.964
Gender (female/male)	20/2	23/2	1.000[1]
Primary malignancy			0.822[2]
Cervical cancer	19 (86.4)	21 (84)	
Endometrial cancer	2 (9.1)	2 (8)	
Rectal cancer	1 (4.5)	1 (4)	
Prostate cancer	0 (0)	1 (4)	
Cumulative radiation dosage (Gy), mean ± SD	80.5 ± 17.3	83.6 ± 20.5	0.842[3]
Latency period (mo), mean ± SD	8.3 ± 0.8	7.2 ± 1.1	0.252[3]
Duration from treatment to end of radiotherapy (mo), mean ± SD	16.3 ± 1.3	14.8 ± 1.6	0.277[3]
Duration of bleeding (month), mean ± SD	7.9 ± 1.0	8.0 ± 1.8	0.466[1]
Score of bleeding, mean ± SD	2.7 ± 0.5	2.0 ± 0.5	< 0.001[3]
Mean hemoglobin (g/L), mean ± SD	60.8 ± 18.1	88.2 ± 19.3	< 0.001
Alb (g/L), (≤ 35/> 35)	6/16	3/22	0.339[1]
BMI (kg/m^2), (≤ 17.5/> 17.5)	3/19	2/23	0.880[1]
Concomitant radiation uropathy	8 (36.4)	6 (24)	0.355
Radiation enteritis	5 (22.7)	2 (8)	0.315[1]
Previous abdominal surgery	6 (27.3)	11 (44)	0.234
Diabetes mellitus	2 (9.1)	3 (12)	1.000[1]
Hypertension	6 (27.3)	7 (28)	0.956

[1]χ^2 test; [2]Fisher exact test; [3]Wilcoxon rank-sum test. BMI: Body mass index; Alb: Albumin.

Table 3 Bleeding remissions in severe radiation proctitis after treatment

Variables	Diverting Colostomy (*n* = 22)	Conservative treatment (*n* = 25)	*P* value
6 mo after treatment			
Remission of bleeding	17/18 (94%)	3/25 (12%)	< 0.001[1]
Remission of refractory perianal pain	8/8 (100%)	0/6 (0%)	< 0.001[2]
Score of bleeding, mean ± SD	1.1 ± 0.5	2.2 ± 0.7	< 0.001[3]
Elevated Hb, mean ± SD	34.1 ± 18.2	-12.3 ± 9.1[4]	< 0.001
Remission of moderate bleeding	8/8 (100%)	6/19 (21.5%)	0.002[2]
Remission of severe bleeding	11/11 (100%)	0/5 (0)	< 0.001[2]
Score of bleeding, mean ± SD	0.8 ± 0.5	2.0 ± 0.9	< 0.001[3]
Post-treatment recto-vaginal fistula	0/22	3/25 (12%)	0.237[2]
Elevated Hb, mean ± SD	40.3 ± 19.3	-1.9 ± 32.5[4]	0.003

[1]χ^2 test; [2]Fisher exact test; [3]Wilcoxon rank-sum test; [4]Represents decreased level.

enemas and transfusions when needed.

Outcomes

During a mean 22 (range: 6-77) mo of follow-up, eight (17%) patients died. The cause of death was recurrent malignancy in seven cases. The other one died of bladder perforation and sepsis that occurred secondary to radiation recto-vesical-vaginal perforation. At 6 mo after treatment, colostomy offered higher remission of bleeding (94% *vs* 12%, $P < 0.001$), higher remission of refractory perianal pain (100% *vs* 0%, $P < 0.001$), decreased scores of bleeding (1.1 *vs* 2.2, $P < 0.001$), and obvious increased hemoglobin levels (34.1 g/dL *vs* -12.3 g/dL, $P < 0.001$), compared to the conservative treatment group. At 1 year after treatment, colostomy achieved still higher remission of both moderate bleeding (100% *vs* 21.5%, $P = 0.002$) and severe bleeding (100% *vs* 0%, $P < 0.001$), acquired lower score of bleeding (0.8 *vs* 2.0, $P < 0.001$), and obviously elevated hemoglobin levels (40.3 g/dL *vs* -1.9 g/dL, $P = 0.003$), than those cases in the conservative treatment group. In addition, three recto-vaginal fistulas were found in the conservative treatment group during follow-up, but no new fistula occurred after the operation in the colostomy group. Patients who did not have bleeding remission continued conservative treatment at home (Table 3).

Stoma closure and complications

Of the eight patients who received colostomy to control severe bleeding, three (37.5%) underwent stoma closure (2 cases at 9 mo and 1 at 10 mo after colostomy). All three had no bleeding and remained well after stoma reversal. Of the remaining five, all obtained bleeding remission and improved hemoglobin levels. However, among these five, one had grade IV New York Heart Association heart failure and could not risk stoma closure. Four were unsuitable for closure

Table 4 Quality of life between diversion group and conservative group in CRP patients by EORTC QLQ-C30 scale

QLQ-C30 scale	Ref. (Normal German population)	Diverting colostomy group (n = 18), mean (SD)				Conservative treatment group (n = 23), mean (SD)			
		Pre-treatment	Follow-up	Δ(FU)-Pre[1]	Significance[2]	Pre-treatment	Follow-up	Δ(FU)-Pre	Significance[2]
Global health	63.2	23.1 (15.1)	64.8 (13.8)	41.7	< 0.001	47.1 (21.5)	62.3 (25.0)	15.2	0.033
Physical function	82.6	50.7 (17.8)	77.8 (16.6)	27.1	< 0.001	78.0 (22.7)	78.6 (26.1)	0.6	0.856
Role function	75.0	34.3 (23.9)	75.9 (27.3)	41.6	< 0.001	77.5 (24.9)	77.5 (29.1)	0.0	0.775
Emotional function	62.2	46.3 (27.4)	73.6 (27.0)	27.3	0.001	75.7 (17.6)	80.8 (23.8)	5.1	0.384
Cognition function	81.3	92.6 (12.7)	93.5 (9.8)	0.9	0.581	94.2 (15.6)	95.7 (9.0)	1.5	0.798
Social function	78.4	43.5 (28.9)	65.7 (31.7)	22.2	0.004	91.3 (20.6)	89.1 (21.1)	-2.2	0.916
Fatigue	34.1	72.8 (12.9)	36.4 (25.9)	-36.4	< 0.001	26.6 (24.1)	23.2 (27.2)	-3.4	0.695
Nausea/vomiting	5.7	4.6 (15.5)	1.9 (7.6)	-2.7	0.109	5.1 (15.4)	6.5 (16.5)	1.4	0.655
Pain	33.1	44.4 (28.3)	14.8 (22.8)	-29.6	0.001	14.5 (21.5)	11.6 (18.4)	-2.9	0.481
Dyspnea	18.8	42.6 (31.0)	18.5 (22.8)	-24.1	0.003	14.5 (19.7)	8.7 (18.0)	-5.8	0.210
Insomnia	38.5	48.1 (35.5)	29.6 (31.2)	-18.5	0.026	21.7 (21.6)	26.1 (31.7)	4.4	0.287
Appetite loss	9.4	22.2 (27.2)	13.0 (19.7)	-9.2	0.125	10.1 (25.5)	8.7 (18.0)	-1.4	0.785
Constipation	9.1	9.3 (14.9)	3.7 (10.5)	-5.6	0.066	8.7 (20.6)	7.2 (22.4)	-1.5	0.414
Diarrhea	9.2	33.3 (33.3)	22.2 (24.8)	-11.1	0.018	11.6 (23.8)	2.9 (9.6)	-8.7	0.078
Financial difficulties	17.1	59.3 (26.2)	50.0 (31.9)	-9.3	0.211	26.1 (31.7)	24.6 (30.5)	-1.5	0.595

[1]Wilcoxon rank-sum test; [2]Point (follow-up) - point (pre-treatment).

because of erythema and telangiectasia at 6 mo after colostomy, and two of these four had not yet reached 1 year follow-up to assess the lesion by colonoscopy.

Stoma complications were found in seven (31.8%) cases, which contained six stoma prolapses and one stoma stricture. Of these six stoma prolapses, four were managed with conservative measures by manual repositions (Grade Ⅱ by Clavien-Dindo classification[28]), and two required stoma rebuilding (Grade Ⅲ). One stoma stricture occurred in a stoma of the descending colon, instead of the transverse colon, and stoma stricture was managed by finger dilatation (Grade Ⅱ).

Quality of life

The quality of life was evaluated in 41 (87.2%) patients by the EORTC QLQ-C30 questionnaires. Because there were no similar reports in the Chinese population, the values were referred to the normal German population. Osoba *et al*[29] suggested that a difference of ⩾ 20 points in global health was considered to be clinically relevant. In this study, when compared to pre-treatment, diverting colostomy improved quality of life, including improved global health (difference = 42, $P < 0.001$), improved functions like physical function ($P < 0.001$), role function ($P < 0.001$), emotional function ($P < 0.001$), social function ($P < 0.001$), and improved symptoms like fatigue ($P < 0.001$), pain ($P = 0.001$), dyspnea ($P = 0.003$), insomnia ($P = 0.026$), and diarrhea ($P = 0.018$). However, conservative treatment did not significantly improve quality of life at follow-up compared with before treatment (Table 4).

Endoscopic and pathological features

Classical endoscopic images prior to colostomy and at stoma closure from three patients with stoma closures were collected (Figure 3). After a mean 9.3 (range: 9-10) mo after colostomy, endoscopic lesions of active bleeding, multiple confluent telangiectasia, congested mucosa, or even ulcer were greatly improved and reached the criteria of stoma closure.

Pathological evaluation of endoscopic biopsy from rectal lesions was conducted at 31 mo after colostomy in Case 3. This patient obtained complete remission of bleeding and the biopsy sites were fully healed according to endoscopic observation at follow-up. Pathologically, diffuse chronic inflammatory cells were observed in the mucosa and sub-mucosa layers. In addition, massive fibrotic collagen depositions were found in the sub-mucosa layer, which revealed fibrosis formation (Figure 4).

DISCUSSION

Endoscopic treatments, such as topical formalin or APC, are extensively used for mild to moderate hemorrhagic CRP worldwide[14,16]. However, these treatments show only limited long-term efficacy for severe CRP[17]. It is also unclear how many patients have received these treatments for serious transfusion-dependent bleeding in previous studies[17]. Meanwhile, serious complications can be caused by these endoscopic treatments[5,17]. Our previous study also suggested that topical formalin should not be applied in CRP with ulcer because of the risk of fistula[24]. In the present study, patients in the conservative treatment group received more topical formalin or APC treatment and developed more fistulas later, than patients in the colostomy group. Therefore, we suggest topical formalin or APC should be selected cautiously for patients with severe CRP. Recently, radiofrequency ablation (RFA) in treating CRP has been introduced, with improvement in hemoglobin level and decrease in clinical symptoms[30,31]. Most RFA studies are based

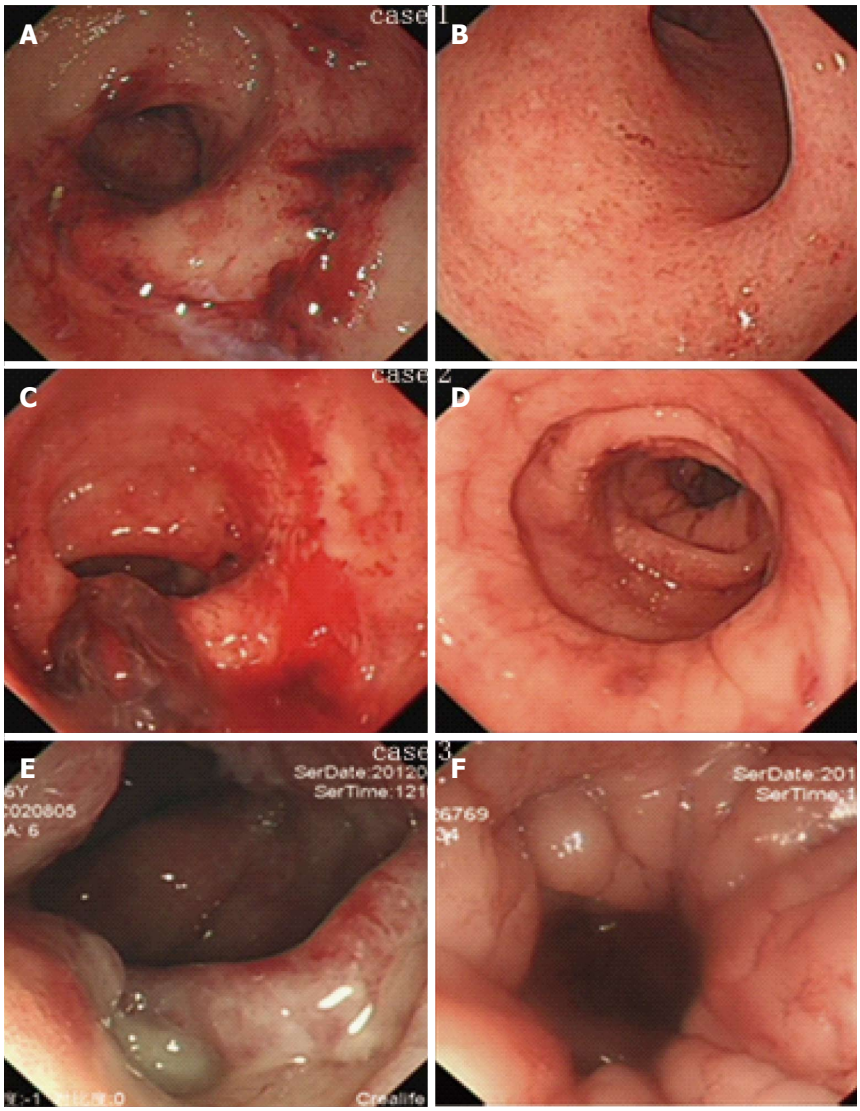

Figure 3 Classical endoscopic images before colostomy and at stoma reversal. Severe active bleeding (A and C), or confluent telangiectasia, edema and ulcer (E) were observed in cases 1-3 before colostomy, while these lesions was greatly improved at stoma reversal (B, D, F).

on retrospective case series without controls and current data are scare, therefore, prospective trials of RFA should be conducted in the future to validate its efficacy and application in severe CRP.

Surgical intervention is often the last resort for severe CRP[3,19]. Rectal resection is controversial, because it is difficult to perform a safe anastomosis in the radiation-injured tissue, and high risks of anastomotic leak and death from postoperative peritonitis are reported[19]. Therefore, a simple and safe procedure to save life and relive symptoms is mandatory. Theoretically, diverting colostomy can reduce bacterial contamination and decrease irritation injury by fecal stream, and colostomy can gain time to subside any radiation reaction to protect injured tissue[32]. Thus, severe bleeding and refractory perianal pain can be controlled. Colostomy can also accelerate the course of fibrosis and relieve severe proctitis rapidly, which may prevent deep ulcers progressing to fistulas. In our recent study[33], we reported typical histopathological features of CRP: telangiectasia, abnormal hyaline-like wall vessels and sporadic radiation fibrocytes in the submucosal layer. In this study, consistently, massive fibrotic collagen depositions were observed in irradiated tissue after colostomy. Collectively, the nature of CRP is a progressive fibrosis course[7]. The efficacy and safety of colostomy have been reported in previous studies[18,19,34]. However, most previous studies did not contain controls, which could not discriminate the efficacy of interventions from the self-remission course. To the best of our knowledge, no previous study has reported quality of life after colostomy. It is also not clear whether colostomy can reduce serious CRP complications.

In this study, diverting colostomy resolved most cases of severe bleeding. No recurrent bleeding and no colostomy-related death were observed. Colostomy

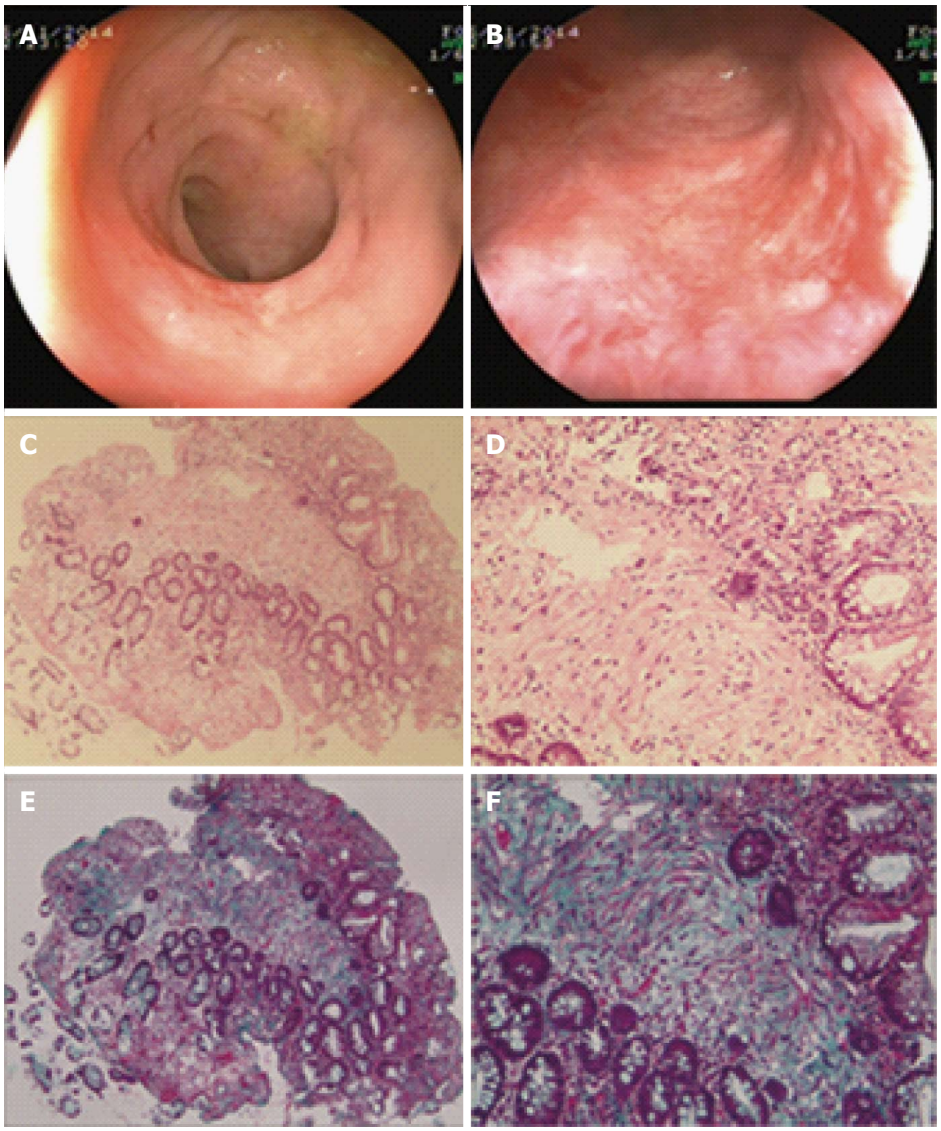

Figure 4 Histo-pathological features after colostomy in case 3. Complete remission of confluent telangiectasia, edema and ulcer were observed 31 mo after colostomy (A and B). Diffused chronic inflammatory cells and rebuilt of mucosal integrity were seen in hematoxylin and eosin-stained slides (C: × 100; D: × 400). Massive fibrotic collagen depositions were observed in the sub-mucosa layer, with green color by Masson staining (E: × 100; F: × 400).

can also decrease serious complications, including remission of long-term perianal pain, transfusion-dependent bleeding and fistula. Deep ulcer with severe bleeding is a contraindication to endoscopic treatment, because it is easy to progress to fistula, due to the poor-healing capacity and impaired blood supply of friable intestinal wall[17,19]. We have previously tried topical revision and skin flap transplantation for some CRP fistulas. However, the efficacy was limited and new fistulas can occur rapidly, due to poor healing capacity of irradiated mucosa and bacterial infection from the fecal stream, which leads to treatment failure. In this study, two patients with deep ulcer and severe bleeding were successfully controlled by colostomy, and no fistula was found at follow-up.

Transverse colostomy is preferred because it provides a greater blood supply by preserving superior rectal and left marginal vessels, and provides more options for later possible recto-sigmoid resection than sigmoid colostomy does, and transverse colostomy is easier to be closed and is more effective[32,34]. In addition, the recto-sigmoid colon is expected to receive a higher radiation dose for pelvic malignancy, while the transverse colon receives the least radiation and damage, because it is located far from pelvic tumors, and thus causes the fewest complications[19,32,35]. Loop ileostomy is not widely used because of the risk of high-volume fluid discharge. Colostomy-related complications were reported, ranging from 21.8% to 40%[19,35]. Consistent with previous studies, colostomy-related complications in this study were 31.8%, including six (85%) cases of Grade Ⅱ and one (15%) case of Grade Ⅲ complications. Among them, stoma prolapse was a common complication.

Colostomy for recto-vaginal fistula and rectal stricture is permanent. However, colostomy for patients

unresponsive to medical treatment can be closed when severe proctitis improves sufficiently. Anseline *et al*[19] reported six (43%) colostomy reversals in 14 CRP patients, who were unresponsive to medical treatment. A similar result was observed in the present study. Three (38%) of eight patients with severe bleeding were closed successfully in a mean 9 mo after colostomy. Because the duration of follow-up after fecal diversion was short, many patients who obtained long-term remission of bleeding after colostomy had great potential to reverse the stoma.

In this study, we used a modified SOMA system, which coordinates subjective bleeding symptoms and objective accurate hemoglobin level. According to the system, we suggest that mild to moderate hemorrhagic CRP can be managed by medical or endoscopic treatment. However, for severe refractory bleeding colostomy should be considered to prevent development of serious complications. The scoring system will guide physicians in primary care to evaluate patient condition according to hemoglobin level, and then choose the appropriate treatment. Having a routine and easy protocol can reduce treatment-related delays and avoid unnecessary morbidity[7].

In this study, 44 (94%) CRP patients enrolled had gynecological cancers, so most fistulas were documented in women. In western countries, patients with prostate cancer are the dominant population receiving pelvic radiation, and CRP is mainly reported in prostate cancer[4,12]. However, prostate cancer receives only external beam radiation such as 3D conformal radiotherapy or intensity-modulated radiotherapy, and it does not receive intra-cavity brachytherapy, thus, fewer fistulas are observed. According to our clinical practice, intra-cavity radiation can result in more fistulas and other severe adverse radiation-related symptoms than external beam radiation can.

Although the colostomy group had more severe bleeding than the conservative treatment group, which could have resulted in selection bias, it achieved dramatically better control of bleeding, higher increased hemoglobin level, and improved quality of life compared with the conservative treatment group. These results have shown the advantages of diverting colostomy in treating severe CRP bleeding. Topical formalin or APC was not used in all the patients in the conservative treatment group, because some patients in China have not sufficient knowledge of CRP, poor compliance with physicians' advice, and poor economic status. Thus, they choose to continue self-enemas at home, when recurrent bleeding occurs. In addition, this study was limited by its non-randomized, retrospective design and small sample size. Additional randomized prospective studies of diverting colostomy are needed to confirm our findings.

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COMMENTS

Background

Chronic radiation proctitis (CRP) occurs in 5%-20% of patients receiving radiotherapy for pelvic malignant tumors. Mild to moderate CRP is usually self-limiting and easy to manage, but severe and refractory bleeding is still problematic, especially in cases requiring blood transfusions and that are life threatening. Furthermore, endoscopic treatment can cause severe side effects and only limited efficacy can be obtained for severe CRP. Thus, a simple and safe treatment with fewer complications to save life and relive symptoms is mandatory.

Research frontiers

Diverting colostomy has been reported previously, mainly for severe CRP complications. However, unlike formalin or argon plasma coagulation, colostomy is now not widely used for severe bleeding in CRP patients. The issue of colostomy is not well studied to date. To the best of our knowledge, no study has compared diverting colostomy to conservative measures in treating severe hemorrhagic CRP.

Innovations and breakthroughs

In this series, the authors reported their experience that diverting colostomy was a simple, effective and safe procedure for severe hemorrhagic CRP. Furthermore, they found that colostomy improved quality of life and reduced serious complications secondary to radiotherapy, while conservative medical and endoscopic treatments did not show efficacy in severe CRP patients.

Applications

Diverting colostomy is a simple and safe procedure that can be performed in most medical centers. The authors also developed a modified Subjective Objective Management Analysis system, which coordinates subjective bleeding symptoms and objective accurate hemoglobin level, to guide physicians in primary care to evaluate patient condition according to hemoglobin level, and then choose the appropriate treatment. Having a routine and easy protocol can reduce treatment-related delays and avoid unnecessary morbidity.

Terminology

The underlying causes of CRP are endarteritis obliterans and progressive submucosal fibrosis due to radiotherapy. Diverting colostomy can reduce bacterial contamination and decrease irritation injury by the fecal stream, and can gain time to reduce any radiation reaction to protect injured tissue. Colostomy can also accelerate the course of fibrosis and relieve severe proctitis rapidly, which may prevent deep ulcers progressing to fistulas.

Peer-review

This is a single center, controlled, and retrospective case series of severe CRP patients who received diverting colostomy. Colostomy can relieve most of severe bleeding rapidly and unexpected, colostomy can also reduce serious CRP complications, including remission of long-term perianal pain, transfusion-dependent bleeding and fistula.

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ORIGINAL ARTICLE

Observational Study

Clinical study of anesthetization by dezocine combined with propofol for indolent colonoscopy

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Abstract

AIM: To assess the use of dezocine combined with propofol for the anesthetization of patients undergoing indolent colonoscopy.

METHODS: A cross-sectional survey of patients undergoing indolent colonoscopy in the Xinjiang People's Hospital was conducted from April 1 to April 30, 2015. The survey collected patient general information and anesthesia data, including overall medical experience and pain management. Thirty minutes after colonoscopy surgery, samples of venous blood were collected and the biochemical indicators of gastrointestinal function were analyzed.

RESULTS: There were 98 female and 62 male respondents. Indolent colonoscopy was found to be more suitable for mid to older-aged patients. The necessary conditions for the diagnosis of digestive diseases were required in 65 of the 73 inpatients. Adverse reactions to the intraoperative process included two cases of body movement and two cases of respiratory depression. Gastrin and vasoactive intestinal peptide levels were slightly increased. However, somatostatin and endothelin levels were slightly decreased.

CONCLUSION: This study revealed that dezocine combined with propofol can be successfully used for the anesthetization of indolent colonoscopy patients without pain and should be widely used.

Key words: Dezocine; Propofol; Colonoscopy; Patient

assessment; Anesthetization; Cross-sectional

Core tip: A cross-sectional survey of patients undergoing indolent colonoscopy was carried out to study the clinical effect of anesthetization by dezocine combined with propofol. Thirty minutes after colonoscopy surgery, samples of venous blood were collected and the biochemical indicators of gastrointestinal function were analyzed. Indolent colonoscopy was found to be more suitable for mid to older-aged patients. Gastrin and vasoactive intestinal peptide levels were slightly increased. However, somatostatin and endothelin levels were slightly decreased. This study revealed that dezocine combined with propofol can be successfully used for the anesthetization of indolent colonoscopy patients without pain and should be widely used.

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INTRODUCTION

Colonoscopy is an endoscopic examination of the large intestine and the distal part of the small intestine. It provides a diagnosis and therapeutic opportunity for colorectal lesions[1-4]. At present, colonoscopy remains the gold standard investigation for inspecting colorectal lesions such as adenomas, cancer or inflammation. In many situations, it remains preferable to other imaging examinations such as computed tomography (CT) colonography or barium enema due to the capacity to intervene and sample or remove the pathology encountered.

Unlike sigmoidoscopy which can get to the distal portion (about 600 mm) of the colon, colonoscopy provides an investigation of the full colon (1200-1500 mm)[5,6]. The pain is not caused by the scope of the insertion but rather by the inflation of the colon to allow the inspection. The scope itself is essentially a long, flexible tube about a centimeter in diameter, *i.e.*, around the diameter of the little finger, which is less than the diameter of an average stool[7-9].

Increasingly, doctors like to operate on totally anesthetized patients because it allows for a calmer examination without perceived pain or discomfort. Compared with general anesthesia, twilight sedation is safer and it also allows the patient to follow simple commands and even to watch the operation on a monitor. Therefore, twilight sedation is generally best and the operator should not rush, even although the examination may cause discomfort. Tens of millions of people need colonoscopies annually and yet many of them refuse because of concerns about the procedure[9]. Propofol is a short-acting, intravenously administered hypnotic/amnestic agent. It is usually used in the induction and maintenance of general anesthesia, sedation for mechanically ventilated adults and procedural sedation. Propofol is also used in veterinary medicine[9-11].

Recently, propofol has replaced sodium thiopental for the induction of anesthesia in many hospitals because anabiosis is faster and "clearer" using propofol. Propofol is not a pain medication so it may be combined with opioids such as fentanyl to alleviate pain. Whether this is always necessary is unclear.

Propofol has been known as "milk of amnesia" (a play on milk of magnesia) because the intravenous preparation has a milk-like appearance. It is on the World Health Organization Model List of Essential Medicines. More than 50 countries have approved it for use and generic versions are available[11-15]. Some patients may develop propofol infusion syndrome (PRIS), a rare syndrome that affects patients who undergo long-term treatment with high doses of propofol (> 4 mg/kg per hour for more than 24 h). It can result in cardiac failure, kidney failure, metabolic acidosis and rhabdomyolysis and is often fatal. High blood potassium, high blood triglycerides and liver enlargement, possibly caused by either direct mitochondrial respiratory chain inhibition or impaired mitochondrial fatty acid metabolism, are also key features. Children are more likely to develop PRIS and critically ill patients who receive glucocorticoids and catecholamines are at high risk. The main treatment is supportive therapy[9,16,17]. Early recognition of the syndrome and discontinuation of the propofol infusion reduces morbidity and mortality.

Dezocine is an opioid analgesic which was first synthesized in 1970. It is a mixed agonist/antagonist of opioid receptors and has the effects of analgesic action and euphoria. However, it is a silent antagonist of the κ-opioid receptor and thus has no side effects such as hallucinations or dysphoria[18]. Dezocine was patented by the American Home Products Corp. in 1978. Clinical trials ran from 1979 to 1985 before its approval by the United States Food and Drug Administration in 1986. The use of dezocine was discontinued in the United States in 2011 but it is commonly used after surgery in China.

This study analyzed the clinical manifestations after the administration of dezocine combined with propofol in the anesthetization of indolent colonoscopy patients to assess its effects on gastrointestinal function. The incidence of adverse reactions and improvement of patient tolerance were also studied.

A cross-sectional survey was used to collect epidemiological evidence. The use of regularly collected data provides large cross-sectional studies at little or no expense[19-21], which is an important advantage

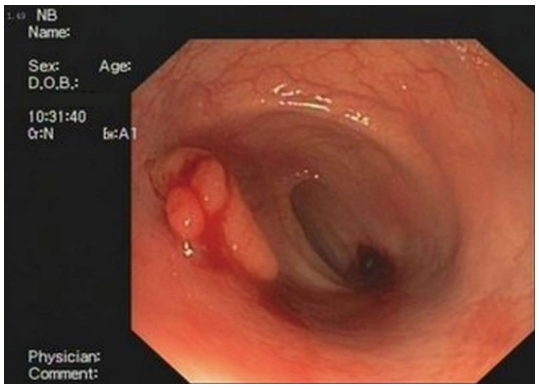

Figure 1 Colonoscopy image.

over other styles of epidemiological studies. A natural progression has been suggested from cheap cross-sectional studies of regularly collected data that can put forward hypotheses. However case-control studies test more specific hypotheses, cohort studies and trials cost much more and take much longer but provide stronger evidence. A cross-sectional survey is conducted to examine a specific group to determine whether alcohol consumption is correlated with cirrhosis being investigated. If alcohol is related to cirrhosis, this would support the hypothesis that alcohol may induce cirrhosis.

MATERIALS AND METHODS

General information

After obtaining institutional review board approval and informed consent from the participants or their guardians, 160 consecutive eligible patients previously diagnosed with indolent colonoscopy were enrolled in the study. All the patients had an ASA physical status of Ⅰ or Ⅱ and were aged between 1 and 68 years old.

All the experiments were performed under the supervision of the Patients' Experimental Ethics Committee of the Beijing Army General Hospital (Beijing, China). The cross-sectional survey, conducted from April 1 to April 30, 2015, recruited patients undergoing indolent colonoscopy in the Xinjiang People's Hospital. Patients with severe organ damage and drug allergies were excluded. This department does not operate on patients aged over 70 or under 1 year, or with a body mass index over 28 or under 18.

General epidemiological data and a history of neurological anesthetic drugs, including intravenous injection or oral drugs, were recorded.

Anesthesia methods

After identification of the patients, intravenous access to the appropriate area was established. All patients received 5 mg of anisodamine, avoiding microvascular spasm. Baseline hemodynamic data were recorded on arrival in the operating room after placement of routine monitors.

Anesthesia was induced with an intravenous injection of 5 mg of dezocine. Ten minutes later, 1.5-2.0 mg/kg of propofol was administered intravenously to induce anesthesia. The dose of propofol was adjusted to maintain the heart rate and blood pressure within 20% of the pre-induction values. No opioids were given intraoperatively. All patients received standardized colonoscopy by a practicing physician.

Evaluation index

The intraoperative index included the time the patient took to lose consciousness (from the induction of anesthesia until the eyelash conditioned reflex disappeared) and the total dosage of propofol.

The postoperative index included the length of time from the end of the colonoscopy surgery to the patient waking up and the patient's pain score in the observation room 30 min after surgery. Adverse reactions were investigated during two phases: the incidence of body movements and respiratory depression in the intraoperative phase and the incidence of vomiting and dizziness or headache in the postoperative phase.

Laboratory testing

Samples of venous blood were collected thirty minutes after the colonoscopy surgery. The biochemical indicators gastrin, vasoactive intestinal peptide, somatostatin and endothelin were tested using the radioimmunoassay (RIA) method. The RIA kits were purchased from the Chinese People's Liberation Army General Hospital, RIA Technology Development Center Institute (East Immunity Institute of Technology).

Statistical analysis

All data are represented as the means x ± SD (x ± s) of three or more independent experiments. Data with a positively skewed distribution were logarithmically transformed into a normal distribution. If the data were homogenous, analysis of variance, the Student-Newman-Keuls test and Pearson's correlation were used. If the data were not homogenous, the Kruskal-Wallis test, Games-Howell test and Spearman's correlation analysis were used. All the analyses were carried out using SPSS17.0 software (SPSS Inc., Chicago, IL, United States). Values below 0.05 were considered to be statistically significant.

RESULTS

Population investigation

From April 1 to April 30, 2015, 160 patients who had undergone dezocine combined with propofol in the anesthetization of indolent colonoscopy took part in the cross-sectional survey. Table 1 summarizes the patients' characteristics. There were 98 females and 62 males; therefore, there were more than 1.6 times

Table 1 Sample characteristics of patients receiving dezocine combined with propofol

Items	Value
Gender	
Male	62
Female	98
Age (yr)	
Median age	43
Maximum age	68
Minimum age	1.3
Average age	48
Modal age	52
Source	
Outpatients	87
Inpatients	73
Feedback from all patients	
Good medical experience	92
Normal medical experience	58
Bad medical experience	9

Table 2 Management survey for operations conducted in this study

	Value
Anesthesiologists' (ASA) Grading	
P 1	89
P 2	62
P 3	9
P 4, 5 or 6	0
Adverse reactions	
Body movement	2
Respiratory depression	2
Vomiting	2
Dizziness	3
Exhaustion and fatigue	12
Pain management	
Level 0-3	132
Level 4-6	28
Above level 7-10	0

as many females as males. They ranged from 1 to 68 years of age, with a median of 43, an average of 48 and a mode of 52 years. These figures indicate that indolent colonoscopy was more common among mid to older-aged patients.

There were 87 outpatients and 73 inpatients. For the purpose of health screening, more people chose indolent colonoscopy, especially the older patients. The necessary conditions for a diagnosis of digestive disease were required in 65 of the 73 inpatients (89.04%).

The patients' feedback about their overall medical experience indicated that 92 had a good medical experience, 58 had an average experience, but 9 had a bad experience and 1 had a very bad experience, which was concentrated in the younger age group. Only one child had no statistical significance.

Management survey for operation

According to the American Society of Anesthesiologists (ASA) grading, 89 patients were classified as P1, 62 as P2, and 9 as P3, but no patient was classified as P4, 5 or 6. All diagnoses met the standard clinical symptoms of the ASA.

The ASA physical status class risk stratification system is dependent on comorbid conditions that are a threat to life or limit activity, thus helping to predicting preoperative risks. They are as follows: (1) P1 - normal healthy patient; (2) P2 - patient with mild systemic disease; (3) P3 - patient with severe systemic disease; (4) P4 - patient with severe systemic disease that is a constant threat to life; (5) P5 - moribund patient who is not expected to survive without the operation; and (6) P6 - declared brain-dead patient whose organs are being removed for donor purposes. All the patients were assessed by the anesthetist before surgery and not by self-reporting.

Among the 160 anesthetized patients, two episodes of postoperative emesis were recorded which occurred after propofol anesthesia. No patient required additional medication for pain in the post-anesthesia care or normal unit. The anesthesia experience perceived by the parents of the children was more appropriate for dezocine combined with propofol. However, three patients reported dizziness 30 min after the operation.

Intraoperative adverse reactions included two cases of body movement and two cases of respiratory depression. The assessment and pain management methods were based on the World Health Organization classification. One hundred and thirty-two patients reported a pain level under 3. No patients reported a higher level of pain; however, 12 patients reported feeling exhausted and fatigued. These results are shown in Table 2.

Length of operation

The mean duration of surgery was 52 ± 24 min, the induction time was 10 ± 4 min and the duration of maintenance was 60 ± 23 min.

Laboratory results

Thirty minutes after the colonoscopy surgery, samples of venous blood were collected and four meaningful biochemical indicators of gastrointestinal function were assessed, as shown in Table 3. The results showed that gastrin and vasoactive intestinal peptide levels were slightly increased, whereas somatostatin and endothelin levels were slightly decreased.

Gastrin can increase gastric mucosal blood flow and protect the gastric mucosa. Vasoactive intestinal peptide can cause the relaxation of the pyloric sphincter. The decrease in somatostatin and endothelin levels helped to inhibit the relaxation of the smooth gastric muscles.

DISCUSSION

General anesthesia refers to a medically induced coma

Table 3 Biochemical indicators of gastrointestinal function

	Detection value	Reference value	Comparison
Gastrin (μg/mL)	88.94 ± 18.77	63.12 ± 28.71	Up
Vasoactive intestinal peptide (pg/mL)	58.33 ± 4.22	35.25 ± 3.12	Up
Somatostatin (pg/mL)	79.42 ± 4.26	108.25 ± 5.12	Down
Endothelin (pg/mL)	36.77 ± 9.12	48.91 ± 10.1	Down

with the lack of protective reflexes caused by one or more general anesthetic agents[1,12,21]. Various kinds of medications may be administered, with the aim of amnesia, analgesia, ensuring unconsciousness, relaxing the skeletal muscles and loss of control of the autonomic nervous system reflexes[9]. The optimal combination of these agents for any given patient and operation is usually chosen by the anesthesiologist or another provider, such as an anesthesiology assistant or nurse anesthetist, in consultation with the patient and the doctor conducting the operation.

Colonoscopy is the standard procedure for the diagnosis, screening, treatment and follow-up of many colorectal diseases[22,23]. Although some patients can tolerate the colonoscopy procedure without sedation or analgesics, the use of drugs in some patients is associated with stress. There are difficulties in determining an optimal dose for sedation and monitoring patients adequately during the procedure[24-27]. Many patients require intravenous benzodiazepines and opiates, which are associated with amnesic, anxiolytic and sedative properties. Combined administration of benzodiazepines and opioids has several undesirable effects, including a delay of several minutes from the time of injection before the drugs exert their effects, amnesia and the risk of respiratory depression[7,28,29].

Hospitals increasingly prefer to use fospropofol for colonoscopy. Fospropofol (trade name Lusedra) is an intravenous sedative-hypnotic agent. It is currently used for the sedation of adult patients undergoing diagnostic or therapeutic operations such as endoscopy. Some water-soluble prodrugs of propofol have been developed, of which fospropofol is known as the most fit for clinical development to date. Fospropofol is often administered combined with an opioid such as fentanyl. As a prodrug of propofol, it is metabolized into propofol by alkaline phosphatases. Theoretically, one millimole (mmol) of propofol may be generated for each mmol of fospropofol sodium administered; 1.86 mg of fospropofol sodium is the molar equivalent of 1 mg propofol[30-33].

Dezocine can be administered intravenously or intramuscularly. Dezocine is an effective painkiller compared with meperidine (pethidine) and a more effective analgesic than pentazocine but may cause greater respiratory depression. It is an effective drug for the treatment of pain but side effects such as dizziness restrict its clinical use and it also causes opioid withdrawal syndrome in patients already using other opioids[34].

A study conducted by the Department of Anesthesia, Xishan People's Hospital of Wuxi City in Jiangsu province explored the clinical effect of dezocine combined with propofol in the anesthetization of 60 patients undergoing indolent enteroscopy between July 2012 and June 2014. The patients were randomly divided into a research group that received dezocine combined with propofol and a control group that received fentanyl combined with propofol[33-36]. The total dosage of propofol in the research group was less than that in the control group ($P < 0.05$), the awakening time in the research group was shorter than in the control group ($P < 0.05$) and the number of adverse effects during and after surgery was lower in the research group than in the control group ($P < 0.05$). Dezocine combined with propofol applied in the anesthetization of indolent enteroscopy can have a remarkable effect, improve operational safety and decrease the occurrence of adverse reactions. Our results were consistent with those of this study.

According to the results of a study by the Department of Anesthesiology at the First Hospital of Quanzhou, age was an important influence on the pharmacodynamics of propofol in patients receiving propofol combined with dezocine while undergoing colonoscopy[37]. However, the results from the Department of Gastroenterology at the Second People's Hospital of Fujian province showed that endoscopic mucosal resection under painless colonoscopy shortened the recovery time, increased the success rate and improved the satisfaction rate of older patients who received propofol and fentanyl. Endoscopic mucosal resection under painless colonoscopy is an easy, safe and effective therapy for colorectal polyps.

Thirty minutes after colonoscopy surgery, samples of venous blood were collected and the biochemical indicators gastrin, vasoactive intestinal peptide, somatostatin and endothelin were tested. There was an increase in the plasma contents of gastrin and vasoactive intestinal peptide ($P < 0.05$) and a decrease in somatostatin and endothelin ($P < 0.05$) compared with the standard reference values[38-40].

Anesthesia with dezocine combined with propofol evidently increases gastric mucosal blood flow, suggesting that it has a regulative effect on gastrointestinal function. The use of dezocine combined with propofol might change plasma brain-gut peptides levels and may be useful.

COMMENTS

Background

Increasingly, doctors like to operate on totally anesthetized patients because it allows for a calmer examination without perceived pain or discomfort. Twilight sedation is safer than general anesthesia and allows the patient to follow simple commands and even to watch the operation on a monitor. Therefore,

twilight sedation is generally best and the operator should not rush, even although the examination may cause discomfort. Tens of millions of adults need colonoscopies annually and yet many refuse because of concerns about the procedure.

Research frontiers

This study assessed the use of dezocine combined with propofol for the anesthetization of patients undergoing indolent colonoscopy for the assessment of gastrointestinal function. A cross-sectional survey of patients undergoing indolent colonoscopy in the Xinjiang People's Hospital was conducted from April 1 to April 30, 2015. The survey collected general information and anesthesia data, including overall medical experience and pain management.

Innovations and breakthroughs

Indolent colonoscopy was found to be more suitable for mid to older-aged patients. Somatostatin and endothelin levels were slightly decreased and patients had a good medical experience.

Applications

Gastrin and vasoactive intestinal peptide levels were slightly increased. However, somatostatin and endothelin levels were slightly decreased. This clinical study of dezocine combined with propofol indicates that it is a successful method for the anesthetization of indolent colonoscopy patients without pain and should therefore be widely used.

Peer-review

In this study, the authors assessed the use of dezocine combined with propofol for the anesthetization of patients undergoing indolent colonoscopy for the assessment of gastrointestinal function. The study collected general information and anesthesia data, including overall medical experience and pain management. This clinical study of dezocine combined with propofol indicates that it is a successful method for the anesthetization of indolent colonoscopy patients without pain and should therefore be widely used.

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CASE REPORT

Successful treatment of ileal ulcers caused by immunosuppressants in two organ transplant recipients

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Abstract

Although gastroduodenal ulcers are common in solid organ transplant patients, there are few reports on multiple giant ulcers in the distal ileum and ileocecal valve caused by immunosuppressants Herein, we report on a liver transplant recipient and a renal transplant recipient with multiple large ulcers in the distal ileum and ileocecal valve who rapidly achieved ulcer healing upon withdrawal of sirolimus or tacrolimus and administration of thalidomide. In case 1, a 56-year-old man with primary hepatocellular carcinoma had received a liver transplantation. Tacrolimus combined with sirolimus and prednisolone was used as the anti-rejection regimen. Colonoscopy was performed because of severe abdominal pain and diarrhea at post-operative month 10. Multiple giant ulcers were found at the ileocecal valve and distal ileum. The ulcers healed rapidly with withdrawal of sirolimus and treatment with thalidomide. There was no recurrence during 2 years of follow-up. In case 2, a 34-year-old man with end-stage kidney disease received kidney transplantation and was put on tacrolimus combined with mycophenolate mofetil and prednisolone as the anti-rejection regimen. Twelve weeks after the operation, the patient presented with hematochezia and severe anemia. Colonoscopy revealed multiple large ulcers in the ileocecal valve and distal ileum, with massive accumulation of fresh blood. The bleeding ceased after treatment with intravenous somatostatin and oral thalidomide. Tacrolimus was withdrawn at the same time. Colonoscopy at week 4 of follow-up revealed remarkable healing of the ulcers, and there was no recurrence of bleeding during 1 year of follow-up. No lymphoma, tuberculosis, or infection of cytomegalovirus, Epstein-Barr virus, or fungus was

found in either patient. In post-transplantation cases with ulcers in the distal ileum and ileocecal valve, sirolimus or tacrolimus should be considered a possible risk factor, and withdrawing them or switching to another immunosuppressant might be effective to treat these ulcers.

Key words: Ileal ulcers; Liver transplantation; Kidney transplantation; Sirolimus; Tacrolimus

Core tip: There are few reports available on ileal ulcers caused by immunosuppressants. Herein, we report a liver transplant recipient and a renal transplant recipient who had multiple large ulcers in the distal ileum and ileocecal valve. Ulcers rapidly healed after withdrawal of sirolimus or tacrolimus and administration of thalidomide. No lymphoma, tuberculosis, or infection of cytomegalovirus, Epstein-Barr virus, or fungus was found in either patient. There was no recurrence of ulcers or organ rejection. In some post-transplantation cases with ileal ulcers, sirolimus or tacrolimus should be considered as a risk factor because of their inhibitory effects on wound healing. Withdrawing them or switching to other immunosuppressants might be effective.

Guo YW, Gu HY, Abassa KK, Lin XY, Wei XQ. Successful treatment of ileal ulcers caused by immunosuppressants in two organ transplant recipients. *World J Gastroenterol* 2016; 22(24): 5616-5622 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5616.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5616

INTRODUCTION

Solid organ transplant recipients are susceptible to a variety of gastrointestinal (GI) complications, one of which is ulcer disease[1-5]. Most of these ulcers are located at the anastomotic stoma or gastroduodenum; ulcers at the intestine or colon are rare. In addition to *Helicobacter pylori* infection and ischemia, infection with cytomegalovirus (CMV), Epstein-Barr virus (EBV), mycobacteria, and fungus can contribute to ulcer disease in transplant recipients[1-5]. A few reports suggest that the use of immunosuppressants, especially high-dose immunosuppression after transplantation, might correlate with impairment of the gastrointestinal tract[6,7]. Herein, we report a liver transplant recipient and a renal transplant recipient with multiple large ulcers in the distal ileum and ileocecal valve who rapidly achieved ulcers healing upon withdrawal of sirolimus or tacrolimus and administration of thalidomide.

CASE REPORT

Case 1

A 56-year-old man with primary hepatocellular carcinoma received orthotopic liver transplantation in our hospital. Prior to the transplantation, he was diagnosed with chronic hepatitis B virus (HBV), Child-C liver function, and elevated blood glucose. No history of renal or cardiac disease or mycobacterium tuberculosis (TB) infection was noted. He denied smoking and drinking. Tacrolimus combined with sirolimus and prednisolone was used as the anti-rejection regimen. The dose of tacrolimus and sirolimus was adjusted according the drug serum concentration. The patient recovered and liver function improved to normal level. Entecavir was prescribed to prevent HBV reinfection.

After administration of the immunosuppressants, the patient began to develop mild peri-umbilical pain and diarrhea, which were tolerable at the time. Probiotics and antispasmodic treatment seemed not so effective. At 10 mo post-operation, he was admitted to our hospital because of severe diarrhea and abdominal pain. There were 7-10 bowel movements per day, with mucous blood stool. On physical examination, blood pressure, heart rate, and temperature were normal. There was slight tenderness on the peri-umbilicus area without rebound tenderness. Complete blood count revealed a leukocyte count of 11.50 × 10^9/L, erythrocyte count of 4.50 × 10^{12}/L, hemoglobin level of 121 g/L, platelet count of 295 × 10^9/L, neutrophils of 76.4%, and lymphocytes of 16.3%. Leukocytes and erythrocytes were found in the stool. The ratio of coccus to bacillus in stool was 1:9. Serum glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, total bilirubin, BUN, creatine, and electrolytes were within normal range. Serum 1-3-β-D dextran was < 10 pg/mL. Blood and stool cultures for fungus and bacteria showed no growth. Chest computed tomography (CT) scan revealed no lesions and serum T-SPOT.TB was negative. Immunoglobulin (Ig)M and IgG of the EB virus were negative. Abdominal enhanced CT scan showed thickening of the distal ileum and ileocecal region, suggesting inflammatory lesions. The spleen was slightly enlarged, muddy stones were found in the common bile duct, and no enlarged lymphonodus was noted. Chronic erosive gastritis with negative *Helicobacter pylori* infection was confirmed through esophagogastroduodenoscopy. Colonoscopy revealed multiple giant and deep ulcers in the ileocecal valve and distal ileum, with polypoid hyperplasia. The length of the largest ulcer was up to 5.0 cm (Figure 1A-C). Histopathology of biopsy specimens revealed benign ulcers and chronic inflammation with non-caseous granulomas (Figure 2A and B), without signs of fungus and parasites infection. The immunohistochemical study was negative for CMV infection. EBV encoded

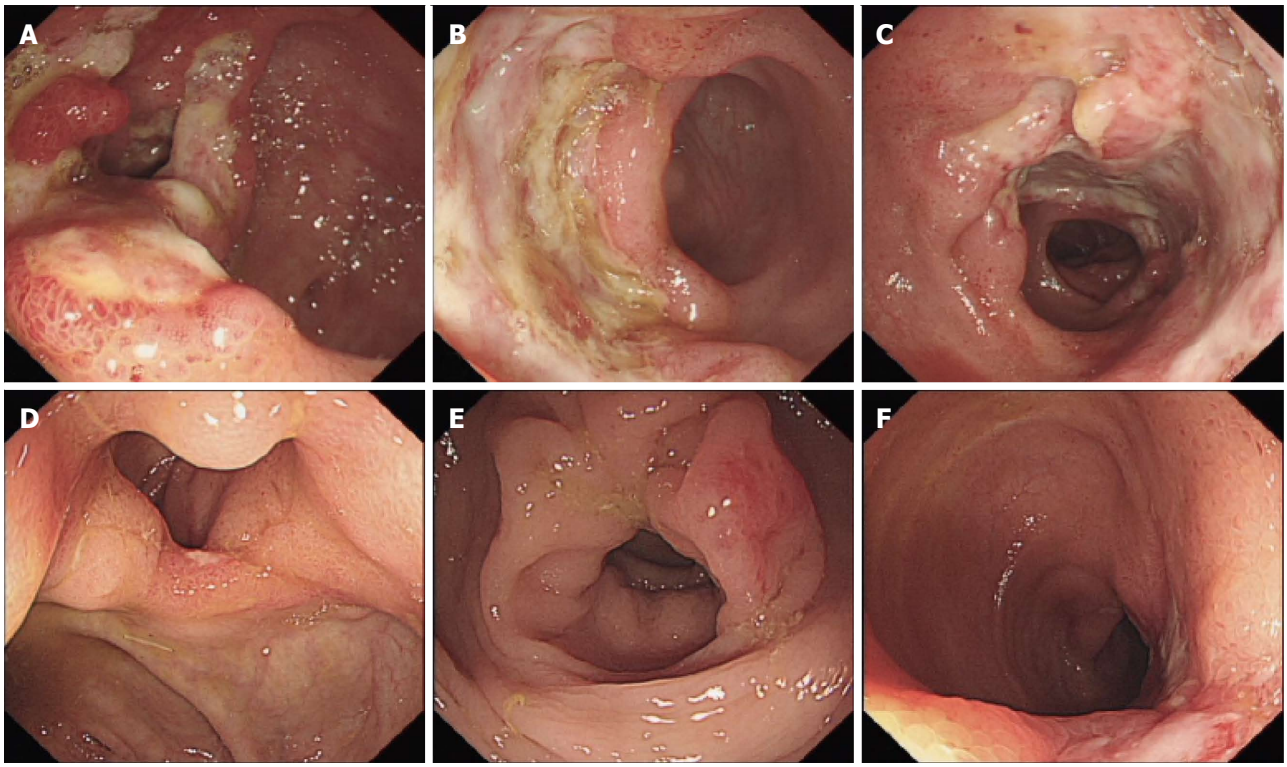

Figure 1 Colonoscopic images of case one. A-C: Multiple giant and deep ulcers in the ileocecal valve and distal ileum, with polypoid hyperplasia; D-F: Rapid healing of the ulcers in the ileocecal valve and distal ileum and only two healing 2 stage ulcers left.

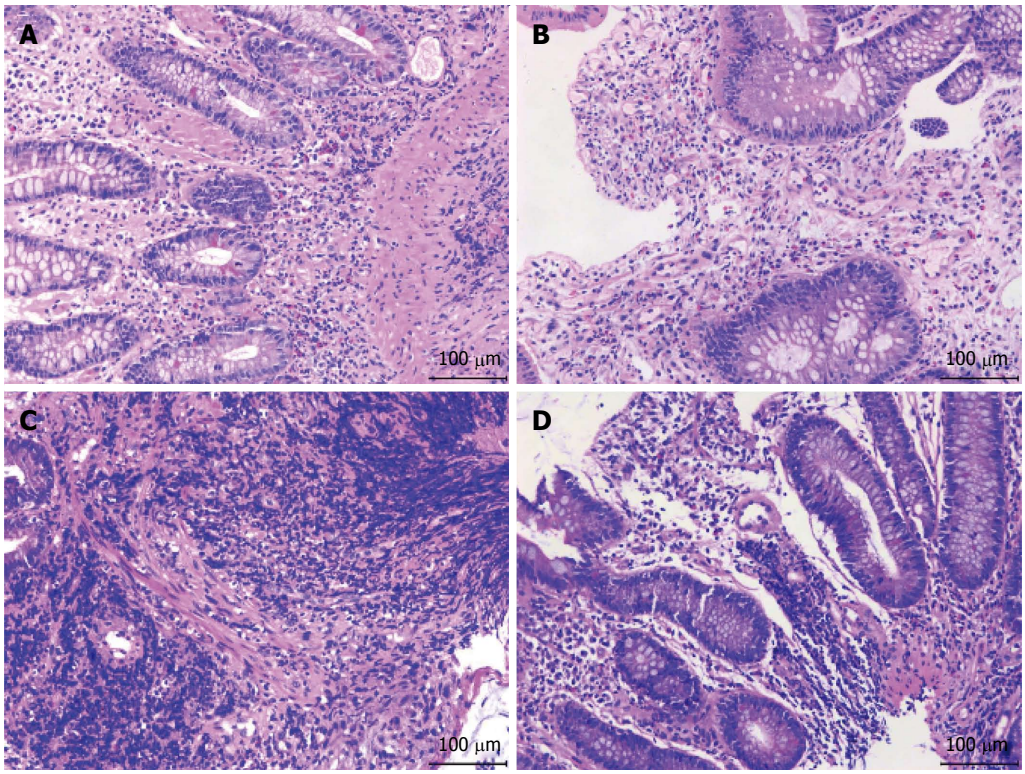

Figure 2 Photomicrograph of biopsy specimens. A and B: Biopsy specimens of ulcers from case one; C and D: Biopsy specimens of ulcers from case two. Hematoxylin-eosin staining, magnification × 200.

early small RNA (EBER) was negative by *in situ* hybridization.

Considering sirolimus to have more gastrointestinal complications than tacrolimus in clinical application,

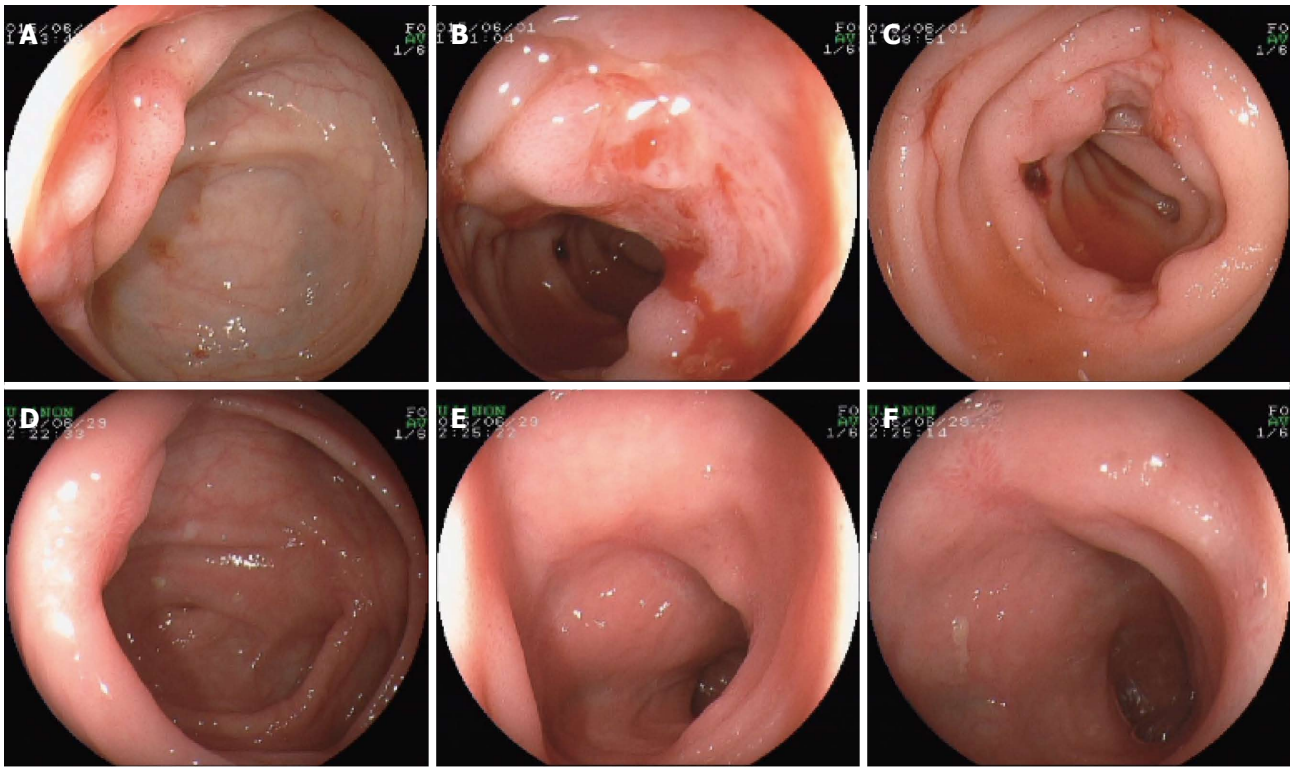

Figure 3 Multiple ulcers in ileocecal valve and distal ileum, with massive fresh blood accumulation (A-C); rapid healing of the ulcers with scar tissue in ileocecal valve and distal ileum (D-F).

sirolimus was withdrawn first. The patient was also put on oral thalidomide at a dose of 100 mg/d for 2 wk and intravenous antibiotics for one week. Diarrhea and abdominal pain were gradually relieved and subsided. Colonoscopy at week 6 of follow-up revealed remarkable healing of the ulcers in the ileocecal valve and distal ileum, and only two healing 2 stage ulcers were found (Figure 1D-F). No organ rejection was noted after withdrawing sirolimus. No recurrence of diarrhea and abdominal pain was noted during the 2 years of follow-up.

Case 2

A 34-year-old man with end-stage kidney disease was admitted to the department of renal transplantation for living-donor kidney transplantation. Except for kidney disease, he had no history of primary liver, heart, or head disease. He denied smoking and drinking. Tacrolimus combined with mycophenolate mofetil and prednisolone was applied as the anti-rejection regimen. The dose of tacrolimus was adjusted according to the drug serum concentration. At the same time, oral ganciclovir, voriconazole, and esomeprazole were used to prevent CMV and fungus infection and esophagogastroduodenal complications. The patient recovered smoothly. Serum creatinine level decreased to 152.0 μmol/L, and urine output was normal.

At postoperative week 12, the patient was admitted to our department because of repeated hematochezia for 2 wk, which was accompanied by dizziness and weakness. No abdominal pain, nausea, or vomiting was observed. On physical examination, blood pressure, heart rate, and temperature were 95/55 mmHg, 102 beats/min, 37.5 ℃, respectively. There was mild tenderness at the peri-umbilicus area and the lower right abdomen, without rebound tenderness. Complete blood count revealed a leukocyte count of 7.45 × 10^9/L, erythrocyte count of 2.05 × 10^{12}/L, hemoglobin level of 57 g/L, and platelet count of 241 × 10^9/L. Mild elevated serum creatinine level of 168.0 μmol/L was noted, with a normal BUN level of 6.8 mmol/L. Prothrombin time, apartprothrombin time, and thrombin time were within the normal range. Laboratory indices about hepatic, cardiac, and respiratory function were all normal. Blood and stool cultures for fungus and bacteria showed no growth. Chest CT scan revealed some pulmonary lesions of previous tuberculosis, and serum T-SPOT.TB was negative. There were no abnormal findings in the abdominal doppler ultrasound. Esophagogastroduodenoscopy was performed first, and only mild gastritis was observed, with negative rapid urease test for *Helicobacter pylori* infection. Colonoscopy revealed multiple ulcers in the ileocecal valve and distal ileum, with massive accumulation of fresh blood. These ulcers were oval and deep, covered with white fur or a blood scab, and the biggest had a diameter of 2.0 cm (Figure 3A-C). Histopathology revealed chronic inflammation with a large number of lymphocytes infiltration (Figure 2C

and D), without signs of fungus or parasite infection. Immunohistochemistry stain for CMV was negative. EBER was also negative by *in situ* hybridization.

The bleeding lessened and eventually ceased after treatment with intravenous somatostatin (1.2 mg/d) and oral thalidomide (100 mg/d) for 5 d. At the same time, because the multiple intestinal ulcers may be due to immunosuppressors, tacrolimus was withdrawn, and cyclosporine combined with mycophenolate mofetil and prednisolone were administered. Additionally, the patient was put on oral thalidomide at a dose of 100 mg/d for 4 wk. Colonoscopy at week 4 of follow-up revealed remarkable healing of the ulcers with scar tissue in the ileocecal valve and distal ileum (Figure 3D-F). There was no recurrent bleeding during the 1 year of follow-up. In addition, no organ rejection was found after withdrawing tacrolimus.

DISCUSSION

It is well known that solid organ transplantation patients are particularly at risk for GI complications. Severe GI complications such as GI bleeding and GI perforation may negatively influence long-term outcome and become deadly. It had been reported that GI bleeding occurred in 2.3%-6.4% patients after liver transplantation[8,9] and GI perforation in 2.9% patients after renal transplantation[7]. Ulcer diseases are an important cause of GI bleeding, and perforation can manifest with symptoms of abdominal pain or diarrhea. Most of these ulcers are located at the anastomotic stoma or gastroduodenum, whereas ulcers at the intestine or colon are rare. In the two cases described here, deep and large ulcers were located in the ileocecal valve and distal ileum with no lesions in the gastroduodenum. In case 1, there was a longer period of diarrhea and abdominal pain, while case 2 mainly presented with acute and massive GI bleeding.

Differentiation of ulcer diseases in post-transplantation patients is always difficult. Common and uncommon pathogenesis such as *Helicobacter pylori* infection, ischemia, infection of CMV, EBV, mycobacteria, and fungus, and post-transplant lymphoproliferative disorders (PTLDs) should be considered. In the present two cases, ulcers were in the ileocecal valve and distal ileum, and *Helicobacter pylori* infection was ruled out as the pathogen. Clinical manifestation and endoscopic characteristics of ulcers were not consistent with a pathogenesis of ischemia. Because of negative the findings on chest CT scan, serum T-SPOT.TB, and histopathology of ulcers, mycobacteria and fungus infection were also excluded. CMV infection is common in patients with solid organ transplantation, and attention should be paid in cases of gastrointestinal ulcers. In a study of renal transplant patients susceptible to a variety of GI complications, such as infections, ulcer disease, and malignancies, CMV infection occurred in 11% of all patients[1]. Although oral ganciclovir preventive strategy was used in case 2, this treatment might be inefficient in some patients, and atypical symptoms might be present[10,11]. Decreased leukocyte count and interstitial pneumonitis are always found in CMV infection, but in both of our cases, no positive signs about CMV infection were noted in leukocyte count or chest CT scan. More importantly, immunohistochemistry stain for CMV of ulcer biopsy tissue was negative, and both patients recovered without further anti-CMV therapy. Therefore, CMV infection was not considered the cause of ulcers in these two cases.

PTLD was most difficult to be excluded in both patients. This was especially true in case 1, where the ulcers in the ileocecal valve and distal ileum appeared deeper and larger, with surrounding proliferative tissue. PTLD is a severe complication after organ transplantation with a cumulative incidence of 1.1% at 18 mo and 4.7% at 15 years, and it is always associated with EBV infection[12]. Lymph nodes, GI tract, and graft liver are the most common sites of involvement[13]. The involvement of the GI tract could result in deadly perforation and hemorrhage. In both patients, no persistent fever, palpable superficial lymph nodes, enlarged liver, or lymph nodes were found on chest and abdominal CT scan or ultrasound; EBER was negative by *in situ* hybridization. Pathology was also not consistent with the characteristics of PTLD. Furthermore, PTLD usually deteriorates quickly and is difficult to treat. However, the present two patients were stable within 1-2 wk, and the ulcers healed rapidly in 4-6 wk. Taken together, the evidence did not support the diagnosis of PTLD.

Finally, we considered that ulcer development was related to the use of immunosuppressants. Current studies have shown that some immunosuppressants, such as mammalian target of rapamycin inhibitors, had inhibitory effects on wound healing. Sirolimus is the most common drug that can lead to impairment of wound healing, and the most common wound complication is skin or dermal eruption[14-17]. Therefore, the immunosuppressant use was considered to be a possible cause of GI epithelium impairment. Fortunately, both patients recovered quickly after withdrawing sirolimus and tacrolimus, supporting our speculation. In Smith *et al*[6], three liver transplant patients taking sirolimus suffered from gastrointestinal hemorrhage due to complications of gastroduodenal ulcers. The ulcers in two patients healed only after discontinuation of sirolimus, and the third patient died of massive gastrointestinal bleeding[6].

Thalidomide has anti-angiogenic properties and seems effective in some cases of GI bleeding, especially angiodysplasia-related bleeding[18-20]. It is also used in some inflammatory and ulcerative diseases like inflammatory bowel disease and some skin and oral ulcers, because of its anti-inflammatory and immunomodulatory effects[20-22]. In our clinical practice, thalidomide is effective in some unexplained

and refractory multiple ulcers of intestine and related GI bleeding. It also seemed to work in our present two patients. Thalidomide was administrated at a dose of 100 mg/d for 2 wk and 4 wk respectively, and no severe side effects were found.

In summary, some types of immunosuppressants, such as sirolimus and tacrolimus, can lead to impairment of the GI track and sometimes to the development of severe ulcers. Withdrawing them or switching to other immunosuppressants might be effective to treat these ulcers.

COMMENTS

Case characteristics

A 56-year-old man presented with severe diarrhea and abdominal pain after orthotopic liver transplantation, and a 34-year-old man presented with hematochezia and severe anemia after living-donor kidney transplantation.

Clinical diagnosis

Multiple giant ulcers in the distal ileum and ileocecal valve were caused by immunosuppressants.

Differential diagnosis

Common and uncommon pathogenesis of gastrointestinal (GI) ulcers in solid organ transplant recipient, such as *Helicobacter pylori* infection, ischemia, infection of cytomegalovirus (CMV), Epstein-Barr virus (EBV), mycobacteria and fungus, and post-transplant lymphoproliferative disorders (PTLDs), should be considered.

Laboratory diagnosis

Blood and stool cultures for fungus and bacteria showed no growth, and serum T-SPOT.TB was negative.

Imaging diagnosis

Computed tomography (CT)scan revealed no current tuberculosis, and there were no abnormal findings on abdominal enhanced CT or doppler ultrasound.

Endoscopic diagnosis

Colonoscopy revealed multiple giant and deep ulcers in the ileocecal valve and distal ileum.

Pathological diagnosis

Histopathology revealed chronic inflammation without signs of fungus or parasite infection, negative immunohistochemistry stain for CMV, and negative stain for EBV encoded early small RNA by *in situ* hybridization.

Treatment

Sirolimus or tacrolimus was withdrawn, and thalidomide was administrated.

Related reports

Most GI ulcers are located at the anastomotic stoma or gastroduodenum in post-transplant recipients, and ulcers at the intestine or colon are rare. Besides ischemia and infection of CMV, EBV, mycobacteria, and fungus, use of immunosuppressants might contribute to the impairment of gastrointestinal tract.

Term explanation

PTLDs are a severe complication of solid organ and hematopoietic stem cell transplantation, including lymphoproliferative entities varying from reactive hyperplasia to malignant lymphoma. EBV is the main pathogen of PTLD.

Experiences and lessons

In some post-transplantation cases with ileal ulcers, sirolimus or tacrolimus should be considered as a risk factor, and withdrawing them or switching to other immunosuppressants might be effective.

Peer-review

This article is very interesting for those in the field of liver and kidney transplantation. Since ulcers in the distal ileum in solid organ transplant recipients are very rare, experience about the management of these patients is very useful.

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