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ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Yoshiaki Iwasaki, MD, PhD, Professor, Health Service Center, Okayama University, Okayama 700-8530, Japan

AIMS AND SCOPE

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The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

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Ze-Mao Gong, Director
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Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
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Fax: +1-925-2238243
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ORIGINAL ARTICLE

Basic Study

Role of *PTPN2/22* polymorphisms in pathophysiology of Crohn's disease

Robert C Sharp, Shazia A Beg, Saleh A Naser

Robert C Sharp, Saleh A Naser, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL 32816, United States

Shazia A Beg, University of Central College of Medicine, Health Center, Orlando, FL 32816, United States

ORCID number: Robert C Sharp (0000-0002-5167-8941); Shazia A Beg (0000-0002-4096-9452); Saleh A Naser (0000-0001-9222-4527).

Author contributions: Sharp RC is the primary author who performed all experiments, collected data and participated in writing the manuscript; Beg SA is the clinical coordinator in this study and has supervised recruitment of subjects, collection of clinical samples and transmitting of relevant data to the investigators. She played a vital role in analyzing the data and revising the manuscript; Naser SA is the leading investigator in the lab and has supervised all aspects of the study including writing and editing of the manuscript.

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Correspondence to: Saleh A Naser, PhD, Professor, Associate Director, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 4110 Libra Drive, Orlando, FL 32816, United States. saleh.naser@ucf.edu
Telephone: +1-407-8230955
Fax: +1-407-8230955

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Abstract

AIM

To establish the relationship of *protein tyrosine phosphatase non-receptor type 2 and 22* (*PTPN2/22*) polymorphisms and mycobacterial infections in Crohn's disease (CD).

METHODS

All 133 subjects' blood samples were genotyped for nine single nucleotide polymorphisms (SNPs) in *PTPN2/22* using TaqMan™ genotyping, while the effect of the SNPs on *PTPN2/22* and *IFN-γ* gene expression was determined using RT-PCR. Detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) *IS900* gene was done by nPCR after DNA extraction from the isolated leukocytes of each subjects' blood samples. T-cells isolated from the patient samples were tested for response to phytohematoagglutonin (PHA) mitogen or mycobacterial antigens by BrdU proliferation assays for T-cell activity.

RESULTS

Out of the nine SNPs examined, subjects with either heterozygous (TC)/minor (CC) alleles in *PTPN2:*

rs478582 occurred in 83% of CD subjects compared to 61% healthy controls (*P*-values < 0.05; OR = 3.03). Subjects with either heterozygous (GA)/minor (AA) alleles in *PTPN22:rs2476601* occurred in 16% of CD compared to 6% healthy controls (OR = 2.7). Gene expression in *PTPN2/22* in CD subjects was significantly decreased by 2 folds compared to healthy controls (*P*-values < 0.05). *IFN-γ* expression levels were found to be significantly increased by approxiately 2 folds in subjects when either heterozygous or minor alleles in *PTPN2:rs478582* and/or *PTPN22:rs2476601* were found (*P*-values < 0.05). MAP DNA was detected in 61% of CD compared to only 8% of healthy controls (*P*-values < 0.05, OR = 17.52), where subjects with either heterozygous or minor alleles in *PTPN2:rs478582* and/or *PTPN22:rs2476601* had more *MAPbacteremia* presence than subjects without SNPs did. The average T-cell proliferation in CD treated with PHA or mycobacteria antigens was, respectively, 1.3 folds and 1.5 folds higher than healthy controls without any significant SNP.

CONCLUSION

The data suggests that SNPs in *PTPN2/22* affect the negative regulation of the immune response in CD patients, thus leading to an increase in inflammation/apoptosis and susceptibility of mycobacteria.

Key words: Crohn's disease; *PTPN2*; *PTPN22*; *PTPN2/22*; *Mycobacteria*; Single nucleotide polymorphisms

Core tip: Knowledge of the pathophysiology of Crohn's disease (CD) is vital in the development of new diagnosis techniques and treatments for the disease. Our study involves the investigation of single nucleotide polymorphisms (SNPs) in *protein tyrosine phosphatase non-receptor type 2 and 22* (*PTPN2/22*) and their effects on susceptibility to mycobacteria species and the elevation of pro-inflammatory cytokines. Our data demonstrates that SNPs in *PTPN2/22* lead to less negative regulation in T-cells and increase susceptibility to mycobacteria, thus increasing inflammation and apoptosis in intestinal tissues. Personalized treatment could be accomplished by genetic testing and antibiotic treatment for mycobacteria in CD patients.

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INTRODUCTION

Single nucleotide polymorphisms (SNPs) play a significant role in the pathogenic process of inflammatory autoimmune disorders. These SNPs affect several immunity genes, leading to an overactive immune system. Consequently, self-tolerance mechanisms fail in a variety of immune cells including T-cells, B-cells, and antigen-presenting cells[1,2]. Along with these genetic defects, environmental factors such as bacterial and viral infections have also been associated with inflammatory autoimmune disorders. These factors trigger phenotypical response to occur in the defected immune cells[1-3]. Some of these diseases, such as Rheumatoid Arthritis (RA), Type 1 Diabetes (T1D), and Crohn's Disease (CD), share some of the same genetic SNPs with each other[1-4]. An example of regulatory immune genes that these diseases share SNPs in are in the *protein tyrosine phosphatases non-receptor type 2* (*PTPN2*) and *type 22* (*PTPN22*) genes[1-5]. *PTPN2/22* are genes found more frequently in T-cells, where they encode enzymatic phosphatase proteins (PTPN2/22) that negatively regulate the T-cell receptor (TCR)[4,5]. *PTPN2* and its protein product (PTPN2) are also found in a majority of epithelial cell types including synovial joint tissue, β-cells, and intestinal tissues, where they control apoptosis and chemokine production[4,5]. SNPs in *PTPN2/22* have been hypothesize to cause a dysregulation of the immune system that is brought upon by overactive T-cells and increased pro-inflammatory cytokine production due to lack of negative regulation[1-5].

With a majority of inflammatory autoimmune disorders sharing the same genetic pre-dispositions, it is possible that the pathogenesis of these disorders could also share some of the same common environmental triggers with each other as well (Figure 1)[6-10]. Recent studies have shown that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infections have been associated with a variety of different inflammatory disorders including CD[4,11-14]. Mycobacterial infections causes problems in these inflammatory autoimmune patients when the patient is genetically predisposed, causing the immune system to become dysregulated[4,11-14]. This dysregulation will lead to high amounts of pro-inflammatory cytokines, production of autoantibodies, and high amounts of apoptosis occurring in a variety of cell types, thus leading to chronic inflammation[4,11-14].

In addition to sharing the same genetic predispositions and environmental triggers, many inflammatory autoimmune disorders share the same medical treatments as well. For instance, anti-TNF-α therapeutics such as adalimumab and infliximab are used for RA and CD[6,7]. However, anti-TNF-α medications along with non-steroid anti-inflammatory drugs (NSAIDs), glucocorticoids, and other disease-modifying drugs cause several side effects[6-10]. These side effects include osteoporosis, hypertension, GI intolerance, autoantibodies against medications, and increased risk of developing opportunistic infections, especially mycobacterial infections[6-10]. With the undesirable

side effects of these medications, it is important that inflammatory autoimmune disorders pathogeneses is thoroughly examined in order to develop more accurate detection of disease and to develop more personal treatment with little side effects.

In this study, we focus on the pathogenesis of CD, where we explore the effect of both the genetic predisposition of SNPs in *PTPN2/22* and the environmental trigger of MAP infection. We hypothesize that SNPs in *PTPN2/22* lead to loss of negative regulation in T-cells and, with a MAP infection, increases production of pro-inflammatory cytokines such as *IFN-γ*. This leads to an increase inflammation and apoptosis in the intestinal tissues of CD patients.

MATERIALS AND METHODS

Clinical samples

A total of 133 consented CD subjects and healthy controls donated two to three 4.0 mL K_2-EDTA coded blood tubes for us in this study. The study was approved by the University of Central Florida Institutional Review Board #IRB00001138. Each subject completed and signed a written consent form before samples were collected. Healthy control subjects completed a survey that question if said subjects had any medical abnormality (CD, T1D, RA or "other diseases"). No healthy control subjects had any type of medical conditions to the best of their knowledge. The severities of the CD subjects' symptoms were scored from moderate to severe symptoms. The average age of CD subjects was 39.6 ± 14.3 with a gender ratio of 48.6% male and 51.4% female. The average age of healthy controls was 30.7 ± 13.4 with a gender ratio of 41.9% male and 58.1% female subjects. Table 1 lists age, gender and other demographic information for all CD subjects in this study. From the blood tubes, the following procedures were done to the samples: *PTPN2/22* genotyping, gene expression profiling, MAP *IS900* nested PCR (nPCR) detection, and T-cell proliferation assays.

PTPN2/22 genotyping

TaqMan™ SNP Genotyping Assays (Applied Biosystems™) were used to genotype nine SNPs in *PTPN2/22* from the isolated DNA from subjects' blood samples. Samples and reagents were sent to the University of Florida Pharmacotherapy and Translational Research Department (Gainesville, FL) to perform genotyping assays. Out of the nine SNPs, four SNPs were specific to *PTPN2* that includes *rs1893217*, *rs2542151*, *rs7234029*, *rs478582* along with five SNPs that were specific to *PTPN22* that includes *rs2476601*, *rs2488457*, *rs33996649*, *rs34209542*, *rs2476599*. Briefly, DNA was extracted from whole blood samples using QIAamp® DNA Blood Mini Kit (Qiagen™) following manufacturer's protocol. TaqMan™ genotyping assays for *PTPN2/22* SNPs were performed on DNA samples following manufacturer protocol (Applied Biosystems™). Briefly, DNA samples and the TaqMan™ SNP Genotyping Assays mixtures (primers with Vic and Fam fluorophore attachment) were transferred into a 384-well plate along with 2 × TaqMan™ Master Mix and 20 × Assay Working Stock in each well. Plates were treated to an RT-PCR protocol consisting of 95 ℃ for 10 min for 1 cycle, 92 ℃ for 15 s and 58 ℃ for 1 min for 50 cycles. The plates were then read for VIC (551 nm) and FAM (517 nm) fluorescence, where VIC or FAM alone determined allele 1 or allele 2 in the samples, while VIC and FAM together determined heterozygous for each allele in the samples.

PTPN2/22 and IFN-γ gene expression

Gene expression of *PTPN2/22* and *IFN-γ* was performed by converting RNA from subjects' whole blood samples to cDNA and performing RT-PCR. RNA from the subjects' blood samples were isolated from peripheral leukocytes *via* TRIzol® Reagent (Invitrogen) per manufacturer's instruction. Briefly, 1.0 mL of whole blood from subjects' samples were transferred into a microcentrifuge tubes and centrifuged for 3000 rpm for 15 min until the leukocytes formed a buffy coat layer, which was then transferred to new 2.0 mL RNase free microcentrifuge tubes. Tubes containing the leukocytes from subjects' samples were then suspended in 1.0 mL of TRIzol®, where the tubes were incubated and gently rocked for 15 min at room temperature. Next, 0.2 mL of chloroform was then mixed in each tube and then incubated at room temperature for 3 min. Tubes were then centrifuged at 11400 rpm for 15 min at 4 ℃, where afterwards the upper aqueous phase containing RNA was transferred to new 2.0 mL RNase free microcentrifuge tubes. Next, 0.5 mL of 100% isopropanol was added to the tubes containing subjects' RNA samples, where they were incubated at room temperature for 10 min. Tubes were then centrifuged at 11400 rpm for 10 min at 4 ℃, where afterwards the RNA pellets were washed in 1 mL of 75% ethanol. Washed RNA pellets were then centrifuged for 8700 rpm for 5 min at 4 ℃ and then air-dried until fully dried. Dried RNA pellets were then suspended in 20 μL of RNase free H_2O and boiled to 60 ℃ for 10 min.

Conversion of RNA to cDNA was done following the iScript™ Reverse Transcription (Bio-Rad®) manufacturer's instruction. RNA concentration from each subjects' samples were first quantified *via* NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific®) and then diluted to 600 ng of total RNA. Next, diluted RNA samples were then added to PCR reaction tubes that contained 0.2 mL PCR reaction, 4 μL of iScript™ Reverse Transcription (Bio-Rad®), and up to 20 μL RNase free H_2O. The PCR reaction tubes then underwent a PCR protocol consisting of 5 min at 25 ℃, 20 min at 46 ℃ and 1 min at 95 ℃, where the final concentration of cDNA for each sample was 30 ng/μL.

For the RT-PCR reaction, 1 μL of cDNA (30 ng) was

Table 1 Demographics and results of *mycobacterium avium* subspecies *paratuberculosis* presence and frequency of *PTPN2: rs478582/PTPN22:rs2476601* in CD subjects

Sample code	Gender	Age	Diagnosis	MAP +/-	*PTPN2:rs478582*	*PTPN22:rs2476601*
RCS1	M	50	CD	-	TC	GA
RCS2	F	25	CD	-	TC	GA
RCS3	F	68	CD	+	TC	GG
RCS4	M	26	CD	+	CC	GG
RCS5	F	56	CD	+	CC	GG
RCS6	NA	NA	CD	+	TC	GG
RCS7	M	60	CD	+	TC	GG
RCS8	M	43	CD	+	TC	GG
RCS9	F	54	CD	-	CC	GG
RCS10	F	31	CD	NA	TC	GG
RCS11	M	21	CD	+	NA	GG
RCS12	M	25	CD	+	CC	GG
RCS13	F	40	CD	+	TC	GG
RCS14	M	36	CD	+	TC	GG
RCS15	NA	NA	CD	-	CC	GA
RCS16	F	25	CD	+	TC	GG
RCS17	F	27	CD	+	TC	GG
RCS18	M	20	CD	-	TT	GG
RCS19	M	25	CD	+	CC	GA
RCS20	F	41	CD	-	TC	GG
RCS21	M	20	CD	-	TT	GG
RCS22	M	40	CD	-	TC	GG
RCS23	M	30	CD	-	TC	GG
RCS24	F	60	CD	+	TC	GG
RCS25	F	39	CD	+	TT	GG
RCS26	F	30	CD	+	CC	GA
RCS27	F	43	CD	+	CC	GG
RCS28	M	30	CD	+	TC	GA
RCS29	M	28	CD	+	TC	GG
RCS30	M	66	CD	+	TT	GG
RCS31	M	53	CD	-	TT	GG
RCS32	M	28	CD	-	TC	GA
RCS33	F	38	CD	+	CC	GG
RCS34	M	44	CD	-	CC	GA
RCS35	M	53	CD	-	TC	GG
RCS36	M	24	CD	+	TC	GG
RCS37	F	51	CD	+	TC	GG
RCS38	F	46	CD	+	TC	GG
RCS39	M	24	CD	-	CC	GG
RCS40	F	63	CD	+	TC	GG
RCS41	F	25	CD	-	TC	GG
RCS42	F	66	CD	-	TC	GG
RCS43	F	27	CD	+	TC	GG
RCS44	F	25	CD	+	TC	GG
RCS45	F	38	CD	+	TC	GG
RCS46	F	26	CD	-	CC	AA
RCS47	M	54	CD	+	TT	GA
RCS48	F	31	CD	+	TC	GG
RCS49	M	56	CD	-	CC	GG
RCS50	F	53	CD	-	TC	GG
RCS51	F	51	CD	-	TT	GA
RCS52	F	23	CD	+	TC	GG
RCS53	M	26	CD	+	TC	GG
RCS54	M	38	CD	-	TT	GG
RCS55	F	31	CD	+	TC	GG
RCS56	M	61	CD	+	TC	GG
RCS57	F	24	CD	+	TC	GG
RCS58	M	57	CD	-	CC	GG
RCS59	F	30	CD	+	TT	GG
RCS60	M	51	CD	-	CC	GG
RCS61	F	55	CD	-	CC	GG
RCS62	F	61	CD	-	TT	GG
RCS63	F	31	CD	+	TC	GG
RCS64	F	56	CD	NA	TC	GG
RCS65	M	25	CD	+	NA	NA

RCS66	F	53	CD	+	NA	NA
RCS67	M	30	CD	-	TC	GG
RCS68	F	49	CD	-	CC	GG
RCS69	M	28	CD	+	TT	GG
RCS70	M	26	CD	+	TT	GG
RCS71	M	26	CD	+	CC	GG
RCS72	M	58	CD	+	CC	GG

CD: Crohn's disease; TT: Homozygous major allele/no SNP; TC: Heterozygous allele; CC: Homozygous minor allele; GG: Homozygous major allele/no SNP; GA: Heterozygous allele; AA: Homozygous minor allele; MAP: *Mycobacterium avium* subspecies *paratuberculosis*.

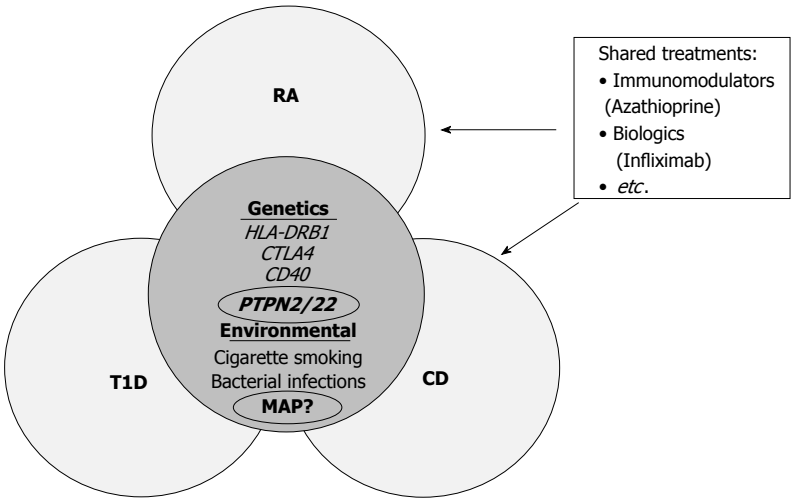


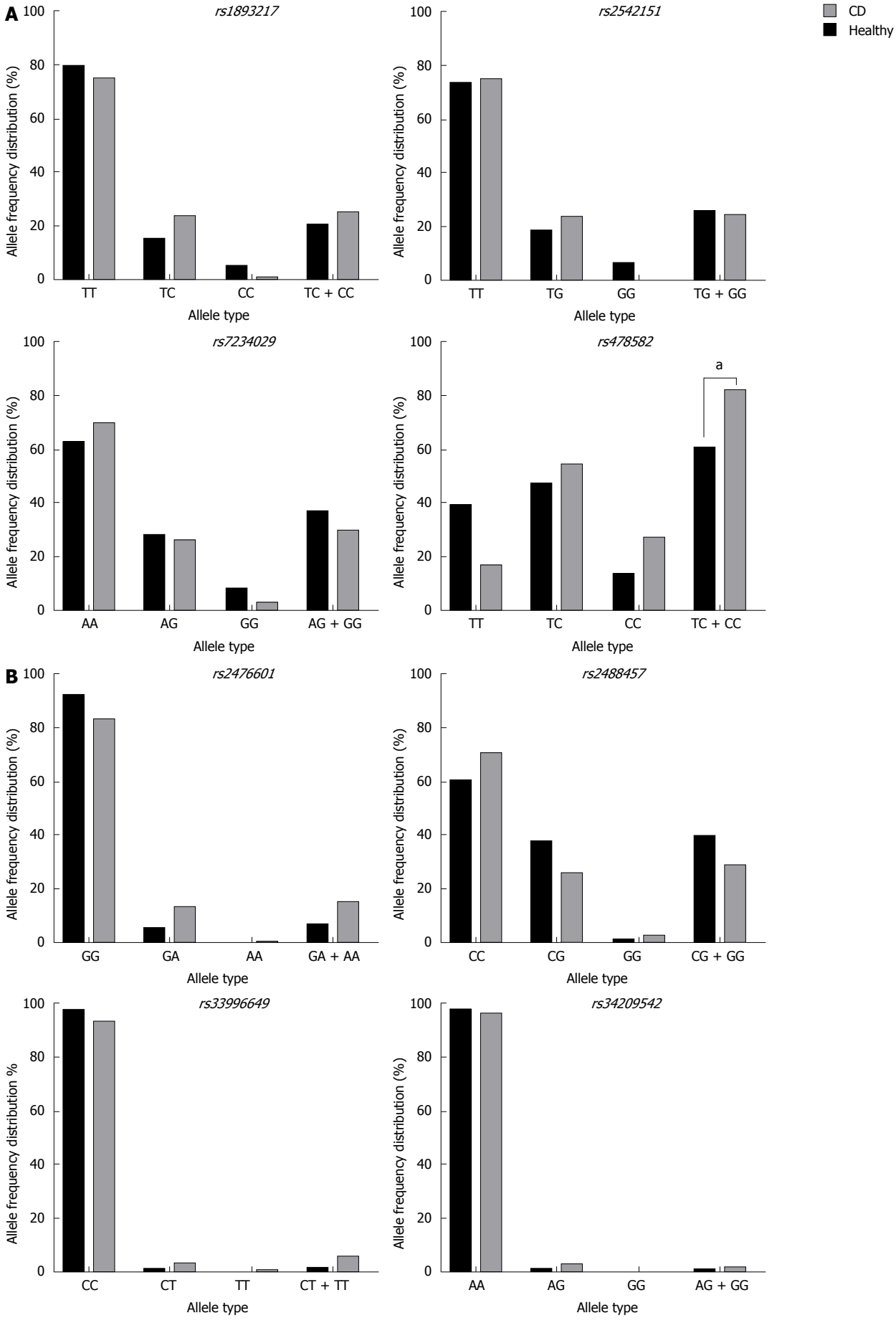
Figure 1 Shared genetic predispositions and environmental triggers between common inflammatory autoimmune disorders. For inflammatory autoimmune disorders, many share the same treatments and some of the same genetic single nucleotide polymorphisms in specific immunity genes. Thus, it is possible that these disorders share the same environmental triggers as well, such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP) bacterial infection. CD: Crohn's disease; *PTPN2: Protein tyrosine phosphatase non-receptor type 2; PTPN22: Protein tyrosine phosphatase non-receptor type 22.*

added to a 96-well microamp plates along with 10 μL of Fast SYBR Green Mastermix (ThermoFisher Scientific®), 1 μL of PrimePCR SYBR Green Assay mix (Bio-Rad®) specific to target gene, and 8 μL of sterile H_2O. For the positive control for the RT-PCR reactions, the 18s RNA gene was the target to determine if the reaction work and to obtain baseline CT readings. The oligonucleotide primers for the 18s RNA gene that were used for the RT-PCR reaction was the following: forward primer: 5'-GTA ACC CGT TGA ACC CCA TT-3' and reverse primer: 5'-CCA TCC AAT CGG TAG TAG CG-3'. RT-PCR reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems®), where relative gene expression levels were calculated using Δ CT (sample gene CT reading-18s RNA gene CT baseline reading) and using the equation $(2^{(\Delta CT)} \times 1000)$.

Detection of MAP IS900 DNA

MAP *IS900* DNA was detected *via* nPCR from cultured peripheral leukocytes that were isolated from the subjects' blood samples as described previously[15]. Briefly, subjects' blood sample tubes were centrifuged for 3000 rpm for 10 min at room temperature, where the buffy coat layer containing peripheral leukocytes was present and transferred to new sterile 2.0 mL microcentrifuge tubes. The peripheral leukocytes were then washed twice by adding double the volume of red cell lysis buffer (ammonium chloride solution, G-Biosciences®) to each tube and incubating/gently rocking for 10 min and then centrifuged at 5000 rpm for 5 min at room temperature. The supernatant from each subjects' samples were then removed and the isolated peripheral leukocyte pellets were re-suspended in Tris-EDTA (TE) buffer. The isolated pellets were then cultured in BD Bactec™ MGIT™ Para-TB medium (Becton, Dickinson and Company©) tubes supplemented with 800 uL of Bactec™ MGIT™ Para-TB Supplement (Becton, Dickinson and Company©) for six months at 37 ℃ in a BD Bactec™MGIT™ 320 Analyzer (Becton, Dickinson and Company©).

After six months of culturing, subjects' cultured samples underwent DNA extraction by using a modified DNAzol® (ThermoFisher Scientific®) extraction protocol as follows. A 2.0 mL sampling of culture from each subjects' tubes were obtained and pipetted into new sterile 2.0 mL microcentrifuge tubes. The tubes were then centrifuged at 13000 rpm for 2.5 min, where afterwards the supernatant was discarded from the tubes and the culture pellets were saved. The subjects' culture pellet tubes were then mixed with 1.0 mL DNAzol® reagent and then mixed with 400 μL of 100% isopropanol. The tubes were then incubated for 15 min at room temperature followed by centrifugation at 8000 rpm for 6 min, where afterwards the supernatant was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rs1893217
rs2542151
rs7234029
rs478582
B
rs2476601
rs2488457
rs33996649
rs34209542
CD
Healthy
Allele frequency distribution (%)
Allele frequency distribution %
Allele type
TT
TC
CC
TC + CC
TG
GG
TG + GG
AA
AG
AG + GG
GA
AA
GA + AA
CG
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CT
TT
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a

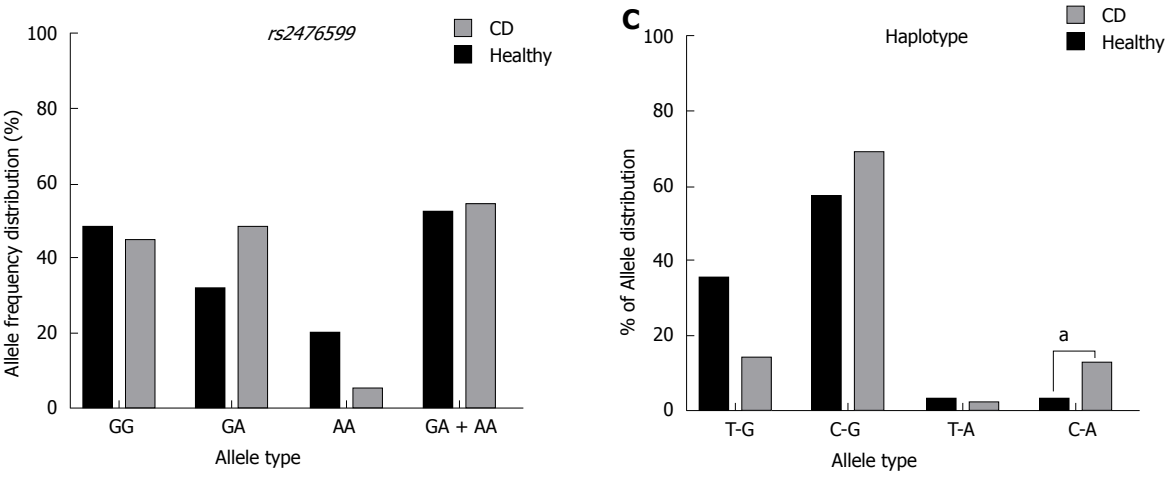

Figure 2 Allele frequency in nine single nucleotide polymorphisms in crohn's disease and healthy control subjects. A: Represents allele frequency of *PTPN2* SNPs: *rs1893217*, *rs2542151*, *rs7234029*, *rs478582*; B: Represents allele frequency of *PTPN22* SNPs: *rs2476601*, *rs2488457*, *rs33996649*, *rs34209542*, *rs2476599*; C: Represents haplotype combinations *PTPN2:rs478582* and *PTPN22:rs2476601*. $^{a}P < 0.05$, healthy *vs* CD. T-G: Major/major; C-G: SNP/major; T-A: Major/SNP; C-A: SNP/SNP; SNPs: Single nucleotide polymorphisms; CD: Crohn's disease; *PTPN2: Protein tyrosine phosphatase non-receptor type 2; PTPN22: Protein tyrosine phosphatase non-receptor type 22.*

discarded, leaving a DNA pellet. DNA pellets from the subjects' samples were then washed once with 500 μL DNAzol® reagent and centrifuged at 8000 rpm for 5 min. Supernatant was then discarded from the tubes and the DNA pellets were then washed again with 1.0 mL of 75% ethanol, where they were centrifuged at 8000 rpm for 5 min. DNA pellets were then dried after supernatant was removed *via* speedvac for 5 min. The dried DNA pellets were then dissolved in 50 μL of TE buffer.

MAP *IS900* DNA was then detected in each subjects' samples by the use of our nPCR protocol and nucleotide primers as described previously[15]. Subjects were considered to have MAP presence when a 298 bp band on a 2% agarose gel is shown after nPCR reaction. The positive MAP DNA control that was used originated from our laboratory cultured clinical strain UCF4, which was isolated from a CD patient. The negative controls for each PCR step that was used contained all PCR reagents except for the DNA template used in the reactions.

T-cell isolation and proliferation assay

T-cells were fully isolated from subjects' whole blood samples by the use of RosetteSep™ Human T-cell Enrichment Cocktail (StemCell™ Technology) as per manufacture's instruction. For the T-cell isolation and proliferation assays, the entire T-cell populations were examined in this study and were not segregated by subpopulations. Briefly, 50 μL/mL of RosetteSep™ Human T-cell Enrichment Cocktail was added to each subjects' whole blood samples and was incubated at 20 min at room temperature. Samples were then diluted with equal volumes of PBS with 2% fetal bovine serum (FBS, Sigma-Aldrich®) and mixed gently. The mixtures from each subjects' samples were then layered on top of a Lymphoprep™ (Axis-Shield®) density medium in a separated tube and centrifuged for 20 min at 2500 rpm at room temperature. Separated T-cells from each subjects' samples were then found on top of the density medium layer and were collected into new sterile 2.0 mL microcentrifuge tubes and washed twice with PBS with 2% FBS.

Subjects' isolated T-cells were then plated on a 96-well plate, where T-cell proliferation assays were done using bromodeoxyuridine (BrdU) labeling proliferation ELISA kit (Roche Molecular Biochemicals®) as described previously[16]. To stimulate the subjects' isolated T-cells, phytohematoagglutunin (PHA) was used as a positive control mitogen. The test mitogen used in the T-cell proliferation assays was purified protein derivative-like (PPD-like) from UCF4 MAP bacterial cultures that were prepared by purification of supernatant from sonicated protein extract. Briefly, 1×10^5 isolated T-cells from each subjects' samples were transferred in triplicates to 96-well plates and incubated in the following conditions: RPMI-1640 (Sigma-Aldrich®) only, PHA (10 μg/mL, Sigma-Aldrich®) or MAP PPD-like (5 μg/mL) along with respected subjects' plasma. The plates were then incubated for 72 h at 37 ℃ and 5% CO_2 and then labeled with 20 μL/well of BrdU and incubated again for 24 h at 37 ℃ and 5% CO_2. The T-cell proliferation assay was done through the Roche BrdU proliferation ELISA kit as described previously[16]. Relative T-cell proliferation levels of samples were compared to the control group (isolated T-cells in RPMI only) by examining the fold change in the absorbance reading of each well at 450 nm.

Statistical analysis

Samples were analyzed for significance using unpaired, two-tailed *t*-tests; unpaired, two-tailed *z*-scores; and odds ratio. GraphPad Prism 7 was used for statistical analysis and creation of graphs. *P*-values < 0.05 were considered significant.

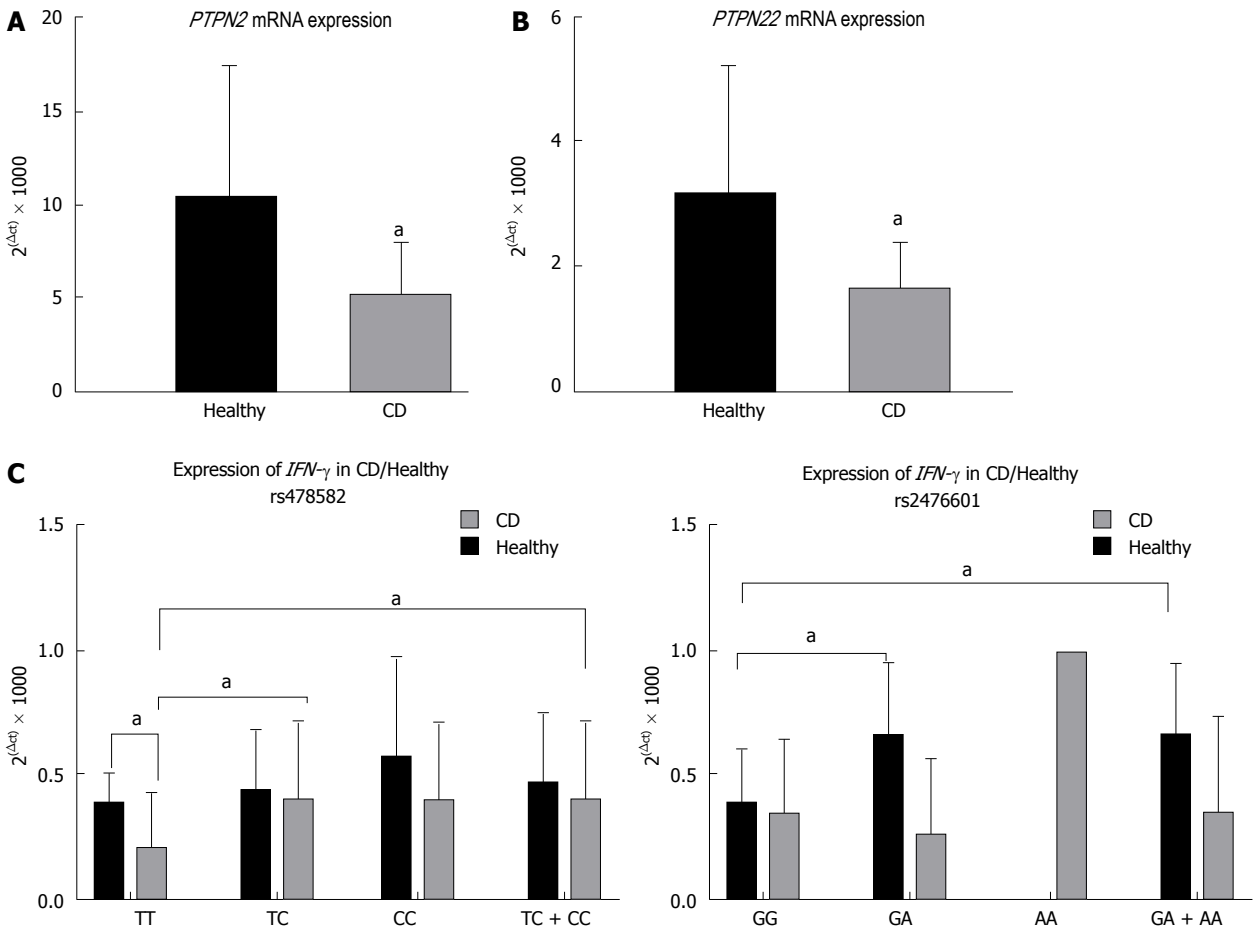

Figure 3 Relative mRNA expression (2($^{-\Delta CT}$) × 1000) of *PTPN2*, *PTPN22* and *IFN-γ*. Relative mRNA expression of *PTPN2* (A) and *PTPN22* (B) in CD and healthy control subjects. Relative mRNA expression of *IFN-γ* was correlated with CD and healthy control subjects with either *PTPN2:rs478582* (C) or *PTPN22*:2476601 (D). *IFN-γ: Interferon-γ*; CD: Crohn's disease; *PTPN2: Protein tyrosine phosphatase non-receptor type 2*; *PTPN22: Protein tyrosine phosphatase non-receptor type 22*. [a]*P* < 0.05.

RESULTS

PTPN2/22 SNP allele frequency in CD

Allele frequency of the nine SNPs examined in *PTPN2/22* found in both CD subjects and healthy controls are shown in Figure 2. All genotyped samples were found in Hardy-Weinberg equilibrium. Out of the four SNPs found in *PTPN2* (*rs1893217*, *rs2542151*, *rs7234029*, and *rs478582*), *rs478582* was significant in the CD, where heterozygous (TC) or minor (CC) alleles when examined together were detected in 57/69 (82.6%) in CD compared to 36/59 (61.0%) healthy controls (OR = 3.03, 95%CI: 1.35-6.84, *P*-values < 0.05, Figure 2A). Specifically, the heterozygous (TC) alleles were detected in 38/69 (55.1%) CD compared to the 28/59 (47.5%) of healthy controls, while homozygous (CC) alleles were detected in 19/69 (27.5%) CD compared to 8/59 (13.6%) healthy controls. SNPs *rs1893217*, *rs2542151*, and *rs7234029* were found to be not significant in CD compared to the healthy controls. Out of the five SNPs specific to *PTPN22* (*rs2476601*, *rs2488457*, *rs33996649*, *rs34209542*, and *rs2476599*), none of SNPs were considered significant in CD compared to the healthy controls (Figure 2B). However, since *PTPN22: rs2476601* is found significantly in various inflammatory autoimmune diseases, we continued to investigate the SNP in more detail along with *PTPN2:rs478582*[3-5,17-19]. For *PTPN22:rs2476601*, CD with either heterozygous (GA) or minor (AA) alleles were detected in 11/70 (15.7%) subjects, while 4/62 (6.45%) was detected in healthy controls (OR = 2.7, 95%CI: 0.81-8.98, *P*-values > 0.05). Specifically, the heterozygous (GA) alleles were detected in 10/70 (14.3%) CD compared to the 4/62 (6.45%) of healthy controls, while homozygous (AA) alleles were rare in all samples.

For confirmation that CD subjects were significant in having SNP alleles for *PTPN2:rs478582* and *PTPN22: rs2476601*, determination of haplotype combinations were done (Figure 2C). Examination of the following haplotype combinations between *PTPN2:rs478582* and *PTPN22:rs2476601* were examined: T-G, C-G, T-A, and C-A. The T-G haplotype (major/major) was found more significantly in the healthy controls (21/59 = 35.6%) than in CD (10/69 = 14.5%, *P*-values < 0.05). The C-G haplotype (heterozygous or minor/major) and the C-A (heterozygous or minor/heterozygous or minor) were found more in CD (48/69 = 69.6%; 9/69 = 13.0%, respectively) than in healthy controls (34/59 = 57.6%; 2/59 = 3.39%, respectively). The C-A haplotype was found more significantly in CD than the healthy controls

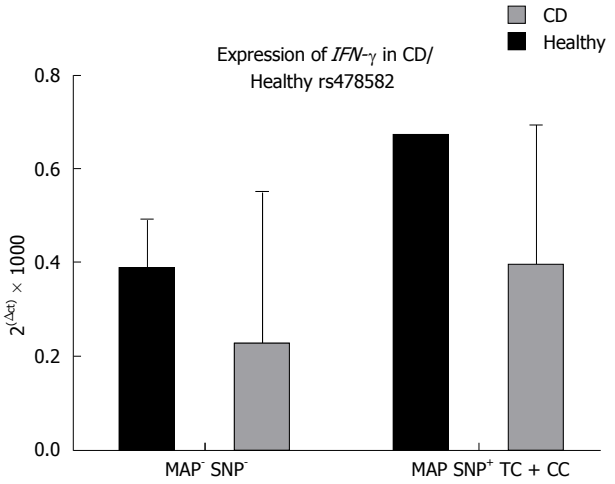

Figure 4 The effect of both *Mycobacterium avium* subspecies *paratuberculosis* and *PTPN2:rs478582* on *IFN-γ* gene expression in Crohn's disease and healthy control subjects. *IFN-γ: Interferon-γ*; CD: Crohn's disease; MAP: *Mycobacterium avium* subspecies *paratuberculosis*; SNPs: Single nucleotide polymorphisms.

(*P*-values < 0.05).

Relationship of PTPN2:rs478582 and PTPN22:rs2476601 on expression of PTPN2/22 and IFN-γ in CD

The average relative gene expression ($2^{(-\Delta CT)} \times 1000$) of *PTPN2*, regardless of SNPs, in CD was significantly lower (5.27 ± 2.68, *n* = 38) than in healthy controls (10.5 ± 6.95, *n* = 30, *P*-values < 0.05, Figure 3A). Similarly, the average relative gene expression of *PTPN22*, regardless of SNPs, was also significantly lower in CD (1.76 ± 1.12, *n* = 38) than in healthy controls (3.24 ± 1.84, *n* = 30, *P*-values < 0.05, Figure 3B). The evaluation of the effect of *PTPN2:rs478582* and *PTPN22:rs2476601* on expression of *PTPN2/22* and *IFN-γ* was determined.

For subjects with either heterozygous (TC) or minor (CC) alleles in *PTPN2:rs478582*, regardless of disease, expression of *PTPN2* did not change when compared to the normal (TT) subjects. However, when examining the CD and healthy control subjects in each allele group, CD overall had a lower average relative gene expression of *PTPN2*. The average relative gene expression in CD with heterozygous (TC) or minor (CC) alleles in *PTPN2:rs478582* was significantly lower (5.34 ± 2.77, *n* = 31) compared to 10.2 ± 7.15 (*n* = 21) in healthy controls with similar SNPs (*P*-values < 0.05). Specifically, when examining subjects with heterozygous (TC) alleles in *PTPN2:rs478582*, CD average relative gene expression was 5.22 ± 2.57 (*n* = 22), which was significantly lower than the healthy controls with heterozygous (TC) alleles (10.5 ± 7.15, *n* = 17, *P*-values < 0.05). When examining subjects with homozygous (CC) alleles in *PTPN2:rs478582*, CD average relative gene expression was 5.64 ± 3.37 (*n* = 9), which was lower than the healthy controls with homozygous (CC) alleles (8.89 ± 8.03, *n* = 4).

For subjects with either heterozygous (GA) or minor (AA) alleles in *PTPN22:rs2476601*, regardless of disease, expression of *PTPN22* did not change when compared to the normal (GG) subjects. However, when examining the CD and healthy control subjects in each allele group, CD overall had a lower average relative gene expression of *PTPN22*. The average relative gene expression in CD with heterozygous (GA) or minor (AA) alleles in *PTPN22:rs2476601* was significantly lower (1.58 ± 0.93, *n* = 6) compared to 3.40 ± 1.19 (*n* = 4) in healthy controls with similar SNPs (*P*-values < 0.05). Specifically, when examining subjects with heterozygous (GA) alleles in *PTPN22:rs2476601*, CD average relative gene expression was 1.48 ± 1.00 (*n* = 5), which was significantly lower than the healthy controls with heterozygous (GA) alleles (3.40 ± 1.19, *n* = 4, *P* < 0.05). Minor (AA) alleles in *PTPN22:rs2476601* was rare in all subjects.

Correlation analyses were performed to determine if expression of relative gene expression of *IFN-γ* changed in subjects with *PTPN2:rs478582* or *PTPN22:rs2476601* (Figure 3C and 3D, respectively). The average relative gene expression of *IFN-γ* in CD subjects with the *PTPN2:rs478582* heterozygous (TC) or minor (CC) allele was 0.41 ± 0.31 (*n* = 38), which was significantly higher compared to the CD subjects with normal (TT) alleles (0.21 ± 0.22, *n* = 12, *P* < 0.05). Specifically, CD subjects with the heterozygous (TC) allele had significantly higher (0.41 ± 0.31, *n* = 24, *P* < 0.05) *IFN-γ* relative gene expression than CD subjects with normal (TT) alleles, while CD subjects with the minor (CC) alleles had higher gene expression as well (0.40 ± 0.31, *n* = 14). There was no significant change in *IFN-γ* relative gene expression in the CD subjects with the *PTPN22:rs2476601* heterozygous (GA) or minor (AA) alleles. However, in healthy controls, subjects with the heterozygous (GA) or minor (AA) alleles had a significantly higher gene expression (0.67 ± 0.28, *n* = 4, *P* < 0.05) than healthy controls with normal (GG) alleles (0.40 ± 0.21, *n* = 20).

Effect of PTPN2:rs478582 and PTPN22:rs2476601 on susceptibility of MAP infection in CD

Overall detection of MAP *IS900* DNA was found in CD and healthy control subjects and were correlated with *PTPN2:rs478582* and *PTPN22:rs2476601* (Table 2). Out of 70 CD subjects, 43 (61.4%) were positive for *MAPbacteremia* compared to only 4/48 (9.33%) of healthy controls (*P* < 0.05, OR = 17.5, 95%CI: 5.65-54.3).

Correlation analyses with *PTPN2:rs478582* and *PTPN22:rs2476601* along with MAP infection was done on CD and healthy controls to see if these SNPs increase MAP susceptibility (Table 2). For CD subjects with heterozygous (TC) or minor (CC) alleles in *PTPN2:rs478582*, 34/56 (60.7%) had *MAPbacteremia* presence compared to only 2/30 (6.67%) in healthy controls with similar SNPs (*P* < 0.05, OR = 21.6, 95%CI: 4.68-100.1).

Specifically, CD subjects with heterozygous (TC) alleles in *PTPN2:rs478582* was 25/37 (67.6%) compared to 0/22 (0.00%) in healthy controls with heterozygous (TC) alleles ($P < 0.05$, OR = 91.8, 95%CI: 5.14-1640.3). The CD subjects with heterozygous (TC) or minor (CC) alleles group (34/56 = 60.7%) and CD subjects with heterozygous (TC) allele group (25/37 = 67.6%) in *PTPN2:rs478582* had higher *MAPbacteremia* compared to CD subjects with normal (TT) alleles (6/12 = 50%).

For CD subjects with heterozygous (GA) alleles in *PTPN22:rs2476601*, 3/10 (30.0%) had *MAPbacteremia* compared to 0/4 (0.00%) in healthy controls with heterozygous (GA) alleles (OR = 4.2, 95%CI: 0.17-101.5). Presence of *MAPbacteremia* was rare in all subjects with the minor (AA) allele.

Correlation of haplotype combinations of *PTPN2: rs478582* and *PTPN22:rs2476601* alleles on susceptibility to *MAPbacteremia* was analyzed, where CD subjects with the C-G haplotype (heterozygous or minor/major) had 31/46 (67.4%) with *MAPbacteremia* presence compared to 2/29 (6.90%) of healthy controls with the C-G haplotype (*P*-values < 0.05, OR = 30.0, 95%CI: 6.3-142.6). The T-A haplotype (major/heterozygous or minor) and the C-A haplotype (heterozygous or minor/heterozygous or minor) was rare in all samples. However, CD subjects with the T-A haplotype had 1/2 (50.0%) with *MAPbacteremia* presence compared to the 0/2 (0.00%) in healthy controls with the T-A haplotype, while CD subjects with the C-A haplotype had 3/9 (33.3%) with *MAPbacteremia* presence compared to the 0/2 (0.00%) in healthy controls with the C-A haplotype.

Relationship of combined MAP presence with PTPN2: rs478582 and PTPN22:rs2476601 on expression of PTPN2/22 and IFN-γ in CD

When examining CD and healthy control subjects with or without *MAPbacteremia* presence alone, there was no change in *PTPN2/22* and *IFN-γ* relative gene expression when examining correlation data. However, *PTPN2* was significantly lower in CD subjects than in the health control subjects regardless of *MAPbacteremia* presence or not. CD subjects who had *MAPbacteremia* presence had an average relative gene expression of 5.25 ± 2.58 ($n = 21$) in *PTPN2* compared to the healthy controls with *MAPbacteremia* presence (11.9 ± 10.5, $n = 3$, $P < 0.05$). CD subjects who had an absence of *MAPbacteremia* presence had an average relative gene expression of 5.28 ± 2.87 ($n = 17$) in *PTPN2* compared to the healthy controls without *MAPbacteremia* presence (10.3 ± 6.71, $n = 27$, $P < 0.05$). For *PTPN22* average relative gene expression, CD subjects with *MAPbacteremia* presence had 1.73 ± 0.97 ($n = 21$) compared to healthy controls with *MAPbacteremia* presence (2.83 ± 1.94, $n = 3$). CD subjects without *MAPbacteremia* presence had an average relative gene expression of 1.81 ± 1.31 ($n = 17$) in *PTPN22* compared to the healthy controls without *MAPbacteremia* presence (3.29 ± 1.86, $n = 27$, *P*-values < 0.05).

The effect of combined *MAPbacteremia* presence and either *PTPN2:rs478582* or *PTPN22:rs2476601* did not significantly change *PTPN2/22* expression in all CD and healthy control samples. However, when examining the combined effects of *MAPbacteremia* presence and either *PTPN2:rs478582* or *PTPN22:rs2476601*, the average relative gene expression of *IFN-γ* does increase in subjects compared to subjects without *MAPbacteremia* presence and no SNPs. For CD subjects with both *MAPbacteremia* and heterozygous (TC) or minor (CC) alleles in *PTPN2:rs478582*, the average relative gene expression of *IFN-γ* was higher (0.40 ± 0.29 $n = 22$) compared to the CD subjects without *MAPbacteremia* and *PTPN2:rs478582* (0.23 ± 0.31, $n = 5$, Figure 4). For CD subjects with both *MAPbacteremia* and heterozygous (GA) or minor (CC) alleles in *PTPN22: rs2476601*, the average relative gene expression of *IFN-γ* was higher (0.42 ± 0.32, $n = 4$) compared to the CD subjects without *MAPbacteremia* and *PTPN22: rs2476601* (0.37 ± 0.31, $n = 18$).

T-cell proliferation response in CD

T-cell functionality when SNPs and *MAPbacteremia* was presented in subjects was determined in five CD and five healthy control subjects. All five CD subjects that had their T-cell response tested had SNPs in either *PTPN2:rs478582* and/or *PTPN22:rs2476601*, while the five healthy control subjects had no observed SNPs present. Overall, when the subjects' T-cells were treated with PHA, the average overall fold change in the CD subjects was 2.22 ± 1.36 ($n = 5$) fold increase compared to the healthy controls (1.67 ± 0.51 fold increase, $n = 5$). Similarly, when the same T-cells were treated with MAP PPD-like, the average overall fold change in CD subjects was 2.01 ± 0.79 ($n = 5$) compared to the healthy controls (1.39 ± 0.24 fold increase, $n = 5$).

Out of the five CD subjects, 3/5 were tested for having *MAPbacteremia* presence. When examining T-cells treated with PHA from CD subjects tested positive for *MAPbacteremia* presence, the average overall fold change was 2.7 ± 1.65 ($n = 3$) compared to the CD subjects' T-cells that were absence of *MAPbacteremia* presence and treated with PHA (1.51 ± 0.51 fold increase, $n = 2$). Similarly, when the same T-cells were treated with MAP PPD-like, the average overall fold change in CD subjects with *MAPbacteremia* was 2.5 ± 0.59 ($n = 3$) compared to the CD subjects' T-cells without *MAPbacteremia* presence (1.27 ± 0.12 fold increase, $n = 2$).

DISCUSSION

The pathogenesis of CD, as with other inflammatory autoimmune disorders, involves both genetic predisposition leading to higher immune responses and an environmental trigger that exacerbates the

immune response. However, with current diagnosis and treatment, it has been difficult to treat CD symptoms due to loss of treatment response and many side effects[6-10]. Thus, understanding the key elements of CD pathogenesis (genetic SNPs and environmental triggers), it is possible to find new treatment targets for the disease and new diagnosis techniques as well. CD pathogenesis is very dependent on the overproduction of pro-inflammatory cytokines such as TNF-α and IFN-γ, which promote chronic inflammation, increased granuloma formation, and increased apoptosis of intestinal tissues[8,9,20,21]. Since the majority of CD medications are blocking pro-inflammatory cytokines such as TNF-α and IFN-γ, other types of targets has been ignored[6-10,20,21]. This study is focused on finding new targets for both diagnosis and treatment of CD, where we looked into the SNPs of negative regulatory genes *PTPN2/22* and their impact on: increased production of pro-inflammatory cytokines, apoptosis, mycobacterial susceptibility, and inflammation. To our knowledge, this is the first study to look into SNPs in both *PTPN2/22* together along with correlation with gene expression and MAP susceptibility in CD.

The effect of SNPs in *PTPN2/22* in CD pathogenesis has been highly debated in the literature, thus we selected nine SNPs that not only was found associated with CD, but with other diseases as well[4,5,17-19,22-24]. Out of the nine SNPs examined in this study, *PTPN2:rs478582* was found to be significant in CD (*P*-values < 0.05, OR = 3.03) compared to the healthy controls (Figure 2A). Although *PTPN22:rs2476601* was found to not be significant to CD ($P > 0.05$, OR = 2.7) compared to the healthy controls, we continued to study the effects of the SNP along with *PTPN2:rs478582* due to *PTPN22:rs2476601* being associated with inflammatory autoimmune diseases in general (Figure 2B)[3-5,17-19,22-24]. Since a diverse population (no restriction on race, place of origin, age, or gender) was used in this study, alterations of allele distribution in the SNPs could possibly happen due to SNPs overall fluctuating between different population groups[3-5,17-19,22-24]. Further isolated population studies on *PTPN2/22* SNPs in CD subjects need to be investigated more. Knowledge of which SNP is more associated with CD could possibly be used as a diagnosis tool for clinicians when examining patients with CD like symptoms.

Gene expression of *PTPN2/22* correlated with the SNPs *PTPN2:rs478582* and *PTPN22:rs2476601* was also done to determine if the SNPs did change *PTPN2/22* levels. Although overall *PTPN2/22* expression was significantly decreased in CD subjects ($P < 0.05$, Figure 3A and 3B), the SNPs *PTPN2:rs478582* and *PTPN22:rs2476601* did not change gene expression between normal, heterozygous, or minor alleles. However, *IFN-γ* gene expression was found significantly higher in both CD and healthy controls ($P < 0.05$) along with an overall increased T-cell activity in subjects that had heterozygous/minor alleles in either *PTPN2:rs478582* and/or *PTPN22:rs2476601* (Figure 3C and 3D). These

Table 2 MAP IS900 nPCR presence and correlation with *PTPN2:rs478582/PTPN22:rs2476601* in clinical subjects

MAP presence			
	Healthy	CD	OR (95%CI)
Overall	4/48 (9.33%)	43/70 (61.4%)[a]	17.5 (5.65–54.3)[a]
rs478582			
TT	2/17 (11.8%)	6/12 (50%)[a]	7.5 (1.17-48.2)[a]
TC	0/22 (0%)	25/37 (67.6%)	91.8 (5.14-1604.3)[a]
CC	2/8 (25%)	9/19 (47.4%)	2.7 (0.43-16.9)
TC + CC	2/30 (6.67%)	34/56 (60.7%)[a]	21.6 (4.68-100.1)[a]
rs2476601			
GG	4/59 (6.78%)	33/59 (55.9%)[a]	17.6 (5.59-54.4)[a]
GA	0/4 (0%)	3/10 (30.0%)	4.2 (0.17-101.5)
AA	0/1 (0%)	0/1 (0%)	1.00 (0.02-92.4)
GA + AA	0/5 (0%)	3/11 (27.3%)	4.53 (0.19-105.8)
Haplotypes			
T-G	2/15 (13.3%)	5/10 (50.0%)[a]	6.5 (0.94-45.1)[a]
C-G	2/29 (6.90%)	31/46 (67.4%)[a]	30.0 (6.3-142.6)[a]
T-A	0/2 (0%)	1/2 (50.0%)	5.00 (0.11-220.6)
C-A	0/2 (0%)	3/9 (33.3%)	2.69 (0.1-73.2)

[a]$P < 0.05$ *vs* healthy.

correlation analyzes shows that the SNPs *PTPN2:rs478582* and *PTPN22:rs2476601* may not necessarily change the regulation of the *PTPN2/22* gene, but could possible disrupt the protein activity of PTPN2/22. For the *PTPN2:rs478582* SNP, a base change (T > C) in intron 3 occurs, where it is theorized that splicing problems could occur during the RNA splicing[25-28]. This could lead to loss of activity in the protein once fully translated[25-28]. The *PTPN22:rs2476601* SNP is a base change (G > A) that occurs in exon 14, which physically changes the amino acid arginine (R) to a tryptophan (W) on the 620 amino acid residue on the catalytic portion of the PTPN22 protein[19,26-28]. It has been highly debated what the R620W does to the PTPN22 protein, but it is suspected to cause the protein to be less active[19,26-28]. Overall, the SNPs *PTPN2:rs478582* and *PTPN22:rs2476601* seem to cause a loss of function in PTPN2/22, thus leading to less negative regulated T-cells. This will lead to a high production of pro-inflammatory cytokines, which will lead to increased inflammation/apoptosis in intestinal tissues in CD subjects. Other SNPs in *PTPN2/22* will need to be studied further to see if those SNPs will alter gene expression of *PTPN2/22* instead of *PTPN2:rs478582* and *PTPN22:rs2476601* just altering protein activity. Although we only examined the effect of PTPN2/22 on the expression of *IFN-γ*, other factors do control *IFN-γ* expression and production. These include cytokines, such as TNF-α and IL-12, which stimulate T-cell production of IFN-γ and cytokines, such as IL-6 and IL-10, which decrease T-cell production of IFN-γ[29]. However, since CD and other inflammatory autoimmune disorders are T-cell mediated, we focused only on PTPN2/22 regulation on *IFN-γ* expression. This is due to PTPN2/22 ultimately acting as negative regulators of T-cell activity and thus controlling IFN-γ production from T-cells. Further investigation of the effect of these other

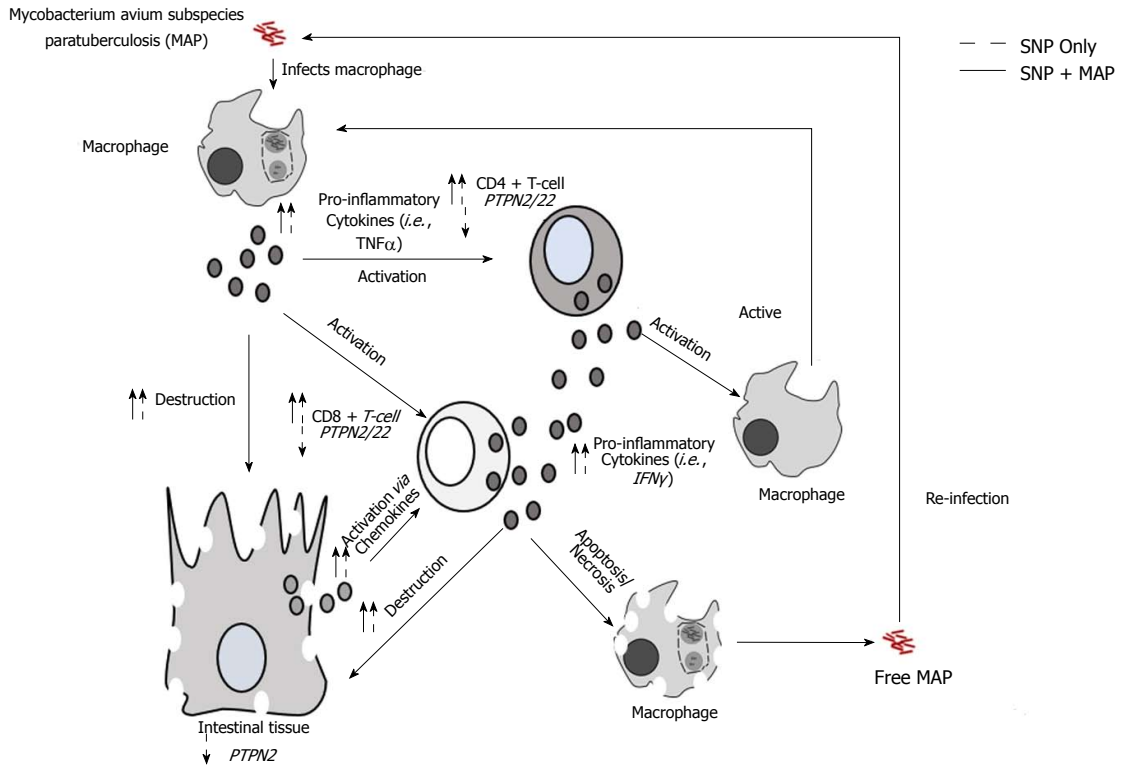

Figure 5 Complex interaction of Crohn's disease pathophysiology. The effect of single nucleotide polymorphisms (SNPs) in *Protein tyrosine phosphatase non-receptor type 2* and 22 (*PTPN2/22*) and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in a dysregulated immune response in crohn's disease (CD).

regulatory IFN-γ production cytokines in subjects with SNPs in *PTPN2/22* is needed.

Although the role MAP has been studied in CD pathogenesis extensively, correlation studies with SNPs in *PTPN2/22* and MAP susceptibility have not been done before until this study[4,9,11-16,20]. Overall, the correlation analyzes of SNPs in *PTPN2/22* and *MAPbacteremia* presence showed that the SNPs might have increased susceptibility in CD subjects (Table 2). Specifically, 60.7% (OR = 21.6, $P < 0.05$) of CD subjects with *PTPN2:rs478582* SNP (heterozygous or minor group) had *MAPbacteremia* presence, while 27.3% (OR = 4.53) subjects with the *PTPN22:rs2476601* SNP (heterozygous or minor group) had *MAPbacteremia*. Limitations however in the detection of MAP *IS900* DNA from the blood of subjects' samples do not provided the information that the MAP bacteria is alive or dead, thus does not show active infection or previous infection. Further culturing of the blood from the subjects is necessary to determine live MAP infection in the subjects examined. The findings found in this study suggest that SNPs in *PTPN2/22* increases susceptibility to *MAPbacteremia*, which is possible due to the lack of negative regulation in the T-cells. Since T-cells control macrophage activity and mycobacterial species such as MAP can survive in infected macrophages, it is important that the T-cells are regulated correctly in order to prevent MAP infection[30-34]. If problems involving the *PTPN2/22* gene regulation or function the PTPN2/22 protein occurs, T-cells will be overactive and in turn will make macrophages overactive as well (Figure 5)[30-34]. This increased activity of macrophages will not only lead to increased pro-inflammatory cytokines like TNF-α, but could allow MAP and other intracellular pathogens to survive and grow faster due to the increased activation of newer macrophages[30-34]. This is why SNPs in *PTPN2/22* and the hyperactivity of T-cells should increase susceptibility to intracellular pathogens such as MAP.

To further test if T-cells from the CD subjects with the *PTPN2:rs478582* and the *PTPN22:rs2476601* were overactive, we induced isolated T-cells from CD subjects with either PHA or MAP PPD-like. Although we did not isolate out total T-cell populations from mucosal intestinal tissues and instead from peripheral blood draws, we believe that T-cell proliferation will be the same regardless of the source of origin. This is possible due to *PTPN2/22* being found in every T-cell population, regardless of the site of isolation, thus SNPs in *PTPN2/22* should affect all T-cells in the body in the same way. Overall, CD subjects with the SNPs proliferated more than healthy controls without the SNPs. In addition, CD subjects who had *MAPbacteremia* presence and SNPs in *PTPN2/22* proliferated more than CD subjects who did not have *MAPbacteremia* presence. These analyzes showed that for T-cells to become overactive, both SNPs in *PTPN2/22* and the presence of *MAPbacteremia* is required to induce the pathogenesis process of CD. This is further evidence that for the pathogenesis of any inflammatory autoimmune disorder, both genetic predisposition and an environmental trigger are needed to cause disease. Further investigation in gene expression of pro-inflammatory cytokines produced (*IFN-γ* for example) by T-cells after being induced

with antigens need to be examined. Along with this, further investigation of subpopulations of T-cell activity is needed to determine which T-cell population is more active in subjects with SNPs in *PTPN2/22*.

Overall, SNPs in *PTPN2/22* lead to overactive T-cell activity and increased susceptibility to intracellular pathogens such as MAP. With genetic testing for SNPs and detection/treatment for mycobacterial infections such as MAP, it is possible for personalized treatment of CD to be an option. Further studies in SNPs in *PTPN2/22* and other immunity specific genes need to be researched and correlated with bacterial infections to improve CD diagnosis and treatment.

ARTICLE HIGHLIGHTS

Research background

Single nucleotide polymorphism (SNPs) and environmental triggers have been associated with a variety inflammatory autoimmune disorders including Crohn's disease (CD). Specifically, SNPs in the negative regulatory immune genes *Protein Tyrosine Phosphatase Non-receptor type 2* and *22* (*PTPN2/22*) have been associated with CD along with mycobacterial infections. Although both elements have been examined separately, correlation analysis have not been done to determine if SNPs in *PTPN2/22* along with a *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infections do cause a dysregulation in the immune system that could lead to CD symptoms.

Research motivation

Due to the flaws of current diagnosis and treatments of CD, new and better methods need to be determined. Investigating the pathogenesis of CD *via* SNPs analysis and MAP presence could lead to the possibility of individualized diagnosis/treatment for CD patients *via* genetic testing and antibiotic treatments. Our research could potentially propose newer routes of CD treatment for clinicians in the near future.

Research objectives

In this study, we examined the allele distribution in nine SNPs found in *PTPN2/22* along with MAP presence in CD and healthy control subjects. Along with this, we determined gene expression of *PTPN2/22* and correlated with both SNPs and MAP presence. Lastly, we examined T-cell proliferation of the subjects and correlated that with both SNPs and MAP presence as well. This study overall examined the effects of both SNPs in *PTPN2/22* and MAP presence in CD subjects.

Research methods

We obtained K_2-EDTA coded blood tubes from both CD and healthy control subjects. Each subjects' blood was examined for *PTPN2/22* genotyping by TaqMan™ SNP genotyping, *PTPN2/22* and *IFN-γ* gene expression by real-time PCR (RT-PCR), MAP presence by MAP *IS900* nested PCR (nPCR), and T-cell proliferation by BrdU treatment.

Research results

We found in this study that the *PTPN2:rs478582* SNP and the haplotype combination of *PTPN2:rs478582* and *PTPN22:rs2476601* SNPs were found significant in CD subjects compared to healthy control subjects. Gene expression of *PTPN2/22* was also found to be decreased significantly in CD subjects as well. *IFN-γ* gene expression was found to be significantly higher in subjects with either *PTPN2:rs478582* or *PTPN22:rs2476601*. MAP presence was found significantly in CD subjects compared to the healthy control subjects, were CD subjects with either *PTPN2:rs478582* or *PTPN22:rs2476601* had higher MAP presence than subjects without SNPs. Overall T-cell proliferation was higher in CD subjects with either SNPs and induced with MAP antigens than subjects who didn't. These findings should provide more background to the pathogenesis of CD. Further studies into the gene expression of pro-inflammatory cytokines produced by T-cells with SNPs in *PTPN2/22* after proliferation needs to be investigated.

Research conclusions

This study was done in order to provide answers on the pathogenesis of CD. We have demonstrated that SNPs found in *PTPN2/22* are found significantly in CD subjects and the SNPs have the following effects on the immune system: increases T-cell proliferation due to loss of negative regulation, increases pro-inflammatory cytokines such as *IFN-γ*, and increases susceptibility to mycobacterial infections. This is further evidence that both a genetic predisposition and an environmental trigger are needed to cause disease in inflammatory autoimmune disorders such as CD.

Research perspectives

This study has provided us with new, possible targets that could be used in diagnosis methods and treatment for CD. With the data found in this study, the possibility of personalized treatment for CD could be possible with genetic testing for SNPs and antibiotic treatments for MAP. Further testing for other immune gene SNPs are needed in order to fully understand the genetic profile of CD patients. Additional research in MAP's relationship with CD pathogenesis is also needed to fully understand the effect of MAP in CD patients.

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ORIGINAL ARTICLE

Basic Study

Health-related quality of life, anxiety, depression and impulsivity in patients with advanced gastroenteropancreatic neuroendocrine tumours

Alexandra R Lewis, Xin Wang, Laurice Magdalani, Paolo D'Arienzo, Colsom Bashir, Was Mansoor, Richard Hubner, Juan W Valle, Mairéad G McNamara

Alexandra R Lewis, Laurice Magdalani, Was Mansoor, Richard Hubner, Juan W Valle, Mairéad G McNamara, Department of Medical Oncology, The Christie NHS Foundation Trust, Manchester M20 4BX, United Kingdom

Xin Wang, Department of Biostatistics, The Christie NHS Foundation Trust, Manchester M20 4BX, United Kingdom

Paolo D'Arienzo, Division of Medical Sciences, Scuola Superiore Sant'Anna, Pisa 56127, Italy

Colsom Bashir, Department of Clinical Psychology, The Christie NHS Foundation Trust, Manchester M20 4BX, United Kingdom

Juan W Valle, Mairéad G McNamara, Division of Cancer Sciences, University of Manchester, Manchester M20 4BX, United Kingdom

ORCID number: Alexandra Rebecca Lewis (0000-0002-6114-488X); Xin Wang (0000-0001-5192-8093); Laurice Magdalani (0000-0002-1363-6011); Paolo D'Arienzo (0000-0002-1581-5443); Colsom Bashir (0000-0002-3077-7641); Was Mansoor (0000-0002-1477-4934); Richard Hubner (0000-0002-7081-8823); Juan W Valle (0000-0002-1999-0863); Mairéad G McNamara (0000-0002-2272-3678).

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Correspondence to: Mairéad G McNamara, MB, BCh, BAO, PhD, Attending Doctor, Senior Lecturer, Department of Medical Oncology, The Christie NHS Foundation Trust, 550 Wilmslow Road, Manchester M20 4BX, United Kingdom. mairead.mcnamara@christie.nhs.uk
Telephone: +44-161-4468106
Fax: +44-161-4463468

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Abstract

AIM

To compare health-related quality of life (HRQoL),

anxiety, depression, and impulsivity scores in patients with and without carcinoid syndrome (CS), and correlated them with serum 5-hydroxyindoleacetic acid (5-HIAA) levels.

METHODS

Patients with advanced gastroenteropancreatic neuroendocrine tumours (GEPNET), with and without CS completed HRQoL QLQ-C30 and QLQ-GI.NET21, Hospital Anxiety and Depression Scale (HADS) and Barratt Impulsivity Scale (BIS) questionnaires. Two-sample Wilcoxon test was applied to assess differences in serum 5-HIAA levels, two-sample Mann-Whitney *U* test for HRQoL and BIS, and proportion test for HADS, between those with and without CS.

RESULTS

Fifty patients were included; 25 each with and without CS. Median 5-HIAA in patients with and without CS was 367nmol/L and 86nmol/L, respectively (P = 0.003). Scores related to endocrine symptoms were significantly higher amongst patients with CS (P = 0.04) and scores for disease-related worries approached significance in the group without CS, but no other statistically-significant differences were reported between patients with and without CS in responses on QLQ-C30 or QLQ-GI.NET21. Fifteen patients (26%) scored $\geqslant$ 8/21 on anxiety scale, and 6 (12%) scored $\geqslant$ 8/21 on depression scale. There was no difference in median 5-HIAA between those scoring < or $\geqslant$ 8/21 on anxiety scale (P = 0.53). There were no statistically significant differences between groups in first or second-order factors (BIS) or total sum (P = 0.23).

CONCLUSION

Excepting endocrine symptoms, there were no significant differences in HRQoL, anxiety, depression or impulsivity between patients with advanced GEPNET, with or without CS. Over one quarter of patients had high anxiety scores, unrelated to peripheral serotonin metabolism.

Key words: Gastroenteropancreatic neuroendocrine tumours; Carcinoid syndrome; Quality of life; Anxiety; Depression; Impulsivity

Core tip: Patients with functioning gastroenteropancreatic neuroendocrine tumours (GEPNETs) may have higher levels of psychological distress than other patients with cancer due to the symptoms of hormone hypersecretion. This study compares 25 patients with advanced GEPNET and carcinoid syndrome (CS) with 25 patients with advanced, but non-functioning GEPNET. Symptoms of anxiety, depression, impulsivity and health-related quality of life were assessed prospectively using symptom scales. Endocrine symptoms were significantly higher in patients with CS. Disease-related worries were more common in those with non-functioning tumours. This is a large study in this rare patient group and further prospective studies are required.

Lewis AR, Wang X, Magdalani L, D'Arienzo P, Bashir C, Mansoor W, Hubner R, Valle JW, McNamara MG. Health-related quality of life, anxiety, depression and impulsivity in patients with advanced gastroenteropancreatic neuroendocrine tumours. *World J Gastroenterol* 2018; 24(6): 671-679 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i6/671.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i6.671

INTRODUCTION

Gastroenteropancreatic neuroendocrine tumours (GEPNETs) are a varied group of neoplasms derived from cells of the diffuse endocrine system, normally distributed in the mucosa of organs originating from the embryological intestine[1]. GEPNETs are rare, representing only around 2% of all gastrointestinal malignancies[2], with an incidence of 5.25/100000/year and a prevalence of 35/100000 / year[3].

Gastroenteropancreatic neuroendocrine tumours are classified according to site and can be further subdivided into functioning and non-functioning. Functioning tumours can cause symptoms due to hypersecretion of hormones, most commonly serotonin, which causes carcinoid syndrome (CS). Approximately 30%-55% of GEPNETs are functioning[3]. CS is associated with flushing, diarrhoea, bronchial constriction and endocardial fibrosis, which can lead to right heart failure[2].

Surgery is the only curative treatment available for patients with GEPNETs. Treatment options for advanced disease include somatostatin analogues (SSAs) which may control the disease[3-5], and result in the reduction of hormone levels and the control of symptoms related to hormone hypersecretion. Other treatment options include everolimus for non-functioning GEPNETs[6] the use of peptide receptor radionuclide therapy (PRRT)[7], and chemotherapy[8]. More recently, among patients with CS not adequately controlled by SSAs, treatment with telotristat ethyl (a tryptophan hydroxylase inhibitor) was reported to be safe and well tolerated and resulted in significant reductions in bowel motion frequency[9].

Despite the availability and use of these treatment options, psychological symptoms may be identified in patients with GEPNETs[10,11]. Psychiatric comorbidity in oncological patients is common. Two German studies including 502 and 4020 patients across all disease stages, have reported that about 30% of these patients can be diagnosed with a mental disorder according to the Diagnostic and Statistical Manual of Mental Disorders criteria[12,13]. The most prevalent mental disorders associated with cancer in these studies were anxiety disorders, with mood and adjustment disorders also being commonly reported, with rates between

Table 1 Summary of studies investigating psychological symptoms in patients with neuroendocrine tumours

Ref.	Primary disease site	Correlation with treatment	Treatment	Number of patients with carcinoid syndrome/total patients	Method of investigation	Key results
Major *et al*[17] 1972	Metastatic carcinoid	No	Not reported	22/22	Not reported	50% displaying depressive symptoms
Larsson *et al*[11] 2001	Midgut carcinoid	Yes – prior to and following 12 mo of treatment with somatostatin analogues	Somatostatin analogues	20/24	Questionnaire – EORTC- QLQC30[1]	Anxiety scores significantly lower at 12 mo than baseline, depression scores significantly higher at 9 mo
Russo *et al*[29] 2003	Metastatic mid-gut carcinoid	No. Experimental tryptophan depletion	12 patients somatostatin analogues, 2 patients no treatment	14/14	Cambridge Neuropsychological tests automated battery (CANTAB): intra-/extra-dimensional shift task, matching to sample visual search, rapid visual information processing and spatial working memory.	Impaired sustained attention. Not mimicking patients with depression
Larsson *et al*[27] 2003	Carcinoid tumour	Yes	Somatostatin analogues or interferon	19/19	Semi-structured interview	Fatigue, diarrhoea, worry about diagnosis and limited physical ability most commonly reported symptoms
Russo *et al*[19] 2004	Mid-gut carcinoid tumour with carcinoid syndrome	No	14 patients on somatostatin analogues, 2 patients on interferon 2 patients no active treatment. 2 patients on somatostatin analogues + interferon	20/20	Semi-structured psychiatric interview	Impulse dysregulation leading to diagnosis of personality change secondary to a medical disorder in 15 patients (75%)

[1]European Organisation for Research and Treatment of Cancer (EORTC) health-related quality of life QLQ-C30 questionnaire.

6.5% and 13.5%. Other studies have reported rates of depression between 18% and 25%[14,15].

Patients with a diagnosis of a neuroendocrine tumour were not considered in these studies, which only addressed the most frequent tumour entities. Patients with advanced GEPNETs experience similar symptoms to those with a diagnosis of gastrointestinal cancers, and there is a perception amongst many treating physicians that patients with functional neuroendocrine tumours, presenting with CS may have higher levels of psychological distress compared to other patients with a cancer diagnosis. However, there is limited data to support this hypothesis[16,17].

It may be, in the case of neuroendocrine tumors, that psychological changes could be related to the effects of biochemical mediators produced by the tumour itself and released into the bloodstream, such as serotonin. For example, in a study by Jacobsen *et al*[18], which included eleven patients with advanced GEPNET and CS, it was reported that psychological distress decreased, and social functioning increased, following one month of treatment with SSAs.

Several studies and reports in the literature dating back to the 1960s, point out that several types of neuroendocrine tumours, can be associated with depression, sleep disturbances, anxiety, aggressive behaviour, psychosis and altered attention span[10,17,19-30]. Studies assessing mood in patients with neuroendocrine tumours are summarised in Table 1.

Previous studies on this topic have included small numbers of patients, without apparent control groups, with varying psychological tests applied, therefore the aim of this study was to compare health-related quality of life (HRQoL), anxiety, depression, and impulsivity in patients with advanced GEPNETs, with and without CS, and to correlate with biochemical markers of

disease activity such as serum chromogranin A and 5-hydroxyindoleacetic acid (5-HIAA).

MATERIALS AND METHODS

Patients with advanced well-differentiated GEPNET tumours with liver metastases, with and without CS attending an outpatient NET clinic at a European Neuroendocrine Tumour (ENET) Centre of Excellence; The Christie NHS Foundation Trust, Manchester, United Kingdom, were asked to complete the European Organisation for Research and Treatment of Cancer (EORTC) health-related quality of life QLQ-C30[31] and neuroendocrine tumour-specific GINET-21 (GINET21) questionnaires[32], the Hospital Anxiety and Depression scale (HADS)[33] and the Barratt Impulsivity Scale (BIS)[34,35]. These were completed at a single time point between April and August 2016. The EORTC QLQ-C30 scores patients on scales that assess global health status, social, physical and emotional functioning and common symptoms. Various disease-specific scales have been developed to work in combination with the QLQ-C30 and the GINET-21 is specific to neuroendocrine tumours. In combination with the QLQ-C30, the GINET-21 questionnaire provides information on neuro-endocrine symptoms including diarrhoea and flushing, treatment side-effects and disease-related worries[32].

The Barratt Impulsivity scale is a well validated score that examines first-order factors including attention, cognitive instability, motor and perseverance, self-control and cognitive complexity. Second-order factors analysed in this scale are attentional, motor and non-planning[34].

Baseline demographic data collected included age, tumour site, Eastern Cooperative Oncology Group performance status (ECOG PS), time from diagnosis, presence of recurrent disease following initial curative-intent surgery, any previous surgery and current and previous treatments, presence of co-morbidities and use of psychoactive medications. Baseline serum chromogranin A and 5-HIAA at initial presentation with advanced disease, and at the time of questionnaire completion were recorded. Patients identified within this study as having significant symptoms of anxiety and depression were offered referral to the psycho-oncology service.

Inclusion criteria for this study were a diagnosis of advanced GEPNET with liver metastases, with or without CS, understanding the English language and the physical ability to complete questionnaires. Patients with neuroendocrine tumours originating from sites other than the gastroenteropancreatic tract and without liver metastases were excluded, as were those with poorly-differentiated tumours. Patients with functional tumours presenting with other syndromes *e.g.*, gastrinoma were also excluded.

The sample size of 25 patients with CS (with matched controls) was selected on the basis of feasibility and as a representative sample in a large ENETs Centre of Excellence. A recent analysis of outcomes of patients with GEPNETs and CS presenting to The Christie over a 28 year period identified 139 patients[36]. With a median overall survival of 65.4 mo in this study, this would therefore suggest that approximately 40 patients would be alive at any one time. Accounting for patients who only attend the Christie for single appointments or experience ill health or language barriers preventing completion of the questionnaires, 25 patients was identified as an achievable patient group.

The local audit committee approved this study (reference number: CE16/1619).

Statistical methods

The median time from diagnosis of advanced disease (radiological or histological) was recorded up to date of completion of questionnaires.

The two-sample Wilcoxon (Mann-Whitney test) was applied to assess differences in serum chromogranin A and 5-HIAA in patients with and without CS.

Responses to the EORTC QLQ-C30 and the QLQ-GINET21 were linearly transformed to a 0-100 scale using EORTC guidelines. Two-sample Mann-Whitney *U* test were applied across scales/items to assess the difference between the patient groups with and without CS. A *P* value less than 0.05 indicated a statistically significant difference. For the HADS, 8 out of 21 was used as the cut-off for both the "Anxiety" and "Depression" category as a level representing clinically-significant symptoms warranting further intervention[33]. The Proportion test was applied to both the "Anxiety" and "Depression" categories to assess whether there was a statistically significant difference in the proportion of "total score $\geqslant$ 8" between patient groups with and without CS. The two-sample Mann-Whitney *U* test was applied to first-order factors, second-order factors and total sum, to compare the patient groups with and without CS.

This study was an exploratory analysis. Detection of a specific effect size (hazard ratio) was not the target, and so power calculations were not used, as detection of an intended hazard ratio was not required.

RESULTS

The median age of all patients was 65.5 years. The majority of patients (88%) had an ECOG PS of 0-1. Most patients had tumours originating in the small intestine (58%) or the pancreas (22%). The most frequent current treatment in all patients was SSAs, either alone or in combination. These results are summarised in Table 2. The median time from initial diagnosis for all patients was 40 mo [95% confidence interval (CI): 22-48 mo]; 45 (95%CI: 20-49) and 36 mo (95%CI: 18-56), *P* = 0.66, in the groups with and without CS respectively. Five patients (10%) were taking prescribed psychoactive medications; two patients with CS. The median serum 5-HIAA at

Table 2 Patient characteristics - advanced gastroenteropancreatic neuroendocrine tumours with and without carcinoid syndrome *n* (%)

	All patients *n* = 50	With carcinoid syndrome *n* = 25	Without carcinoid syndrome *n* = 25	*P* value
Age (median)	65.5	67	62	0.19
95%CI	(61.5-68.5)	(61.3-70.9)	(58.0-68.9)	
Gender	F:21 (42) M:29 (58)	F:9 (36) M:16 (64)	F: 12 (48) M: 13 (52)	0.39
Median time since diagnosis (mo)	39	45	36	0.66
(95%CI)	(21.54-48.46)	(20.10-48.90)	(18.21-56)	
Prior Surgery	24 (48)	9 (36)	15 (60)	0.09
Recurrent disease following previous curative intent treatment	8 (16)	3 (12)	5 (20)	0.44
Primary disease site				
Stomach	1 (2)	0 (0)	1 (4)	
Small bowel	29 (58)	18 (72)	11 (44)	
Pancreas	11 (22)	1 (4)	10 (40)	
Large bowel	3 (6)	1 (4)	2 (8)	
Unknown GI tract	6 (12)	5 (20)	1 (4)	
Median Ki-67				
% (95%CI)	3 (2-4.8)	2 (2-5)	3 (2-7.7)	0.55
ECOG PS *n* (%)				
0	14 (27)	7 (28)	7 (28)	
1	30 (60)	16 (64)	14 (56)	
2	4 (8)	1 (4)	3 (12)	
3	2 (4)	1 (4)	1 (4)	
Current treatment				
Nil	5 (10)	2 (8)	4 (16)	
Best supportive care	1 (2)	0 (0)	1 (4)	
Chemotherapy	5 (10)	0 (0)	5 (20)	
Interferon + Somatostatin analogue	2 (4)	2 (8)	0 (0)	
Peptide receptor radionuclide Therapy	2 (4)	1 (4)	1 (4)	
Peptide receptor radionuclide therapy + Somatostatin analogues	2 (4)	1 (4)	1 (4)	
Somatostatin analogues	28 (56)	8 (72)	9 (36)	
Tryptophan hydroxylase inhibitor + Somatostatin analogues	1 (2)	1 (4)	0 (0)	
mTOR inhibitor	4 (8)	0 (0)	4 (16)	
Use of psychoactive medications				
Nil	44 (88)	22 (88)	22 (88)	
Selective serotonin Reuptake inhibitors	3 (6)	1 (4)	2 (8)	
Benzodiazepine	1 (2)	1 (4)	0 (0)	
Selective serotonin reuptake inhibitors + benzodiazepine	1 (2)	0 (0)	1 (4)	
Unknown	1 (2)	1 (4)	0 (0)	

CI: Confidence interval; ECOG PS: Eastern Cooperative Oncology Group performance status.

diagnosis of advanced disease was 367.0 nmol/L (95%CI: 271.57-1127.89) and 124.0 nmol/L (95%CI: 73.05-200.98) in those patients with and without CS respectively ($P < 0.001$). The median serum 5-HIAA at the time of questionnaire completion was 367.00 nmol/L (95%CI 176.7-855.5) and 86 nmol/L (95%CI: 66.8-123.9) in those patients with and without CS respectively ($P < 0.001$) (normal range 0-140 nmol/L), indicating biochemical differences between groups at baseline and at time of questionnaire completion.

The median baseline chromogranin A at diagnosis of advanced disease was 268 ng/mL (95%CI: 151.6-381.4) and 116 ng/mL (95%CI: 57.77-275.51) in those patients with and without CS respectively ($P = 0.09$). The median chromogranin A measurement at the time of questionnaire completion was 322 ng/mL (95%CI: 215.2-456.5) and 198ng/mL (95%CI: 68.8-392.0) in patients with and without CS respectively ($P = 0.13$) (normal range 0-91 ng/mL).

In the HRQoL QLQ-GI.NET21 questionnaire, scores related to endocrine symptoms (flushing and night sweats) were significantly higher in those with CS ($P = 0.04$). Disease-related worries (related to tumour progression, health in the future and test results) appeared more prominent in the group without CS and the difference approached statistical significance ($P = 0.05$). There were no significant differences in responses between those patients with and without CS for all other symptoms in both health-related quality of life questionnaires (QLQ-C30 and QLQ-GI.NET21). These results are summarised in Tables 3 and 4.

Out of the total of 50 patients, fifteen patients (30%) scored $\geqslant$ 8/21 on the HADS anxiety scale; 8 had CS, and 6 (12%) scored $\geqslant$ 8/21 on the HADS depression scale; 3 with CS. There was no difference in the median serum 5-HIAA between those scoring < or $\geqslant$ 8/21 on the anxiety scale ($P = 0.53$). The Proportion test was not statistically significant between

Table 3 Comparison of responses in patients with and without carcinoid syndrome in the HRQoL QLQ-C30 questionnaire

	Without carcinoid syndrome		With Carcinoid syndrome		Difference between groups	*P* value
	Mean score	(95%CI)	Mean score	(95%CI)		
Global health status	63	52.3-73.7	61.7	49.6-73.7	1.3	0.92
Physical functioning	79.9	73.0-86.8	77.9	67.9-87.9	2.0	0.79
Role functioning	74	61.3-86.7	70.7	56.3-85.0	3.3	0.77
Emotional functioning	73	63.0 – 83.0	74.7	63.7-85.6	-1.7	0.73
Cognitive functioning	76.7	66.3-87.0	74.0	61.0-87.0	2.7	0.93
Social functioning	70	58.6- 81.4	79.3	67.2-91.5	-9.3	0.15
Fatigue	34.7	23.6-45.8	37.8	23.9-51.7	-3.1	0.84
Nausea and vomiting	11.3	4.0-18.7	8.00	1.4-14.6	3.3	0.31
Pain	25.3	11.8-38.8	26.7	13.5-39.8	-1.3	0.81
Dyspnoea	12.5	1.7-23.3	24.0	10.5-37.5	-11.5	0.15
Insomnia	36	22.9-39.1	22.7	10.9-34.3	13.3	0.12
Appetite loss	18.7	8.1-29.2	22.7	9.7-35.7	-4	0.81
Constipation	9.3	1.9-16.8	9.30	1.9-16.8	0	1.00
Diarrhoea	24	9.4-38.6	32.0	17.4-46.6	-8.0	0.27
Financial difficulties	13.3	3.6-23.1	8.00	-18.2	5.3	0.30

HRQoL: Health-related quality of life.

Table 4 Comparison of responses in patients with and without carcinoid syndrome in the HRQoL QLQ-GI.NET21 questionnaire

	Without carcinoid syndrome		With Carcinoid syndrome		Difference between groups	*P* value
	Mean score	(95%CI)	Mean score	(95%CI)		
Endocrine symptoms	16.7	8.4-24.9	28.4	18.1-38.8	-11.8	0.04
Gastrointestinal symptoms	18.9	12.7-25.3	24.0	16.5-31.5	-5.1	0.37
Treatment-related symptoms	17.5	8.6-26.4	10.1	4.1-16.2	7.4	0.20
Social functioning	60.0	50.0-70.0	68.4	58.7-78.1	-8.4	0.19
Disease-related worries	56.9	43.0-70.8	38.7	26.2-51.2	18.2	0.05
Body image	15.3	4.3-26.2	13.3	2.1-24.6	1.9	0.57
Weight gain	18.7	7.4-30.0	13.3	2.1-24.6	5.3	0.30
Muscle/bone pain	41.3	28.6-54.1	46.7	31.3-62.1	-5.3	0.62
Information	10.7	2.0-19.3	16.0	4.7-27.3	-5.3	0.51
Sexual functioning	60.8	35.6-85.9	68.4	47.4-89.5	-7.6	0.82

groups with and without CS for anxiety (P = 0.76) or depression (P = 1.0). There were no statistically-significant differences between groups with and without CS in first or second-order factors (BIS) or total sum (P = 0.23).

DISCUSSION

In this study, there were no significant differences identified in the majority of health-related quality of life responses, anxiety, depression or impulsivity in patients with advanced GEPNET, despite observed significant differences in median serum 5-HIAA between those patients with and without CS. There were more endocrine-related symptoms, specifically flushing and sweating, in patients with CS and those without CS had more disease-related worries (progression, test results and the future). The reasons for increased disease-related worries in those without CS may be due to masking of these symptoms in patients with CS, where the worry may be shifted due to endocrine symptoms.

In patients with GEPNETs, serotonin produced peripherally by the tumour, cannot cross the blood-brain barrier and, as a result, is unable to exert direct effects on the central nervous system (CNS)[29]. However, it has been hypothesised that diversion of dietary tryptophan to peripheral serotonin production, thereby causing a relative CNS depletion may be a cause of emotional disturbances in these patients[29]. Various studies have demonstrated cognitive changes when healthy control subjects have induced tryptophan depletion[37-43]. In a study testing the blockade of the enzyme tryptophan hydroxylase as a method of reducing CS symptoms[43], depression was a severe side-effect. The study investigating the effects of telotristat ethyl for symptom control in patients with CS specifically focused on depression as an "adverse event of special interest", and did report increased rates of depression in the group taking 500 mg three times daily compared to those taking 250 mg three times daily. However, this did not delay treatments or require the introduction of anti-depressant therapy[9].

The lack of difference in scores on the majority of the symptom scales of the questionnaires completed in this study may be related to a long median time from diagnosis, and so many symptoms may be controlled on treatment, and thus there would be a reduced impact on the patient's psychological distress. A meta-analysis of studies which included patients with early-stage and advanced disease reported a mean prevalence of depression amongst patients with cancer of 18%, and reported that rates tended to be lower for those greater

than 1 year from diagnosis[14]. Similarly in a study by Larsson *et al*[11], patients with advanced GEPNETs completed the EORTC QLQC30 at baseline, 3, 6, 9 and 12 mo from commencement of treatment with interferon or SSAs. The authors reported that the scores for physical functioning decreased, whereas those for emotional role and cognitive function improved during the study.

In addition, it has previously been reported that global health scores may not be particularly sensitive in this patient population[32,44,45]. Similarly, with regards to physical symptoms, despite there being increased flushing reported, diarrhoea was not reported as a significant symptom in patients with CS in our study, and again could be a reflection of the impact of disease control on psychological state.

In the study by Russo *et al*[19], formal psychiatric interviews were used to assess patients with CS. The authors identified a significant proportion of patients with impulse dysregulation leading to reduced social functioning. The authors were able to make a diagnosis of "Personality change secondary to a medical condition" in some of these patients. In the study by Larsson *et al*[27] which examined distress and strategies for maintaining good mood in patients with carcinoid tumours, some factors contributing to distress in their study are not covered by either the EORTC-QLQ30 or the HADS questionnaire used in the current study. Therefore, it is possible that the questionnaires used in the current study were not sufficiently sensitive to detect personality changes or some more subtle features of psychological distress.

In the current study, a high number of patients with and without CS had high scores on the anxiety and depression scale. The rate of depression in the current study was 12%, and since the median time from diagnosis was 40 mo in this study, the rate for depression seems proportional to the general cancer population[14].

However, the rates of anxiety reported in the current study (approximately 25% of the patients assessed) were significantly higher than those of 8%-11%[12,13] reported elsewhere in patients with cancer. It is of note that our study included only patients with advanced GEPNET, whereas the studies by Mehnert *et al*[12] and Singer *et al*[13] included patients with early stage and advanced disease.

A limitation of this study was that it included only fifty patients and it may be that it was not large enough to detect small differences in psychological state between the patients with and without CS. Another limitation is that patient questionnaires were completed at a single time point only, and this may not have been a representative view of the patient's psychological symptoms over the course of their disease. There was also variation in time from diagnosis to completion of surveys which could have an impact on psychological effects. Furthermore, there may have been impact from treatments on results. Patients in the group without CS had undergone more prior lines of treatment (mean 2.12 *vs* 1.87 in the group with CS) and had received more systemic treatments including mTOR inhibitors and chemotherapy that are associated with toxicities, which may have an impact on psychological state. However, GEPNETs are a rare tumour group and most studies reported to date included an average of twenty patients without control groups, and so the current study is comparably more robust and informs on a poorly studied topic.

Limitations could potentially be overcome by conducting a prospective study with repeated questionnaire sampling from the time of diagnosis of advanced GEPNET and matching of controls by treatment.

In conclusion, this study contributes to the limited evidence base regarding psychological symptoms for this rare disease group, and includes a relatively large cohort of patients with a diagnosis of GEPNET, with and without CS, reviewed at a tertiary referral centre. This study also highlights the importance of recognition by treating physicians of psychological distress in patients with advanced GEPNET, and the need for input from the psycho-oncology services. Prospective multi-centre studies are required to further enhance the understanding of psychological distress in this disease group and the relation, if any, with biochemical abnormalities and indeed their therapeutic management.

ARTICLE HIGHLIGHTS

Research background

Psychological issues in patients with gastroenteropancreatic neuroendocrine tumours (GEPNETs), with or without carcinoid syndrome (CS), are rarely studied. There is a physician perception of higher levels of psychological distress amongst patients with CS. There is some data to support this in the form of case reports, but not large comparative studies and it is unclear whether this takes the form of anxiety, depression or even impulsivity. The aim of this study was therefore to compare health-related quality of life, anxiety, depression, and impulsivity in patients with advanced GEPNET, with and without CS, and to correlate with biochemical markers of disease activity.

Research motivation

An improved understanding of the psychological issues for these patients will help better inform management of their symptoms.

Research objectives

To assess whether patients with advanced GEPNET and CS have increased levels of anxiety, depression, impulsivity or worse quality of life than patients with advanced GEPNET, but non-functioning tumours.

Research methods

Patients with advanced GEPNET with and without CS were invited to fill out questionnaires at outpatient clinics at The Christie NHS Foundation Trust, which is a European centre of excellence for neuroendocrine tumours. Patients completed the hospital anxiety and depression scale (HADS), the EORTC QLQ-C30 and GINET-21 quality of life scales and the Barrett Impulsivity scale (BIS) at a single time point. Demographic information with regards to gender, time from diagnosis and treatment history was collected from casenotes, as

were serum markers of disease [5-hydroxyindoleacetic acid (5-HIAA)].

Research results

Fifty patients were included; 25 each with and without CS (CS). Median serum 5-HIAA in patients with and without CS was 367nmol/L and 86nmol/L, respectively (P = 0.003). Scores related to endocrine symptoms were significantly higher amongst patients with CS (P = 0.04) and scores for disease-related worries approached significance in the group without CS, but no other statistically-significant differences were reported between patients with and without CS in responses on QLQ-C30 or QLQ-GI.NET21. Fifteen patients (26%) scored ⩾ 8/21 on anxiety scale, and 6 (12%) scored ⩾ 8/21 on depression scale. There was no difference in median 5-HIAA between those scoring < or ⩾ 8/21 on anxiety scale (P = 0.53). There were no statistically significant differences between groups in first or second-order factors (BIS) or total sum (P = 0.23).

Research conclusions

There were no significant differences between groups with regards to anxiety, depression or impulsivity. Serum 5-HIAA and endocrine symptoms were more prevalent in the group with CS as would be expected. Disease-related worries were higher in the group without CS. Results may have been impacted by a long time from diagnosis in the CS group or limited sensitivity of screening tools to detect subtle differences in mental state. Results may also have been impacted by small sample size however in this rare disease group the sample size is robust in a comparative study. Levels of anxiety and depression were high in both groups and may be higher in patients with GEPNET than with other solid tumours. This is one of few comparative prospective studies in this patient group and established methods of assessment were used and correlated with serum biochemistry. More sensitive tools need to be developed to assess psychological symptoms in these patients.

Research perspectives

This prospective comparative study included single time point assessment of symptoms. Studies with questionnaires distributed at varying times would be helpful to assess the impact of changes throughout the patient journey.

Futures prospective studies are needed, ideally involving multiple centres with considered methodology such as psychiatric interviews including the use and development of more selective psychological assessment tools.

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ORIGINAL ARTICLE

Basic Study

Analysis of hepatitis B virus preS1 variability and prevalence of the rs2296651 polymorphism in a Spanish population

Rosario Casillas, David Tabernero, Josep Gregori, Irene Belmonte, Maria Francesca Cortese, Carolina González, Mar Riveiro-Barciela, Rosa Maria López, Josep Quer, Rafael Esteban, Maria Buti, Francisco Rodríguez-Frías

Rosario Casillas, David Tabernero, Irene Belmonte, Maria Francesca Cortese, Carolina González, Rosa Maria López, Francisco Rodríguez-Frías, Liver Pathology Unit, Departments of Biochemistry and Microbiology, Hospital Universitari Vall d' Hebron, Universitat Autònoma de Barcelona, Barcelona 08035, Spain

Rosario Casillas, Josep Gregori, Maria Francesca Cortese, Josep Quer, Liver Unit, Liver Disease Laboratory-Viral Hepatitis, Vall d'Hebron Institut Recerca-Hospital Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona 08035, Spain

David Tabernero, Josep Gregori, Mar Riveiro-Barciela, Josep Quer, Rafael Esteban, Maria Buti, Francisco Rodríguez-Frías, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Instituto de Salud Carlos III, Madrid 28029, Spain

Josep Gregori, Roche Diagnostics SL, Sant Cugat del Vallès 08174, Spain

Mar Riveiro-Barciela, Rafael Esteban, Maria Buti, Liver Unit, Department of Internal Medicine, Hospital Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona 08035, Spain

ORCID number: Rosario Casillas (0000-0002-6758-6734); David Tabernero (0000-0002-1146-4084); Josep Gregori (0000-0002-4253-8015); Irene Belmonte (0000-0002-8675-8343); Maria Francesca Cortese (0000-0002-4318-532X); Carolina González (0000-0002-0169-5874); Mar Riveiro-Barciela (0000-0001-9309-2052); Rosa Maria López (0000-0002-8450-6986); Josep Quer (0000-0003-0014-084X); Rafael Esteban (0000-0001-5280-392X); Maria Buti (0000-0002-0732-3078); Francisco Rodríguez-Frías (0000-0001-9058-4641).

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Correspondence to: David Tabernero, PhD, Research Scientist, Liver Pathology Unit, Departments of Biochemistry and Microbiology, Hospital Universitari Vall d'Hebron (HUVH), Passeig Vall d'Hebron 119-129, clinical laboratories, Barcelona 08035, Spain. david.tabernero@ciberehd.org
Telephone: +34-932-746897

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Abstract

AIM

To determine the variability/conservation of the domain of hepatitis B virus (HBV) preS1 region that interacts with sodium-taurocholate cotransporting polypeptide (hereafter, NTCP-interacting domain) and the prevalence of the rs2296651 polymorphism (S267F, NTCP variant) in a Spanish population.

METHODS

Serum samples from 246 individuals were included and divided into 3 groups: patients with chronic HBV infection (CHB) (*n* = 41, 73% Caucasians), patients with resolved HBV infection (*n* = 100, 100% Caucasians) and an HBV-uninfected control group (*n* = 105, 100% Caucasians). Variability/conservation of the amino acid (aa) sequences of the NTCP-interacting domain, (aa 2-48 in viral genotype D) and a highly conserved preS1 domain associated with virion morphogenesis (aa 92-103 in viral genotype D) were analyzed by next-generation sequencing and compared in 18 CHB patients with viremia > 4 log IU/mL. The rs2296651 polymorphism was determined in all individuals in all 3 groups using an in-house real-time PCR melting curve analysis.

RESULTS

The HBV preS1 NTCP-interacting domain showed a high degree of conservation among the examined viral genomes especially between aa 9 and 21 (in the genotype D consensus sequence). As compared with the virion morphogenesis domain, the NTCP-interacting domain had a smaller proportion of HBV genotype-unrelated changes comprising > 1% of the quasispecies (25.5% *vs* 31.8%), but a larger proportion of genotype-associated viral polymorphisms (34% *vs* 27.3%), according to consensus sequences from GenBank patterns of HBV genotypes A to H. Variation/conservation in both domains depended on viral genotype, with genotype C being the most highly conserved and genotype E the most variable (limited finding, only 2 genotype E included). Of note, proline residues were highly conserved in both domains, and serine residues showed changes only to threonine or tyrosine in the virion morphogenesis domain. The rs2296651 polymorphism was not detected in any participant.

CONCLUSION

In our CHB population, the NTCP-interacting domain was highly conserved, particularly the proline residues and essential amino acids related with the NTCP interaction, and the prevalence of rs2296651 was low/null.

Key words: Hepatitis B virus; Hepatitis B virus preS1 region; Sodium-taurocholate co-transporting polypeptide; NTCP-interacting domain; Virion morphogenesis domain; SNP rs2296651; Next-generation sequencing; Real-time PCR melting curves

Core tip: Simultaneous analysis of both viral and host features important for hepatitis B virus (HBV) entry into hepatocytes provided locally relevant preliminary information in a population previously uncharacterized in this regard. In-house developed next-generation sequencing was successfully used to investigate the variability of the preS1 region of the HBV large envelope protein, and real-time PCR melting curve analysis to detect the rs2296651 polymorphism (NTCP variant, S267F) in the HBV cellular receptor, NTCP. Results in a limited sample indicate that the features analyzed would not decrease the effectiveness of new therapies to block NTCP and avert HBV binding to hepatocytes in our particular CHB population.

Casillas R, Tabernero D, Gregori J, Belmonte I, Cortese MF, González C, Riveiro-Barciela M, López RM, Quer J, Esteban R, Buti M, Rodríguez-Frías F. Analysis of hepatitis B virus preS1 variability and prevalence of the rs2296651 polymorphism in a Spanish population. *World J Gastroenterol* 2018; 24(6): 680-692 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i6/680.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i6.680

INTRODUCTION

Hepatitis B virus (HBV) infection remains a major health threat, with around 240 million chronically infected individuals worldwide[1]. Persistently infected people are at high risk for the development of cirrhosis and hepatocellular carcinoma, and about 1 million people die each year due to HBV-associated liver disease[2,3]. Currently, the available anti-HBV treatments include conventional or pegylated interferon-α (IFN-α) and nucleos(t)ide analogs (NAs)[4]. Both types of treatments have drawbacks: IFN-based therapies cause significant side effects and yield long-term clinical benefits in less than 40% of treated patients[5], whereas first-line NAs suppress viral activity in more than 80% of patients, but viral eradication is rare[4,6,7].

Based on extensive research in the HBV lifecycle and virus-host interactions, several new agents are under development to achieve a functional cure for HBV infection[8,9]. In this sense, identification of sodium-taurocholate cotransporting polypeptide (NTCP), encoded by the *SLC10A1* gene and located on chromosome 14[10,11], as a receptor for HBV infection[12] has provided valuable information for the development of inhibitors of HBV entry. NTCP is a multipletrans-

Table 1 Demographic characteristics of the 246 patients included

	Group A, CHB (n = 41)	Group B, Resolvers (n = 100)	Group C, Controls (n = 105)	P value[1]
Gender, n males (%)	22 (54)	54 (54)	54 (51)	NS
Age, yr, mean ± SD	54.8 ± 19.1	62.1 ± 13.0	60.3 ± 13.5	NS
Origin, n (%)				
Caucasian	30 (73)	100 (100)	100 (100)	-
Asian	5 (12)	0 (0)	0 (0)	-
Sub-Saharan	6 (15)	0 (0)	0 (0)	-

[1]P-value χ^2 test for gender (qualitative variable), and t-test for age (quantitative variable). CHB: Chronic hepatitis B; NS: Not statistically significant.

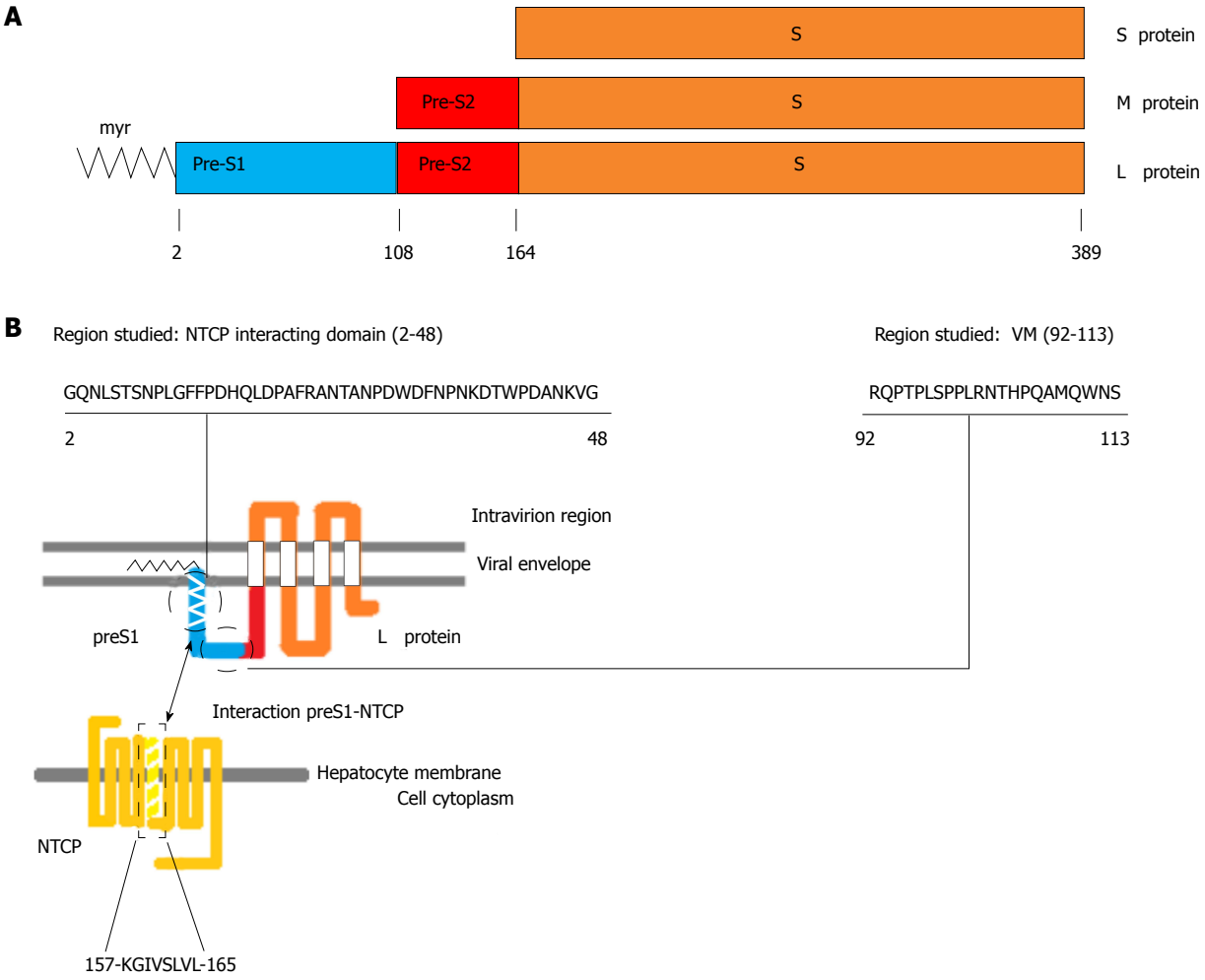

Figure 1 Model of interaction between large envelope proteins and the sodium-taurocholate cotransporting polypeptide. A: Schematic diagram of hepatitis B virus envelope proteins: Small (S), Middle (M) and Large (L) envelope proteins. B: Representation of the interaction between the viral preS1 protein and its host receptor in hepatocytes, sodium-taurocholate cotransporting polypeptide (NTCP), modified from the model proposed by Urban[53]. The 2 domains analyzed in this study, the NTCP-interacting and virion morphogenesis (VM) domains, are indicated in the L protein. Numbering is based on the HBV genotype D consensus sequence. myr: Myristic acid; HBV: Hepatitis B virus.

membrane protein that is predominantly expressed at the basolateral membrane of hepatocytes. The primary role of NTCP is to transport bile salts from the portal blood into hepatocytes[10,11]. Interactions of viral particles with this receptor are mediated by the hepatitis B surface antigen (HBsAg), which is formed by three viral envelope proteins (large, middle, and small) that differ in length at the N-terminal region and share the same C-terminal S region[13,14]. The HBV large envelope proteins (LHBs), which include the preS1, preS2, and S regions of the surface open reading frame of the HBV genome, interact with NTCP through specific binding of a 47 amino acids (aa) domain in the N-terminal end of the preS1 region[15-18] (hereafter referred to as the NTCP-interacting domain), as is shown in Figure 1.

It is reasonable to think that the degree of sequence variability in the NTCP-interacting domain, which is an indication of the extent to which sequence conservation

Table 2 Individual demographic, biochemical and virological characteristics of the 18 patients from group A (chronic hepatitis B patients) in whom the preS1 region was analyzed

ID	Gender	Age	Origin	HBV Genotype[1]	HBV DNA (logIU/mL)	ALT (IU/L)	HBeAg
1	Female	68	Caucasian	D	5.0	29	Negative
2	Male	55	Caucasian	A	5.8	46	Negative
3	Female	40	Caucasian	D	4.2	29	Negative
4	Male	47	Caucasian	F	5.8	88	Negative
5	Female	47	Caucasian	D	6.3	170	Negative
6	Female	28	Caucasian	D	5.4	22	Negative
7	Male	28	Asian	C	> 8	94	Positive
8	Female	41	Caucasian	A	4.7	18	Negative
9	Male	39	Caucasian	D	5.0	29	Negative
10	Male	45	Caucasian	F	> 8	51	Positive
11	Female	35	Asian	C	> 8	56	Positive
12	Female	46	Caucasian	H	5.3	23	Negative
13	Male	37	Sub-Saharan	E	4.3	37	Negative
14	Female	54	Caucasian	A	4.3	21	Negative
15	Female	36	Asian	C	> 8	19	Positive
16	Female	20	Sub-Saharan	E	5.1	22	Positive
17	Male	73	Caucasian	B	> 8	221	Negative
18	Male	49	Asian	B	5.6	181	Negative

[1]HBV genotype determined by Sanger sequencing of the preS1 region (the same region as was analyzed by next-generation sequencing). ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

is important to maintain its function[19], may have an impact on the response to inhibitors of HBV entry based on synthetic myristoylated lipopeptides[20] that share the same aa sequence with the NTCP-interacting domain (*i.e.*, therapy to block NTCP). For example, sequence variability in the NTCP-interacting domain might change its affinity to attach to NTCP, changing the dynamics of the competition with synthetic myristoylated lipopeptide analogues, and making them less effective in patients showing such variation.

Furthermore, several single nucleotide polymorphisms (SNPs) that may change the physiological function of NTCP as the key transporter for bile salt homeostasis and affect HBV entry have been identified in *SLC10A1*[21], and most of them show an ethnicity-dependent profile[22,23]. The rs2296651 SNP in *SLC10A1* (g.69778476G>A, GenBank accession number NC_000014.9), which causes the S267F variant, has been identified in Asian populations at a prevalence of 3.1% to 9.2%[22]. Previous studies focusing on rs2296651 and HBV infection have yielded conflicting results[24,25], and the role of this SNP in cirrhosis and hepatocellular carcinoma remains uncertain[26]. However, as synthetic myristoylated lipopeptides share the same aa sequence with the NTCP-interacting domain, it is reasonable to think that the presence of this SNP might influence the effectiveness of these therapies. This variant has not been found in some Caucasian populations (European Americans and Hispanic Americans)[22], but to our knowledge, there are no studies investigating the prevalence of rs2296651 in our country (Spain), where a Caucasian population mainly of European Mediterranean origin is prevalent.

The main aims of this study were to analyze the variability and conservation of the HBV preS1 region NTCP-interacting domain, involved in HBV entry into hepatocytes, and to determine the prevalence of the rs2296651 SNP, causing the S267F NTCP variant, in an HBV patient population from our area (Barcelona, Spain).

MATERIALS AND METHODS

Patients and samples

The study included 246 individuals recruited from the population attending the outpatient clinic of Vall Hebron University Hospital (Barcelona, Spain). The study was approved by the hospital ethics committee and all individuals gave informed written consent for participation at enrollment. Participants were divided into 3 groups: group A, patients with chronic hepatitis B infection (n = 41, CHB); group B, patients testing negative for HBsAg, but positive for antibodies against the hepatitis B core antigen (anti-HBc) (n = 100, Resolvers); and group C, an HBV-uninfected control group (HBsAg-negative, antiHBc-negative) (n = 105, Controls). Participants from groups B and C were all Caucasian. Ethnic heterogeneity was greater in Group A, with 30/41 (73%) Caucasian, 5/41 Asian (12%), and 6/41 sub-Saharan (15%) participants (Table 1).

Two main analyses were performed. First, the variability and conservation of the preS1 region of LHBs was analyzed in the 18 patient samples from Group A with a viral load greater than > 4 logIU/mL. This is the sensitivity limit of the PCR to amplify that region. Then we determined the sequence of preS1 by next-generation sequencing (NGS) based on ultra-deep pyrosequencing (UDPS) on the GS-Junior platform (454 Life sciences-Roche, Branford, United States). All 18 patients were treatment-naive and

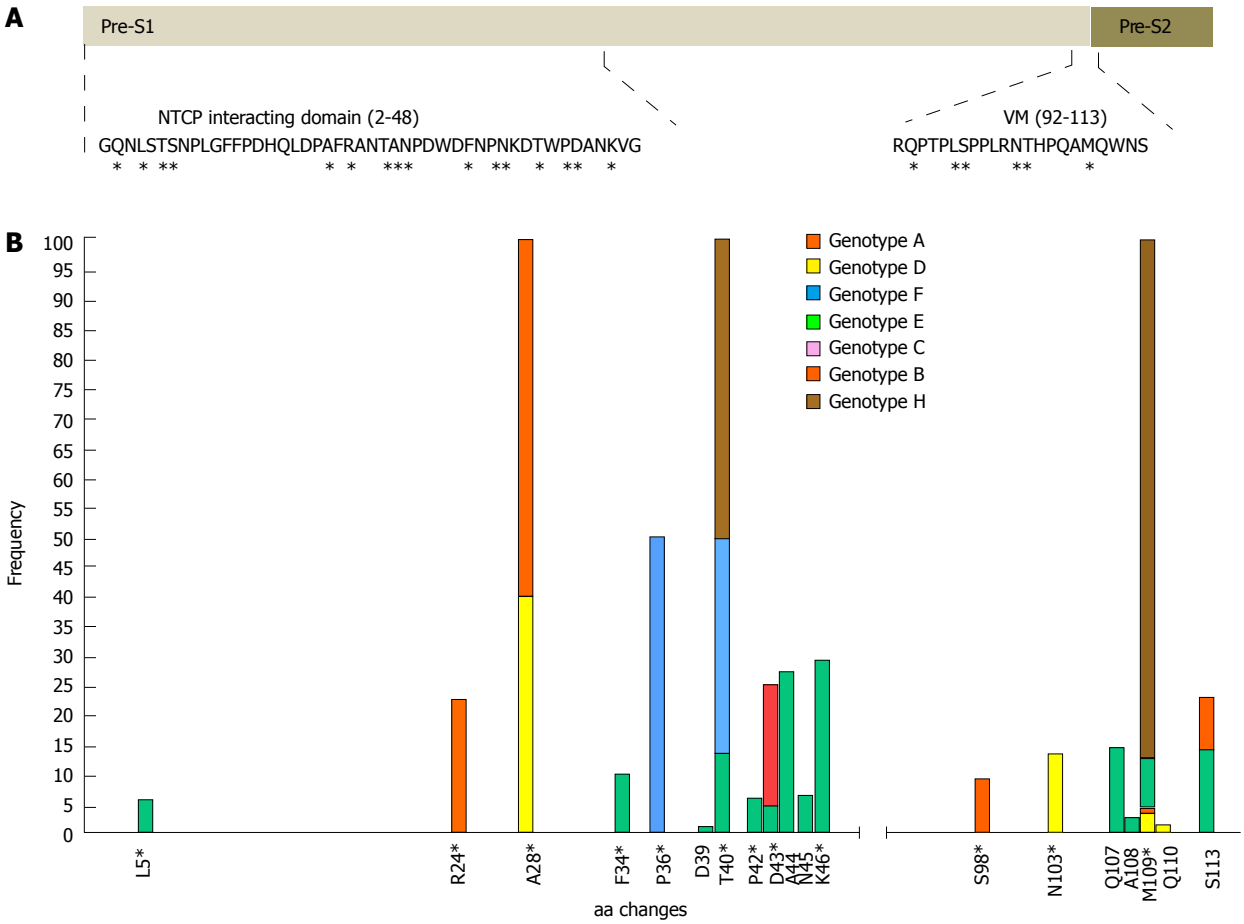

Figure 2 Frequency of amino acid changes in each position in the two domains studied. In order to simplify the variations due to HBV genotype, the numeration of aa positions in both domains and their consensus sequences is presented according to genotype D (reference sequences obtained from GenBank, accession numbers provided in Supplementary Table 1). Asterisks indicate positions where the wild-type aa varies according to HBV genotype. A: Schematic diagram where the two regions studied are represented: the sodium-taurocholate cotransporting polypeptide (NTCP)-interacting domain from residues 2 to 48 of the N-terminal end of preS1, and the virion morphogenesis (VM) domain from residues 92 to 108 of the C-terminal end of preS1 and the first 5 residues from the N-terminal end of preS2. B: Barplot representing the frequency of aa changes (above 1% of HBV quasispecies) within each HBV genotype in the NTCP interaction and virion morphogenesis domains (Specific aa changes are shown in Supplementary Table 2).

tested negative for hepatitis D virus (HDV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV). The demographic, biochemical, and virological characteristics of these patients are shown in Table 2. Second, the rs2296651 SNP (S267F) was determined in all 246 individuals comprising the 3 groups following a previously described in-house developed protocol to detect SNPs in human serum samples[27]. To avoid the discomfort of additional blood drawing, 1 mL of serum from samples obtained for routine clinical analysis was used for all analyses.

Serological and virological determinations

Serological markers for HBV (HBsAg, HBeAg, anti-HBe, and anti-HBc) and anti-HCV antibodies were tested using commercial enzyme immunoassays mounted on a COBAS 8000 analyzer (Roche Diagnostics, Rotkreuz, Switzerland). Antibodies against HDV were tested using the HDV Ab kit (Dia.Pro Diagnostic Bioprobes, Sesto San Giovani, Italy), and anti-HIV antibodies were tested by the Liaison XL murex HIV Ab/Ag kit (DiaSorin, Saluggia, Italy). HBV-DNA was quantified by real-time PCR with a detection limit of 20 IU/mL (COBAS TaqMan HBV V2.0, Roche Diagnostics, Mannheim, Germany). HBV genotypes were determined by Sanger sequencing and phylogenetic analysis with reference sequences from HBV genotypes A to H of the preS1 region (Supplementary Table 1), and supported by NGS analysis of the same region.

Analysis of the HBV preS1 region by NGS

In this study, we explored a fragment of the HBV genome including the entire preS1 region and the N-terminal end of preS2. The HBV genotype-specific insertions and deletions occurring along the HBV genome change the nucleotide (nt) numbering of the fragment. Hence, the fragment includes nt positions 2844 to 56 (434 bp) in genotype A (genome size 3221 nt), positions 2838 to 56 in genotypes B, C, D, E and H [434 bp in B, C, and H (genome size 3215 nt in all of

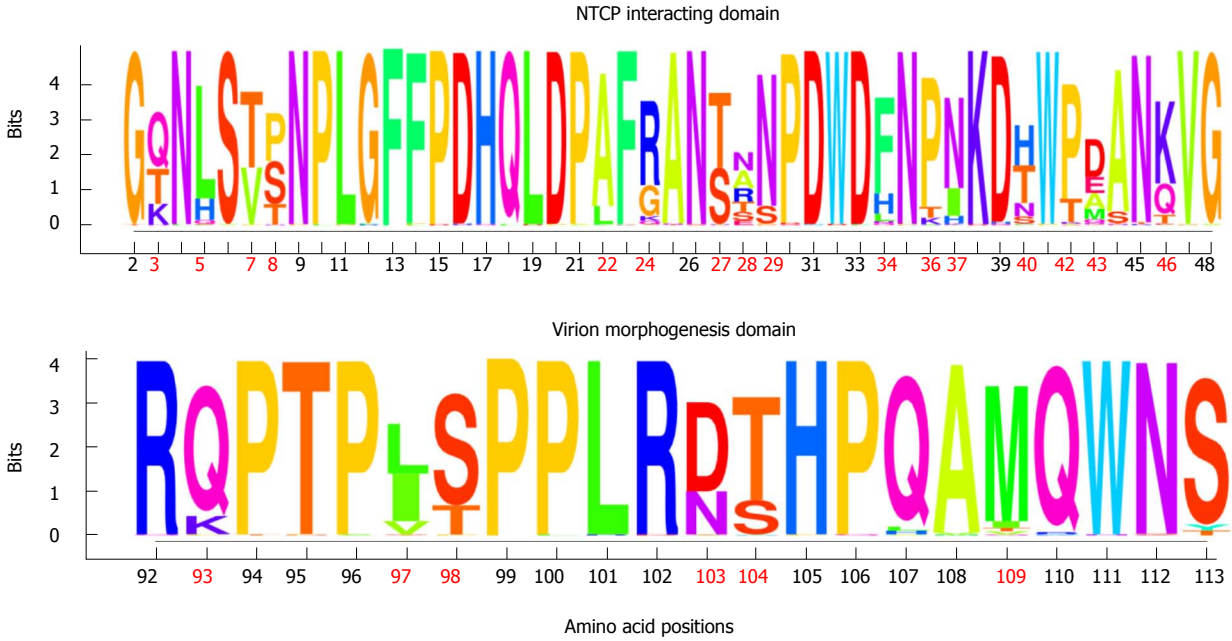

Figure 3 Sequence logos showing the information content of amino acid positions from the sodium-taurocholate cotransporting polypeptide-interacting domain and the virion morphogenesis domain, in all the haplotypes obtained by next-generation sequencing. In order to simplify variations due to HBV genotype, the numeration of aa positions from both domains is presented according to genotype D: NTCP-interacting domain from residues 2 to 48 of the N-terminal end of preS1, and the virion morphogenesis domain from residues 92 to 108 of the C-terminal end of preS1 and first 5 residues from the N-terminal end of preS2. Positions where the wild-type aa varies according to HBV genotype have been highlighted in bold and red. aa: Amino acid; NTCP: Sodium-taurocholate cotransporting polypeptide.

them), 401 bp in D (genome size 3182 nt), and 431 bp in E (genome size 3212 nt)] and positions 2837 to 56 (434 bp) in genotype F (genome size 3215 nt).

A detailed description of the molecular amplification, NGS procedures carried out, and subsequent bioinformatics filtering of the sequencing data is provided in the Supplementary Materials and Methods (Supplementary Protocol 1). Briefly, molecular amplification was performed by nested PCR. In the final PCR products (amplicons), the technique incorporated M13 universal adaptor sequences (forward and reverse), a unique identifier that enabled grouping of the sequences derived from each sample [multiplex identifier sequences (MID)], and sequences A and B (adaptors for the elements of the UDPS system). The amplicons were purified and pooled at equimolecular concentrations. The pool was UDPS-analyzed following the manufacturer's protocol. The sequencing data underwent a bioinformatics filtering procedure based on an in-house-developed pipeline[28], with all computations done in the R environment and language[29]. UDPS reads (sequences from each individual amplicon) were demultiplexed according to their MID sequence, and primers were trimmed. After a quality filter step, reads with the same nt sequence were collapsed into haplotypes, that is, unique sequences covering the full amplicon observed on the clean set of sequences[30]. We then selected haplotypes covering the full amplicon and common to both the forward and reverse strands whose sequences were present in frequencies of > 0.25% of the complete set of sequences.

Amino acid variability/conservation in the preS1 region

The variability/conservation of the aa sequence in the NTCP-interacting domain was compared to that of a conserved domain with a pivotal function in virion morphogenesis[31]. This domain, located in the preS1 C-terminal (Figure 1), was selected for comparison purposes as a "sequence conservation control" because of its high degree of conservation in other orthohepadnaviruses, such as woodchuck hepatitis virus[31].

The NTCP-interacting domain includes 47 aa from the N-terminal end of the preS1 region (aa 13-59 in HBV genotypes A, B, C, F and H, aa 2-48 in genotype D, and aa 12-58 in genotype E), whereas the virion morphogenesis domain includes 22 aa lying between the C-terminal end of preS1 and the first 5 aa from the N-terminal end of preS2 (aa 103-124 in HBV genotypes A, B, C, F and H, aa 92-103 in genotype D, and aa 102-123 in genotype E).

The first step of this analysis was to classify the haplotypes from each patient according to their HBV genotype by phylogenetic analysis in order to differentiate sequence variations from genotype-related polymorphisms. First, we selected 86 full-length HBV genome sequences representative of HBV genotypes A to H, obtained from GenBank (accession numbers in Supplementary Table 1) and extracted the region from nt 2837, 2838, or 2844 (depending on viral genotype) to 56. We then determined the maximum genetic distances between sequences from the same HBV genotype in this region and the minimum genetic distances between sequences from different genotypes, in order to set a

sequence identity threshold. The threshold was then used to cluster the individual haplotypes from each patient; sequences with an identity above the identity threshold were grouped together in the same cluster and their frequencies were added up. The master sequence (the most abundant haplotype) from each cluster and the sequences obtained from the 86 full-length genomes then underwent phylogenetic analysis to determine the HBV genotype. Genetic distances between master sequences and the remaining sequences in their clusters were below the minimum distance between sequences from different genotypes; thus, all the sequences in the cluster were considered to belong to the same HBV genotype as the master sequence. All individual haplotypes were then separated into different fasta files according to the genotype assigned to each of them and translated into aa sequences. The aa haplotypes with the same sequence were recollapsed and their frequencies were updated.

The multiple alignments of aa haplotypes obtained were used to detect point mutations, each of which was considered a separate variant, and to determine their abundance in the two regions studied.

Finally, an overall image of aa conservation/variation in preS1 for each HBV genotype was obtained by calculating the information content (IC) of each position. The IC of an aa position is related to the number of binary decisions (number of questions with a yes/no answer) required to find the correct aa in a given position among a set of 20 possibilities per position in the multiple alignment of haplotypes. For example, if the probabilities of finding any of the 20 aa (p_a) were equal in a given position, the number of binary questions required to find the correct aa in this position would be $\log_2(20) = 4.322$. However, since the different aa have different unknown probabilities of occurring in each position of the multiple alignment, an uncertainty measure, $-\sum_{a\in aa} p_a \log_2(p_a)$[32], also known as "entropy", must be added to the IC calculation. Thus the IC of each aa position was calculated using the following equation: $IC_{aa}(\text{Site}) = \log_2(20) - \sum_{a\in aa} p_a \log_2(p_a)$ If only one aa were found in a given position from the multiple alignment (maximum conservation), there would be no uncertainty in that position and the probability of finding that aa would be 20/20 thus $-\sum_{a\in aa} p_a \log_2(p_a) = 0$ and $IC_{aa} = \log_2(20) - 0 = 4.322$. On the other hand, if all aa had an equal possibility of occurring at a given position (maximum variability), the degree of uncertainty of that position would be the highest and the probability of finding any aa in that position would be 1/20 thus $-\sum_{a\in aa} p_a \log_2(p_a) = 4.322$ and $IC_{aa} = \log_2(20) - 4.322 = 0$. These calculations in the regions of NTCP interaction (47 aa) and virion morphogenesis (22 aa) were also represented as sequence logos created using the R language package, motifStack[33].

The statistical and bioinformatics methods used in this study were reviewed by Dr. Josep Gregori from the liver disease-viral hepatitis laboratory (Vall d'Hebron Institut Recerca-Hospital Universitari Vall d'Hebron), CIBERehd and Roche Diagnostics SL.

Determination of the SLC10A1 gene rs2296651 (S267F) polymorphism

The rs2296651 SNP in the *SLC10A1* gene, causing the NTCP S267F variant, was determined using a new in-house developed real-time PCR method based on fluorescence resonance energy transfer (FRET) probes, on the LightCycler 2.0 analyzer (Roche Diagnostics, Rotkreuz, Switzerland). PCR primers were designed to flank a 111-bp region of *SLC10A1*, including the SNP. The results obtained by this method were validated by direct sequencing (Sanger method) in genomic DNA extracted from blood in 18 samples from the 3 groups: A (12), B (4), and C (2). A detailed explanation of the rs2296651 detection procedure by both real-time PCR and Sanger sequencing is provided in the Supplementary Materials and Methods (Supplementary Protocol 2).

RESULTS

Identity threshold between sequences of the same HBV genotype in reference sequences from the region studied

In the regions from nt 2837, 2838, or 2844 (depending on viral genotype) to 56, extracted from the 86 full-length HBV genome sequences representative of genotypes A to H, analysis of the maximum genetic distance in each genotype and the minimum genetic distance between different genotypes (data not shown) resulted in a sequence identity threshold between genotypes of 95%. Therefore, for each patient, haplotypes with a sequence identity > 95% were clustered together, and the master sequence from each cluster was included in the phylogenetic analysis to determine the HBV genotype.

preS1 variability and/or conservation

The NTCP-interacting domain (47 aa) and virion morphogenesis domain (22 aa) were analyzed in the 18 patients in group A with viremia levels > 4 logIU/mL. In total, 118779 quality-filtered sequences were obtained from the samples, and a median of 5546 (1903 to 13126, interquartile range, 3585) sequences containing both domains were analyzed per sample.

In general, aa residues from the NTCP-interacting domain were conserved within each HBV genotype among the different viral genomes examined. Only 12 of 47 (25.5%) aa positions showed genotype-unrelated changes occurring at > 1% of the HBV quasispecies, a somewhat lower percentage than in the virion morphogenesis domain (7 of 22 aa positions, 31.8% with genotype-unrelated changes) (Figure 2B). Interestingly, according to the consensus sequences of HBV genotypes A to H of both domains, obtained from the 86 full-length HBV genome sequences from

GenBank, the virion morphogenesis domain had a lower percentage of aa positions with genotype-associated changes (viral polymorphisms) than the NTCP-interacting domain (6 of 22, 27.3% *vs* 16 of 47, 34%, respectively) (Figure 2A). On analysis of conservation throughout the NTCP-interacting domain, we observed a high degree of conservation between aa positions 2 to 21 (viral genotype D numeration), in which no aa changes in proportions greater than 1% were found, except in one genotype E patient who showed the H5Q variant in 11.8% of haplotypes (representing 5.9% of all genotype E haplotypes obtained) (Supplementary Table 2). Conservation was especially relevant between aa 9 and 21: very few changes were seen within each HBV genotype (none at > 1%) and wild-type aa were the same in the different genotypes (Figures 2 and 3).

According to the sequence logos layout, which shows the IC analysis for each aa position, HBV genotype C displayed the highest level of conservation: unaltered in the NTCP-interacting domain and only 1 aa change at position 124 in the virion morphogenesis domain (112 according to viral genotype D numeration), in the 4 patients included. HBV genotype E showed the highest variability, although it should be remembered that only 2 patients had this genotype (Supplementary Figure 1). HBV genotypes D and A (the most prevalent in our area)[34] showed moderate variability, with aa 28, 103, and 109 being particularly variable in genotype D, and aa 54 and 120 (43 and 109 according to genotype D numeration, respectively) particularly variable in genotype A (Supplementary Figure 1).

Of note, the sequence logos, including all haplotypes regardless of viral genotype (Figure 3) also indicated that proline (P) residues were highly conserved. Nonetheless, the P residue at position 30 of the NTCP-interacting domain showed a change to lysine (K) in HBV genotype F and to threonine (T) in genotype H, and the P residue in position 36 changed to T in genotype E. Furthermore, there was a notable complete preservation of the five P residues located in the virion morphogenesis domain. Interestingly, serine residues (S) in this domain showed changes to T (positions 109 and 124 in HBV genotype B and positions 98 and 113 in genotype D) or tyrosine (Y) (position 123 in HBV genotype E and position 113 in genotype D) (Figure 3 and Supplementary Figure 1); all of these have similar physicochemical characteristics, being the residues most commonly phosphorylated[35]. In general, the S residues of the NTCP-interacting domain were conserved within each viral genotype, but different aa were sometimes seen in this position between the different genotypes (Supplementary Figure 1).

Of note, 4 of our patients, harboring haplotypes classified into HBV genotypes A, D, E and H, showed a significant percentage of changes in the preS2 initial methionine residue (position 109 in genotype D) (Supplementary Table 2 and Supplementary Figure 1). Other relevant aa changes in the NTCP-interacting and virion morphogenesis domains of the patients and their frequencies are shown in Supplementary Table 2.

SLC10A1 gene rs2296651 (S267F) polymorphism

The *SLC10A1* gene rs2296651 SNP (S267F) was determined in all 246 individuals recruited (95.53% Caucasian, 2% Asian, and 2.47% Sub-Saharan), which constituted a representative population of patients from the outpatient clinic of our hospital. None of them presented the SNP. Real-time PCR results were also confirmed by direct sequencing in 18 blood samples from the 3 groups: A (12), B (4), and C (2).

DISCUSSION

Since it was first identified as a specific receptor enabling HBV entry into human hepatocytes, the NTCP bile salt transporter has gained significant attention as a target in antiviral therapy for HBV and HDV (which share the same entry mechanism)[12,36,37]. The interaction between viral particles and this transporter is mediated by the region between aa 2 and 48 (HBV genotype D numeration) of the preS1 N-terminal end of LHBs[15-18], where aa 2 (glycine) must be bound to myristic acid (N-myr) for the viral particles to be infective[38,39]. Identification of this interaction has allowed the development of drugs that can block HBV entry by targeting NTCP[20]. An example of these drugs is Myrcludex-B, a synthetic myristoylated 47-aa lipopeptide derived from aa 2 to 48 of preS1, which has proven to effectively block HBV cellular entry *in vitro*[18,40], *in vivo*[41-43], and in patients included in clinical trials[44,45]. In a recent report, Tsukuda *et al*[46] explored an alternative strategy for blocking HBV entry based on proanthocyanidin and its analogs, which directly act on LHBs. Hence, inhibition of viral cellular entry is becoming consolidated as a viable new therapeutic approach against HBV infection. In this line, analysis of the preS1 N-terminal NTCP-interacting domain and the presence of the rs2296651 SNP (S267F) may be relevant as prognostic markers of the response to this new therapy because of their potential roles in the interaction that enables HBV to infect hepatocytes.

To our knowledge, this is the first study in which the variability/conservation of the essential preS1 NTCP-interacting domain has been investigated by NGS. The results show a high degree of conservation between preS1 aa positions 2 to 21 (HBV genotype D numeration): aa changes in proportions greater than 1% were found in a single position in only 1/18 patients. Of note, among the 20 aa in the N-terminal end of preS1, wild-type aa were found to be conserved between positions 9 and 21 in all genotypes included. These observations suggest an essential function of this segment of preS1, which would agree with the results of a study by Glebe *et al*[17] In that report, hepatitis B attachment site mapping by infection-inhibiting amino-terminally acylated preS1-derived lipopeptides

highlighted the essential role of aa 9 to 18 for viral particle binding to NTCP. In addition, the findings from that study[17] indicated that aa sequences in positions 29 to 48 would play an accessory role, a fact that seems to justify the more significant variability in this region observed in our NGS analysis. Therefore, conservation/variability is not homogenous throughout the entire preS1 NTCP-interacting domain: whereas segments with a previously described essential role in the NTCP interaction show a highly conserved sequence, positions with accessory roles that are nonetheless needed for strongest blocking show greater variability. preS1 structural simulations would be needed to help clarify the contribution of each segment in NTCP interactions and the sequence conservation requirements for their respective functions.

After their translation, approximately 50% of LHBs undergo posttranslational topological reorientation, in which their N-terminal end is translocated to the endoplasmic reticulum lumen[47]. This gives rise to two types of LHBs: those in which the preS1 region has an external position in the viral particles and those in which this region has an internal position. Whereas the N-terminal region of external LHBs is involved in NTCP interactions[15], the C-terminal part of internal LHBs between aa 92 and 113 (HBV genotype D numeration) has a pivotal function in virion morphogenesis by directly contacting the nucleocapsid during viral particle budding[31]. Moreover, this latter domain is reported to be highly conserved among Orthohepadnavirus[31], a concept that is supported by the lower percentage of HBV genotype-associated viral polymorphisms in the virion morphogenesis domain than in the NTCP-interacting domain (27.3% *vs* 34%, respectively) in consensus sequences of genotypes A to H obtained from the 86 HBV genome sequences downloaded from GenBank. However, in the present study, HBV genotype-unrelated changes above 1% of the quasispecies were found in 25.5% of NTCP *vs* 31.8% of C-terminal positions, with both domains being most highly conserved in genotype C and most highly variable in genotype E (a limited finding because this high variation was observed in 1 of only 2 genotype E patients included). Thus, while the NTCP-interacting domain seems to be more highly conserved within each genotype, the C-terminal virion morphogenesis domain seems more highly conserved between different HBV genotypes. The high proportion of conserved aa positions in both domains within the same viral genotype and between different genotypes seems to confirm that they conduct essential functions for HBV replication. Again, structural simulations would likely be helpful to understand the reasons for the sequence conservation and variability in these two domains.

Interestingly, P and S residues generally showed a high degree of conservation, particularly in the C-terminal virion morphogenesis domain. It must be kept in mind that P is often found at the end of the α helix or in turns or loops, and it contributes to protein folding by stabilizing these structures[48,49], thus being associated with essential structural protein motifs. Conservation of most P residues in the two preS1 domains suggests their structural preservation, which would facilitate interactions between external LHBs and the NCTP cell receptor or between internal LHBs and nucleocapsids in the same manner as a lock and key. In the virion morphogenesis domain, in positions where S was the wild-type aa, we observed that changes with > 1% prevalence within the same HBV genotype or between different genotypes were either to T or to Y. Bearing in mind that S, T, and Y are the main targets for phosphorylation in eukaryotic cells[35], the tendency to keep these specific aa in specific positions of the virion morphogenesis domain suggests that they could be phosphorylated. This phosphorylation could be important for the functionality of the virion morphogenesis domain. Site-directed mutagenesis experiments, with modification of the P and S residues, could clarify the function of these two conserved aa in both the preS1 essential domains analyzed.

In the present study, we also assessed the prevalence of the *SLC10A1* gene SNP, rs2296651 (S267F), in all 246 participants. The effect of this SNP on CHB is controversial. Most studies have reported that it is associated with CHB resistance, as it might interfere with ligand binding, thereby preventing HBV from cellular entry[24,26]. Nonetheless, one study has shown that rs2296651 is associated with enhanced HBV infection[25]. The prevalence of rs2296651 varies greatly between different ethnicities and geographic locations[22,23], being identified prevalently in Asian populations, with the highest rates in southern China and Vietnam[23]. This polymorphism has not been detected in Americans from European, African or Hispanic origins[22]. However, its prevalence in our area (typically classified as having an intermediate HBsAg prevalence)[50] where Caucasian populations mainly of European Mediterranean origin are predominant, has never been assessed. In the present study, rs2296651 was not found in any of the HBV patients or controls studied. Considering the substantial prevalence of this SNP in Asian populations where HBV infection is endemic, the low prevalence found in our area may be associated with the lower incidence of HBV infection[51].

Although the rs2296651 SNP seems to be absent in the chronic HBV-infected population attended in our setting, multiple SNPs in *SLC10A1* have been found at relatively high allele frequencies in certain ethnic populations[21-26,52]. As an example, Ho *et al*[22] reported 6 additional ethnicity-dependent SNPs in the 5 exonic regions of *SLC10A1* (3 were non-synonymous and caused aa changes: I223T, I279T, K314E). The functionally relevant NTCP polymorphisms would be expected to modify bile acid homeostasis and HBV cellular entry; hence, it cannot be excluded that Caucasian individuals might have additional, still undescribed NTCP SNPs that could interfere with HBV infectivity. Therefore, further studies should be

performed to characterize and determine the prevalence of NTCP SNPs in different ethnic populations and their implications in NTCP function.

This study includes a representative sample of CHB patients attended in our center and includes most HBV genotypes (A to F and H). Necessarily, the number of patients carrying the less prevalent genotypes in our area was low. This limitation may have biased the conservation/variability findings in some genotypes due to individual variability in some patients, as was likely the case of the variability in genotype E. Thus, the findings should be considered preliminary, requiring confirmation in further studies with larger populations. Another potential limitation is that the NGS technology used in this study (ultra-deep pyrosequencing in the GS-Junior platform, 454/Roche) has been discontinued by the supplier. When the study was designed, this technique was selected as the best available one for our purposes, as it enabled inclusion of the preS1 regions of NTCP interactions and virion morphogenesis in the same sequence read, using a single amplicon. A suitable alternative to enable future studies aimed at confirming and expanding the results of the present report, and in general to analyze the viral quasispecies, could be Sequencing By Synthesis technology on MiSeq platforms (Illumina, San Diego, United States), as this technique can generate sequence reads with a length similar to that of the fragment analyzed in this study.

In conclusion, NGS analysis of preS1 domains in strains from 7 of the 8 main HBV genotypes showed a high degree of conservation of essential amino acids related with the NTCP interaction. Proline residues in both domains and potential phosphorylation targets in the virion morphogenesis domain were also highly conserved. Given the low to null prevalence of the rs2296651 SNP of the *SLC10A1* gene in our patient population, we would not expect interference from NTCP in its interaction with preS1 or preS1-derived synthetic lipopeptide molecules. Thus, these preliminary results indicate that inhibition of HBV entry by NTCP block therapies would be a suitable treatment in our CHB population.

The findings from this exploratory study are locally relevant as they provide preliminary information on a population previously uncharacterized in this regard. However, they have limited robustness for generalizations. Nonetheless, the study illustrates the value of NGS to investigate the variability of the preS1 region of the LHBs, and real-time PCR melting curve analysis to detect the rs2296651 polymorphism causing S267F variant in NTCP, the HBV cellular receptor. Future studies with larger patient samples are needed to support the preS1 results and investigate additional NTCP polymorphisms. Finally, functional and structural studies will help decipher the implications of the high degree of conservation of the preS1 domains on HBV activity.

ARTICLE HIGHLIGHTS

Research background

The preS1 region of the hepatitis B virus (HBV) large envelope protein interacts with its cellular receptor, sodium-taurocholate cotransporting polypeptide (NTCP), to enable HBV infectivity. Identification of this interaction has led to the development of drugs that can block HBV entry by targeting NTCP, such as synthetic myristoylated lipopeptides derived from the domain of the preS1 N-terminal end which interacts with NTCP (hereafter, NTCP-interacting domain), that can dock to this receptor, blocking the HBV entry mechanism. Several clinical trials are currently testing this type of HBV entry inhibitor; for example, Myrcludex-B. Furthermore, HBV cellular entry may also be impaired by the single nucleotide polymorphism (SNP) rs2296651 in the SLC10A1 gene. This SNP causes the NTCP S267F variant, which can affect interactions between the receptor and viral particles. Study of these viral and host features may be relevant to understand the interaction of NTCP with the preS1 NTCP-interacting domain and, reasonably, to provide an indication of the potential effectiveness of treatments with synthetic myristoylated lipopeptides derived from this domain.

Research motivation

Sequence variability in the NTCP-interacting domain might change its affinity to attach to NTCP, and this would alter the dynamics of competition with synthetic myristoylated lipopeptide inhibitors of HBV entry, potentially weakening the effectiveness of these treatments. The presence of the rs2296651 SNP causing the S267F NTCP variant could also impair the effectiveness of HBV treatment, as synthetic myristoylated lipopeptides have the same amino acid (aa) sequence as the NTCP-interacting domain. These factors could be analyzed in specific patient populations to determine the potential effect they may have on treatment with the new therapies designed to block NTCP and avert HBV binding to hepatocytes. This was done in the chronic hepatitis B (CHB) patient population of our area (Barcelona, Spain), mainly Caucasians of European Mediterranean origin, using robust methods that can be applied in other patient populations.

Research objectives

The main objectives of the study were to determine the variability/conservation of NTCP-interacting domain and the prevalence of the rs2296651 SNP (S267F NTCP variant) in chronically infected HBV patients. Using a high-throughput analytical protocol based on next-generation sequencing (NGS) and an in-house developed PCR using fluorescence resonance energy transfer (FRET) probes, these objectives were realized in an exploratory sample of HBV patients from our setting. Analysis of these viral and host features could be relevant to understand the interactions between HBV and its receptor, and to determine their applicability as prognostic markers of response to treatment strategies based on NTCP blocking.

Research methods

We performed two main analyses in serum samples from 246 individuals in 3 groups: patients with CHB, patients with resolved HBV infection, and HBV-uninfected individuals. First, the variability/conservation of aa sequences in the NTCP-interacting domain was analyzed and compared to that of a highly conserved preS1 C-terminal domain associated with virion morphogenesis. Comparison between the NTCP-interacting domain and the highly conserved virion morphogenesis domain gave an idea of the magnitude of sequence conservation of the former. To perform this analysis, we developed a high-throughput protocol based on NGS. The raw sequencing data obtained underwent a bioinformatics filtering and analysis procedure based on an in-house-developed pipeline with all computations done in R environment and language. The conservation/variation of these sequences was analyzed by calculating the information content of each position, which was represented graphically using sequence logos, as explained in detail in the Materials and Methods section of the main article.

Second, to estimate the prevalence of the rs2296651 polymorphism in our patient population, this SNP was determined in all samples from the patients

included. To accomplish this aim we designed a new in-house real-time PCR method based on FRET probes.

Research results

High-throughput NGS analysis yielded viral sequences for most HBV genotypes (A to F and H). In general, the NTCP-interacting domain showed a high degree of conservation, which depended on viral genotype, particularly the sequence between aa 9 to 21. In comparison to the virion morphogenesis domain, the NTCP-interacting domain showed a smaller percentage of HBV genotype-unrelated changes, but greater variability between different HBV genotypes, according to consensus sequences from the GenBank patterns of genotypes A to H. Interestingly, proline residues showed a high degree of conservation in both domains, and serine residues were also particularly conserved in the virion morphogenesis domain, where changes above 1% of the quasispecies were always to potentially phosphorylatable aa. Finally, we demonstrated a low to null prevalence of the rs2296651 SNP in HBV patients from our area. The high degree of conservation of the NTCP-interacting and virion morphogenesis domains should to be confirmed in larger patient series, and the role of proline and potentially phosphorylatable residues and their implications on HBV activity should be clarified. The potential effect of additional NTCP polymorphisms on interactions between HBV and this receptor should also be investigated.

Research conclusions

This study describes high-throughput NGS analysis of the preS1 NTCP-interacting domain, which showed overall high conservation that depended on HBV genotype, particularly between aa 9 to 21. These findings concur with previous in vitro results demonstrating that these aa are essential for HBV infectivity. In the comparison with the virion morphogenesis domain, we focused on the proline and serine residues in the two domains: proline showed a high degree of conservation and changes in > 1% of the quasispecies in serine residues were always to potentially phosphorylatable aa in the virion morphogenesis domain. Based on the physical-chemical properties of these aa, we hypothesized that proline residues could stabilize the structure of these two preS1 domains, and the tendency to keep phosphorylatable aa in specific positions of the virion morphogenesis domain suggested that they may be phosphorylated. In addition, we developed an in-house real-time PCR method that allowed us to estimate the prevalence of the rs2296651 SNP in our HBV patients, which turned out to be low to null. Bearing in mind that rs2296651 is reported to be prevalent in Asian populations, where HBV infection is endemic, we hypothesized that the low presence of this SNP in our area may be associated with the lower incidence of HBV infection. Taken together, the findings from this exploratory study suggest that inhibition of HBV entry by NTCP block therapies would be suitable treatment in our CHB patient population.

Research perspectives

The present study illustrates the value of NGS to investigate the variability/conservation of the preS1 region of the LHBs. The reasons for the high degree of sequence conservation of the NTCP-interacting and virion morphogenesis domains should be investigated by preS1 structural simulations and site-directed mutagenesis experiments, in particular with modification of the proline and serine phosphorylatable residues. However, it should be borne in mind that the sequence conservation results found in both domains should be confirmed in larger patient samples. In addition, the new in-house real-time PCR method based on FRET probes used here provided fast, reliable detection of the rs2296651 SNP in serum samples of all patients, and revealed a low to null prevalence of this SNP in our patient population. Nonetheless, it cannot be excluded that Caucasian individuals might have additional functionally relevant NTCP SNPs, which would be expected to modify bile acid homeostasis and HBV cellular entry. Therefore, further studies should be performed to determine the prevalence of NTCP SNPs in different populations, and characterize their implications in NTCP function.

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ORIGINAL ARTICLE

Basic Study

Inhibitory effects of patchouli alcohol on stress-induced diarrhea-predominant irritable bowel syndrome

Tian-Ran Zhou, Jing-Jing Huang, Zi-Tong Huang, Hong-Ying Cao, Bo Tan

Tian-Ran Zhou, Jing-Jing Huang, Zi-Tong Huang, Bo Tan, The Research Center for Integrative Medicine, School of Basic Medical Sciences, Guangzhou University of Chinese Medicine, Guangzhou 510006, Guangdong Province, China

Hong-Ying Cao, School of Chinese Materia Medica, Guangzhou University of Chinese Medicine, Guangzhou 510006, Guangdong Province, China

ORCID number: Tian-Ran Zhou (0000-0001-9602-2690); Jing-Jing Huang (0000-0002-2533-6059); Zi-Tong Huang (0000-0002-1288-8655); Hong-Ying Cao (0000-0003-0960-9536); Bo Tan (0000-0003-0614-7567).

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Correspondence to: Bo Tan, MD, PhD, Associate Professor, The Research Center for Integrative Medicine, School of Fundamental Medical Sciences, Guangzhou University of Chinese Medicine, 233 Waihuan Dong Rd, Guangzhou 510006, Guangdong Province, China. tannyhy@gzucm.edu.cn
Telephone: +86-20-39358806

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Abstract

AIM

To elucidate the mechanism of patchouli alcohol (PA) in treatment of rat models of diarrhea-predominant irritable bowel syndrome (IBS-D).

METHODS

We studied the effects of PA on colonic spontaneous motility using its cumulative log concentration (3×10^{-7} mol/L to 1×10^{-4} mol/L). We then determined the responses of the proximal and distal colon segments of rats to the following stimuli: (1) carbachol (1×10^{-9} mol/L to 1×10^{-5} mol/L); (2) neurotransmitter antagonists including N^{ω}-nitro-L-arginine methyl ester hydrochloride (10

μmol/L) and (1R*, 2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (1 μmol/L); (3) agonist α,β-methyleneadenosine 5′-triphosphate trisodium salt (100 μmol/L); and (4) single KCl doses (120 mmol/L). The effects of blockers against antagonist responses were also assessed by pretreatment with PA (100 μmol/L) for 1 min. Electrical-field stimulation (40 V, 2-30 Hz, 0.5 ms pulse duration, and 10 s) was performed to observe nonadrenergic, noncholinergic neurotransmitter release in IBS-D rat colon. The ATP level of Kreb's solution was also determined.

RESULTS

PA exerted a concentration-dependent inhibitory effect on the spontaneous contraction of the colonic longitudinal smooth muscle, and the half maximal effective concentration (EC_{50}) was 41.9 μmol/L. In comparison with the KCl-treated IBS-D group, the contractile response (mg contractions) in the PA + KCl-treated IBS-D group (11.87 ± 3.34) was significantly decreased in the peak tension ($P < 0.01$). Compared with CCh-treated IBS-D rat colon, the cholinergic contractile response of IBS-D rat colonic smooth muscle (EC_{50} = 0.94 μmol/L) was significantly decreased by PA (EC_{50} = 37.43 μmol/L) ($P < 0.05$). Lack of nitrergic neurotransmitter release in stress-induced IBS-D rats showed contraction effects on colonic smooth muscle. Pretreatment with PA resulted in inhibitory effect on L-NAME-induced (10 μmol/L) contraction ($P < 0.05$). ATP might not be the main neurotransmitter involved in inhibitory effects of PA in the colonic relaxation of stress-induced IBS-D rats.

CONCLUSION

PA application may serve as a new therapeutic approach for IBS-D.

Key words: Patchouli alcohol; Colonic longitudinal smooth muscles; Diarrhea-predominant irritable bowel syndrome; Enteric nervous system; Cholinergic nerves; Non-adrenergic non-cholinergic; Potassium channel

Core tip: We reported the results from an isolated colonic smooth muscle experiment in a chronic wrap-restraint stress-induced rat model of diarrhea-predominant irritable bowel syndrome (IBS-D). The model enabled us to study the possible mechanisms underlying IBS-D and the inhibitory effects of patchouli alcohol (PA) on an isolated IBS-D rat colon. This study demonstrated for the first time that the PA was involved in cholinergic and nonadrenergic, noncholinergic neurotransmitter regulation in the enteric nervous system (ENS) *in vitro*. PA acts as a neurotransmitter agent in ENS. The results suggest that PA is a new treatment option for IBS-D.

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INTRODUCTION

Pogostemonis Herba is the dry aerial part of *Pogostemon cablin* (Blanco) Benth, which is a well-known medicinal herb in Asian countries and has been widely used to treat functional gastrointestinal disorders[1]. Patchouli alcohol (PA; its structure is shown in Figure 1) is a tricyclic sesquiterpene extracted from Pogostemonis Herba; this compound is also the major active ingredient of Pogostemonis Herba. In European countries, PA is widely used in products for daily use and cosmetics[2]. Previous studies on PA have shown its component's pharmacological effects, including immunomodulatory, anti-inflammatory, antioxidative, antimicrobial, and antitumor activities; however, the use of PA in the medical field has not been reported[3]. In two previous works[4,5], PA affects the calcium ion antagonism and exerts antiemetic properties by ameliorating the excessive contraction in the digestive organ smooth muscle. These results implied that PA might play a role in neurotransmission regulation of the digestive system smooth muscle. However, no further research on the pharmacological effects of PA in neurotransmission regulation has been reported.

Irritable bowel syndrome (IBS) is a prevalent functional bowel disorder, and diarrhea-predominant IBS (IBS-D) is a major subtype of this disease. This subtype not only inflicts a significant socioeconomic burden[6], but also severely decreases the quality of life in patients with this condition[7]. At present, the precise pathophysiological mechanism of IBS-D remains unclear. Psychosocial stress, neuroendocrine abnormality, and disturbed gastrointestinal motility are potential modes of pathogenesis[8]. Recent studies[9,10] have shown that the abnormal changes in peripheral nerve factors can directly affect the normal movement and secretion of the gastrointestinal tract and sensitivity of gastrointestinal wall to mechanical or chemical stimuli. The myenteric plexus in the intestinal tract regulates the intestinal motility. Neuropsychological factors have been given serious attention in clinical and basic medical studies on IBS-D. Several drugs that target the neurotransmitter receptors, such as loperamide, eluxadoline, alosetron, and some antidepressants, are considered as the treatment options for patients with IBS-D[11]. Additionally, medical food, medications, and psychological therapies can alleviate the symptoms of IBS-D to a certain extent[12]. However, a standard treatment algorithm has not

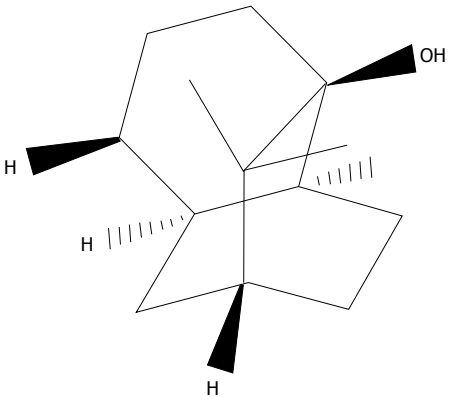


Figure 1 Structure of patchouli alcohol.

been established for IBS-D[13]. The present *in vitro* study was based on our *in vivo* model. Our *in vivo* study showed that compared with the control group, significant visceral hypersensitivity in rat model rats was observed by the abdominal withdrawal reflex score on the 21st day to the 28th day of the model establishment. The frequency of defecation in model rats increased significantly from the 14th day to the 28th day of model establishment. The stool of the model rats was mostly mushy. HE staining indicated that the intestine tissue samples in model rats showed no evident pathological changes compared with rats in the control group.

Therefore, based on the literature and our previous work, we hypothesize that the action mechanism of PA to treat IBS-D is related to the drug regulation of the neural pathways in the enteric nervous system (ENS) *via* its influence on neurotransmitter release. Therefore, the present study aims to further explore the effects of PA on isolated IBS-D rat colon *in vitro*. Our results also indicated that PA plays a specific role in neurotransmitter release regulation in ENS.

MATERIALS AND METHODS

Animal preparation

Male Sprague-Dawley (SD) rats aged four weeks and weighing 100 ± 10 g were purchased and cared for in strict compliance with the Guide to Animal Use and Care published by the Research Center for Laboratory Animals (Guangzhou University of Chinese Medicine, China). Rats were maintained under a constant 12 h/12 h light/dark cycle at an environmental temperature of 20 ℃ to 25 ℃ and humidity of 50% to 70%. The Animal Care and Use Committee of the Guangzhou University of Chinese Medicine approved all procedures used in this study.

Animal model of IBS-D

After the 7-day adaptation, the male SD rats were randomly divided into two groups as follows: control and model groups. Model group rats were subjected to a 2 h wrap-restraint period daily for 14 d. Then, these rats underwent a fourteen-day rest period.

Chemicals and reagents

In our previous studies, we confirmed PA (purity > 99.0%) by its melting point, infrared spectrometry, ^{1}H and ^{13}C nuclear magnetic resonance, and mass spectrometry analyses[14,15]. The following drugs were used. Dimethyl sulfoxide (DMSO) was used as medium to dissolve PA (DMSO < 0.2% in all experiments). Carbachol (CCh; Sigma-Aldrich, United States), N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma-Aldrich, United States), α,β-methyleneadenosine 5′-triphosphate trisodium salt (α,β-MeATP; Tocris Bioscience, United Kingdom), (1R*,2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS 2500; Tocris Bioscience, United Kingdom), potassium chloride (Sigma-Aldrich, United States), and an ATP determination kit (ThermoFisher, United States) were also utilized. All these reagents were dissolved in distilled water except for KCl, which was distilled in Kreb's solution. All other reagents used were of analytical grade.

Tissue preparation

After euthanasia by CO_2 asphyxiation, the rats were weighed, and colons and jejuna were excised and placed in 37 ℃ Kreb's solution (NaCl, 120 mmol/L; KCl, 5.9 mmol/L; $NaHCO_3$, 25 mmol/L; $Na_2HPO_4{\cdot}12H_2O$, 1.2 mmol/L; $MgCl_2{\cdot}6H_2O$, 1.2 mmol/L; $CaCl_2$, 2.5 mmol/L; and dextrose, 11.5 mmol/L). At the end of the experiments, the weight of each tissue from the colon and jejunum was recorded after blotting on filter paper.

Measurement of the longitudinal smooth muscle contraction

Full-thickness colonic and jejunal segments were isolated from the control and IBS-D model rats. Hung in the direction of the longitudinal muscle, one end of each tissue was attached to a fixed hook, whereas the other end of each strip was attached to a flexible hook with surgical suture. Each surgical suture was affixed in a force transducer that measured the isometric tension (Harvard Apparatus, United States). Afterward, the tissues were transferred to an organ bath containing Kreb's solution, maintained at 37 ℃, and continuously gassed with carbogen (950 mL/L O_2 + 50 mL/L CO_2). After a tension-free adaptive treatment for 0.5 h, the tissues from the colon and jejunum were stretched under 1.4 g of tension and equilibrated for 1 h. Force measurements were displayed on a strip chart recorder (Harvard Apparatus, United States; LabChart, New Zealand) and were digitally acquired by computer (LabChart software).

After equilibration, PA of various concentrations (3×10^{-7} mol/L to 1×10^{-4} mol/L) were added to each bath

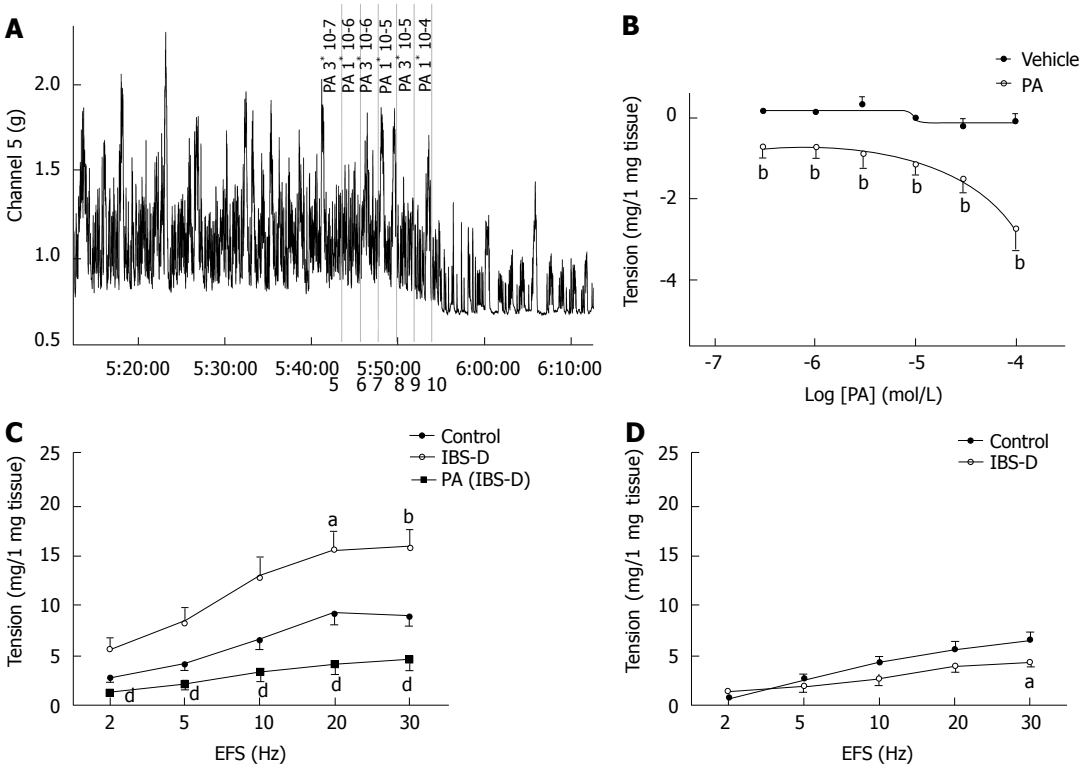


Figure 2 Inhibitory effects of patchouli alcohol on the spontaneous and EFS-induced contractions of the isolated colonic longitudinal smooth muscle. A and B: mechanical recording and linear regression curve of the cumulative log concentration-response of PA-induced (3×10^{-7} mol/L to 1×10^{-4} mol/L) relaxation of the spontaneous contraction in the colonic longitudinal smooth muscles of the control rats (n = 8; $^{b}P < 0.01$ *vs* vehicle DMSO group; unpaired t test). C: Relaxation effect of PA on EFS-induced contraction response of the colonic longitudinal smooth muscle in control, IBS-D, and PA-treated (100 μmol/L) IBS-D rats (control group: n = 38, IBS-D group: n = 31, PA-treated IBS-D group: n = 8. $^{a}P < 0.05$ *vs* control, $^{b}P < 0.01$ *vs* control, $^{d}P < 0.01$ *vs* group IBS-D; ANOVA). D: Contraction response to EFS of the jejunal longitudinal smooth muscle (control group: n = 9, IBS-D group: n = 6. $^{a}P < 0.05$ *vs* control; unpaired t test). Data are expressed as mean ± SE. PA: Patchouli alcohol; EFS: Electrical field stimulation, PA: Patchouli alcohol.

to observe the drug effects on spontaneous contraction of the colonic longitudinal smooth muscle. At the end of each experiment, the colonic contractile responses to KCl (120 mmol/L) in the control and IBS-D model rats were compared.

In this *in vitro* study, CCh (1×10^{-9} mol/L to 1×10^{-5} mol/L) was added to the organ bath, and the contractile responses in the control and IBS-D model rats were compared. After the contractile response of CCh reached a plateau, the strips were washed thrice and equilibrated for 1 h in the next experiment.

After equilibration, electrical field stimulation (EFS; 40 V, 2 Hz to 30 Hz, 0.5 ms pulse duration, 10 s) was performed to elicit the nerve-mediated contraction of the colonic tissue strips. EFS was achieved through the platinum electrodes connected to a stimulator (Harvard Apparatus, United States). The inhibitory or excitatory neurotransmitter agents, such as L-NAME (10 μmol/L), α,β-MeATP (100 μmol/L), and MRS 2500 (1 μmol/L), were separately treated in each bath before EFS.

Tissues were pretreated at a single PA concentration of 100 μmol/L to observe its drug effects on the functions of the neurotransmitter agents, including CCh (10^{-9} mol/L to 10^{-5} mol/L), L-NAME (10 μmol/L), MRS 2500 (1 μmol/L), and high-extracellular-concentration KCl (120 mmol/L), separately during the spontaneous or EFS-induced contraction of the intestinal segments. In this *in vitro* study, each tissue was subjected to a 1 h equilibration before the next test.

ATP release measurements

After EFS, Kreb's solution in each bath was collected (400 μL), frozen immediately in liquid nitrogen, and then stored at -80 ℃ until assay for ATP. The ATP levels in samples were determined by the luciferin-luciferase ATP bioluminescence assay kit (ATP Determination Kit, Thermo Fisher, United States). To calculate ATP release, we corrected the amounts detected in samples using the standard curve.

Statistical analysis

Data are expressed as means ± SEs around the mean. Non-pairwise comparisons were performed using the Student's t test. ANOVA was used to test three or more variables for statistical significance. Nonlinear and linear regression analyses were also

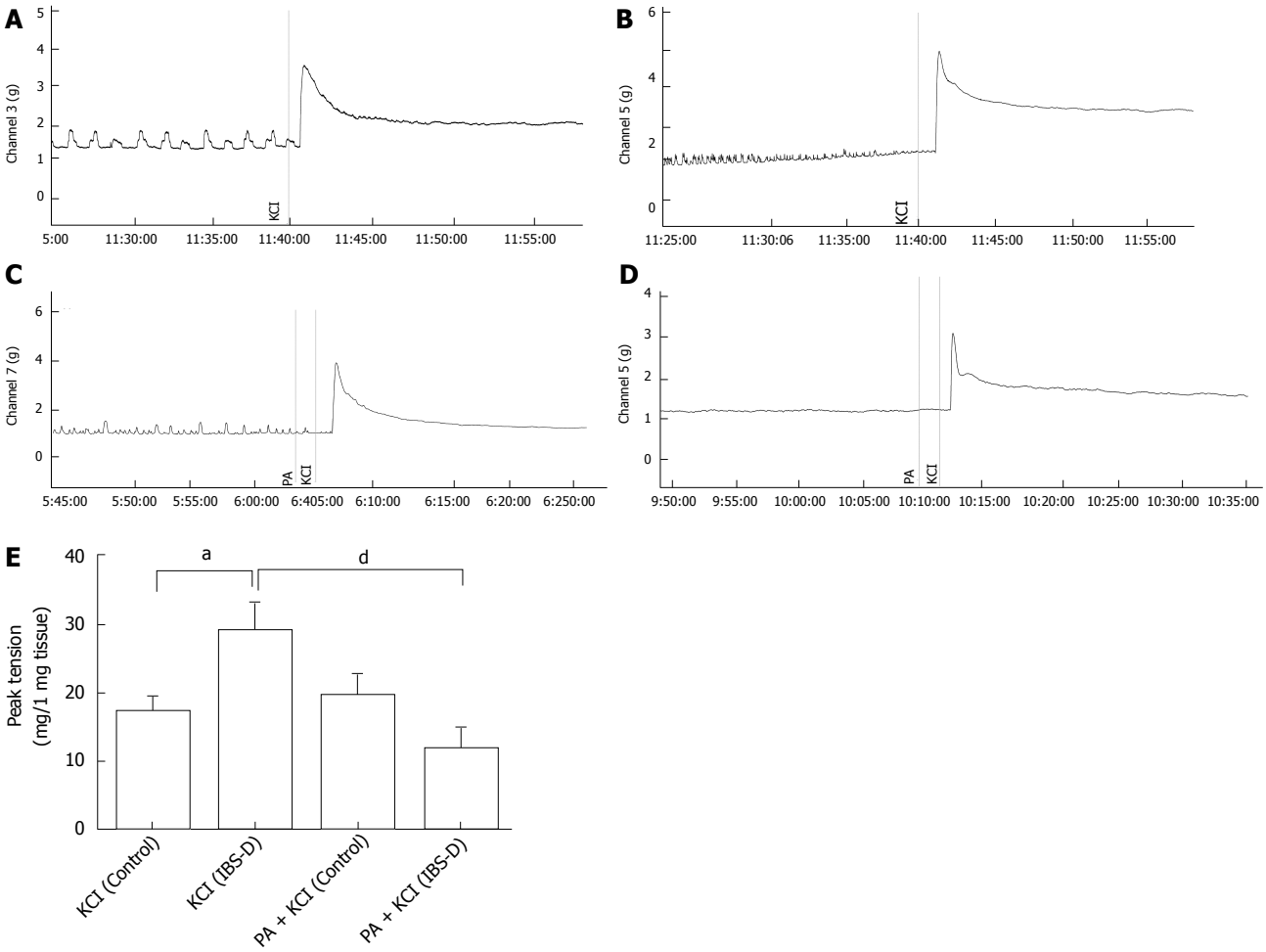


Figure 3 Inhibitory effects of patchouli alcohol on contractions induced by high extracellular KCl levels in isolated colonic longitudinal smooth muscle. Mechanical recording (A to D) and histogram (E) of relaxation effect of PA (100 μmol/L) on colonic contractions induced by high extracellular KCl levels in IBS-D rats [KCl-treated control group A: n = 27 , KCl-treated IBS-D group B: n = 23, PA + KCl-treated control group C: n = 8, PA + KCl-treated IBS-D group D: n = 8. $^{a}P < 0.05$ *vs* KCl-treated control group, $^{d}P < 0.01$ *vs* KCl-treated IBS-D group; unpaired t-test]. Data are expressed as mean ± SE.

utilized as appropriate. Calculations were performed using SPSS 20.0 based on the number of individual tissue segments. A P value of < 0.05 was considered statistically significant.

RESULTS

Inhibitory effects of PA on the spontaneous and EFS-induced contraction of the isolated colonic longitudinal smooth muscle

After equilibration, colonic muscle strips developed spontaneous basal tension for several minutes. A total of 6 cumulative PA concentrations (3×10^{-7} mol/L to 1×10^{-4} mol/L) caused a notable concentration-dependent decrease in the basal tension of the isolated rat colonic longitudinal muscle, and the half maximal effective concentration (EC_{50}) was 41.9 μmol/L (P < 0.01). PA (3×10^{-6} to 1×10^{-4} mol/L) lowered the amplitude of the spontaneous contraction in the longitudinal smooth muscles of the control rats relative to that in the vehicle strips; however, the difference was insignificant (P > 0.05). The effect of PA on the frequency of the spontaneous contraction of the colonic smooth muscle did not show significant differences between groups (P > 0.05). Compared with the control group, the EFS-induced contraction of the colonic longitudinal smooth muscles in the IBS-D group significantly increased at 20 and 30 Hz (P < 0.05). In contrast, the colonic tissues of the PA-pretreated group (100 μmol/L, 1 min) showed a significantly lower EFS-induced contraction than that of the IBS-D group (P < 0.01). Meanwhile, the jejunal tissue tension was significantly lower at 30 Hz in the IBS-D group than in the control group (P < 0.05) (Figure 2).

Inhibitory effects of PA on the isolated colonic longitudinal smooth muscle contraction induced by high extracellular KCl concentration

Contractile responses to KCl (120 mmol/L) were tested at the end of each experiment; the results showed significant differences between the control and IBS-D groups. The KCl peak contractile responses (mg contractions) reached 17.05 ± 2.47 and 29.00 ± 4.38 in the control and IBS-D rat colons, respectively (P <

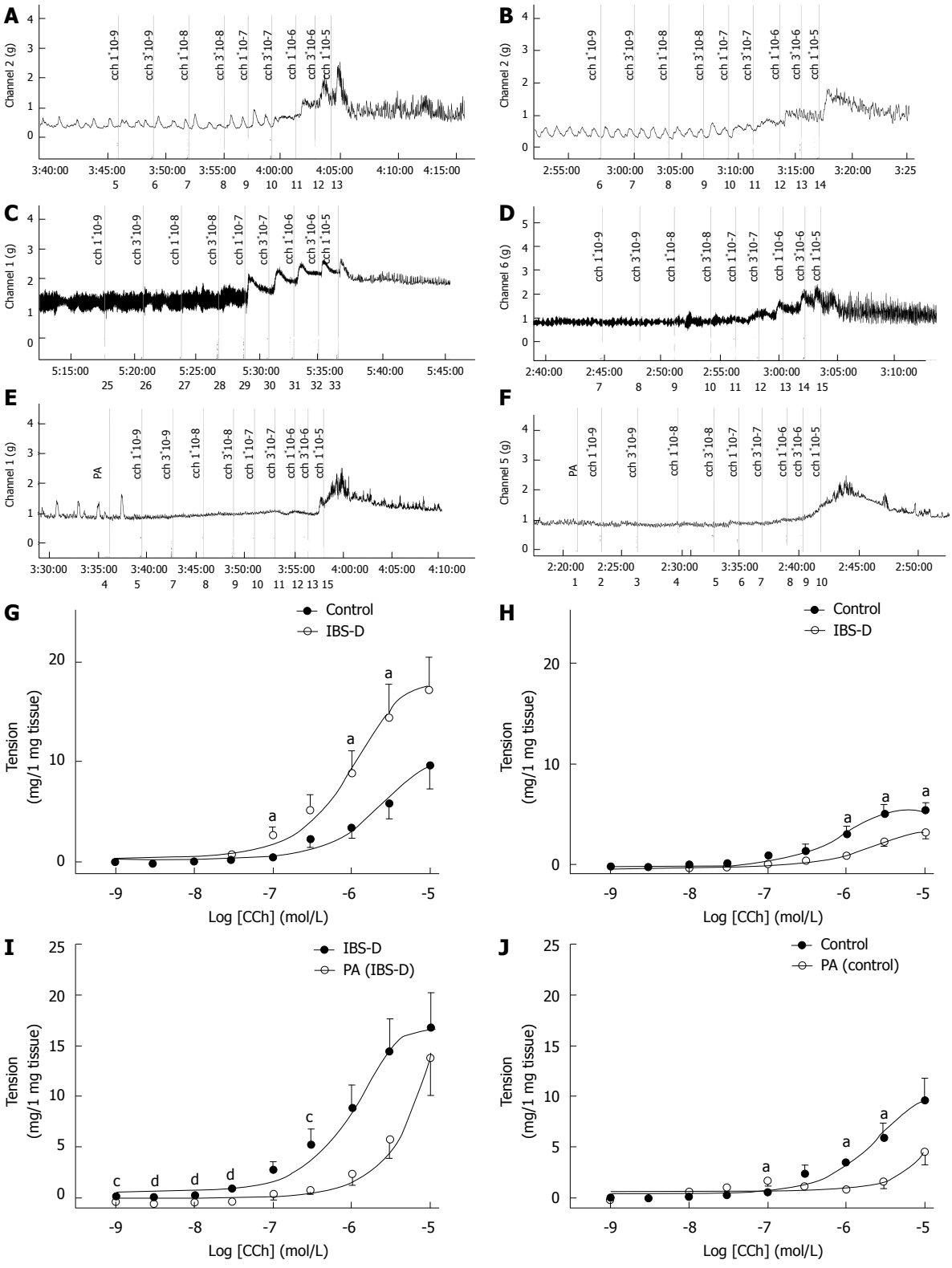


Figure 4 Inhibitory effects of PA on CCh-induced contraction in the isolated colonic longitudinal smooth muscle. Mechanical recording (A to F) and cumulative log concentration-response curve (G) of CCh-induced contraction of the smooth muscle in IBS-D rats. Colonic tissues (control group [A]: n = 14, IBS-D group [B]: n = 16; $^{a}P < 0.05$ *vs* control group, unpaired t test). Jejunal tissues (H) (control group [C]: n = 14, IBS-D group [D]: n = 11; $^{a}P < 0.05$ *vs* control group, unpaired t test). Cumulative log concentration-response curve (I) of the effect of PA (100 μmol/L) on CCh-induced contraction of smooth muscle in IBS-D rats. Colonic tissues (IBS-D group: n = 16, PA-treated IBS-D group [F]: n = 8; $^{c}P < 0.05$ *vs* group IBS-D, $^{d}P < 0.01$ *vs* group IBS-D, unpaired t test). Cumulative log concentration-response curve (J) of the effect of PA (100 μmol/L) on CCh-induced contraction of the smooth muscle in control rats. Colonic tissues (control group: n = 14, PA-treated control group [E]: n = 8. $^{a}P < 0.05$ *vs* control group, $^{b}P < 0.01$ *vs* control group, unpaired t test). Data are expressed as mean ± SE. CCh: Carbachol, IBS-D: Irritable bowel syndrome; PA: Patchouli alcohol.

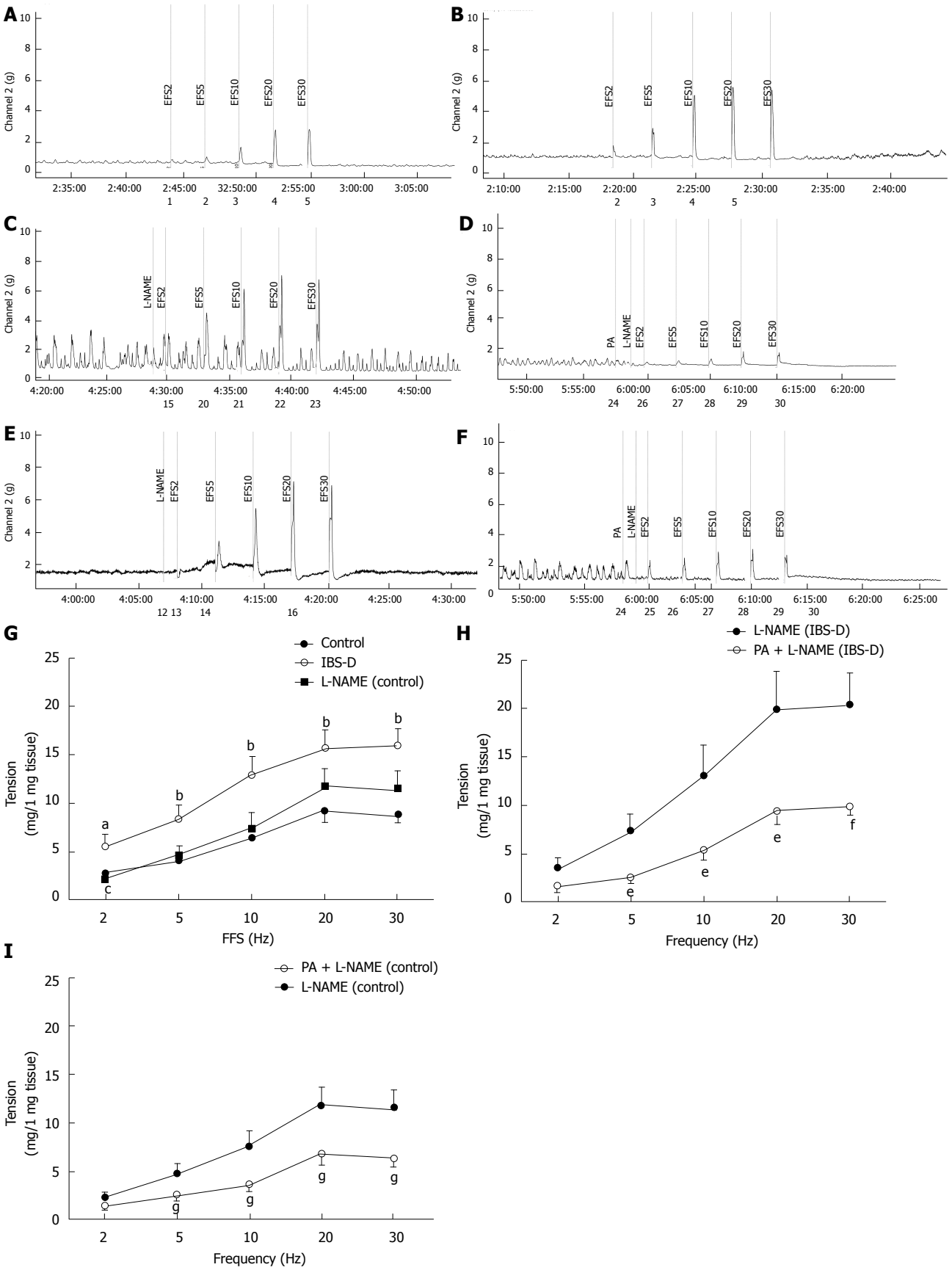
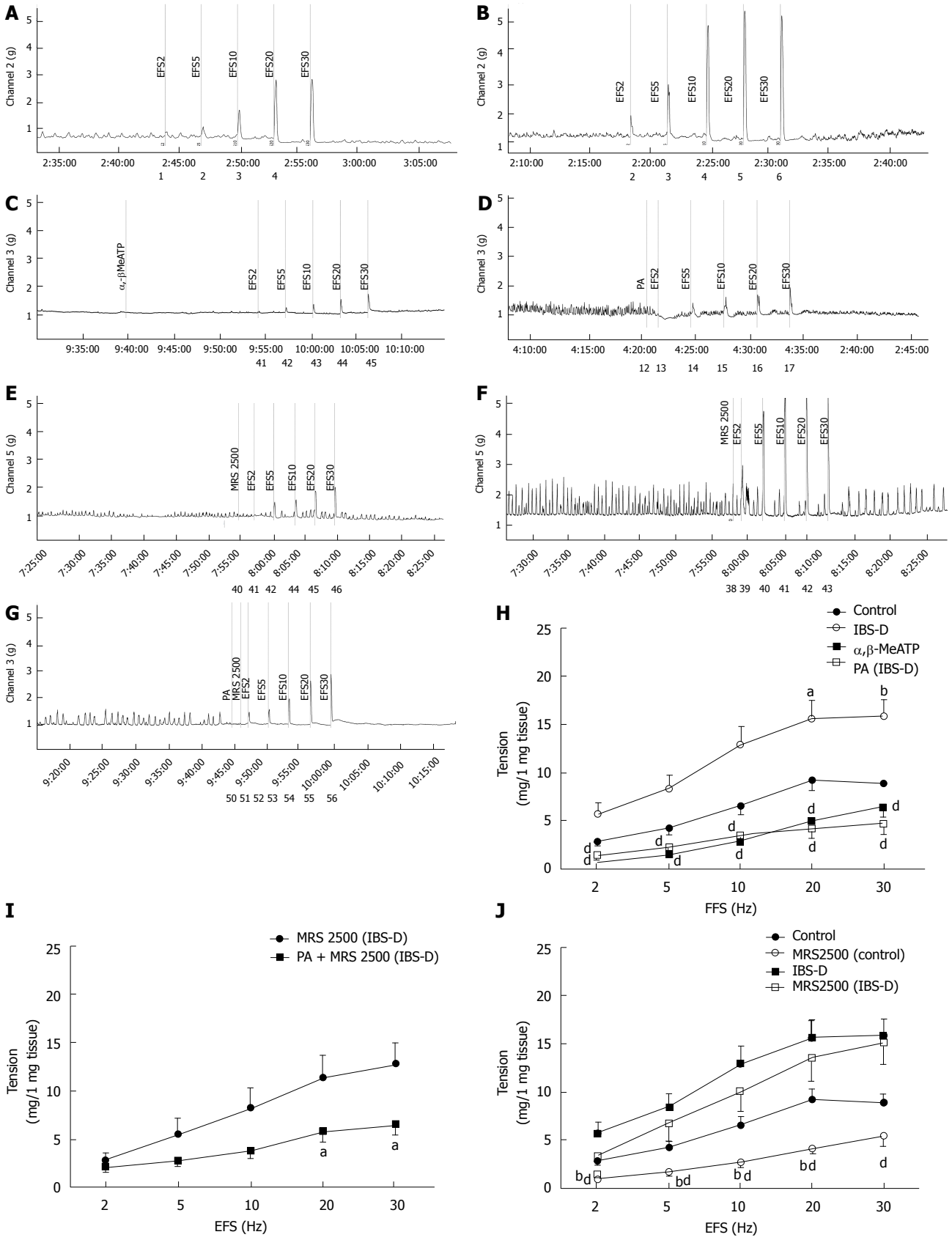


Figure 5 Inhibitory effects of patchouli alcohol on EFS-induced nitrergic contractile responses of isolated IBS-D rat colonic longitudinal smooth muscle. A-F: Mechanical recording and line chart of the mechanism and inhibitory effect of PA (100 μmol/L) on nerve-mediated contraction of stress-induced IBS-D rat colon; G: Effect of L-NAME on EFS-induced contraction of control rat colon [control group (A): n = 32, IBS-D group (B): n = 31, L-NAME-treated control group (C): n = 12; ^{a}P < 0.05 *vs* control group, ^{b}P < 0.01 *vs* control group, ^{c}P < 0.05 *vs* group IBS-D; ANOVA]; H: Effect of PA on L-NAME-induced contraction of IBS-D rat colon [L-NAME-treated IBS-D group (E): n = 14, PA + L-NAME-treated IBS-D group (F): n = 15; ^{e}P < 0.05 *vs* L-NAME-treated IBS-D group, ^{f}P < 0.01 *vs* L-NAME-treated IBS-D group, unpaired t-test]; I: Effect of PA on L-NAME-induced contraction of control rat colon [L-NAME-treated control group: n = 12, PA + L-NAME-treated control group (D): n = 16. ^{g}P < 0.05 *vs* L-NAME-treated control group; unpaired t-test]. Data are expressed as mean ± SE. L-NAME: N^{ω}-nitro-L-arginine methyl ester hydrochloride, EFS: Electrical field stimulation; IBS-D: Irritable bowel syndrome; PA: Patchouli alcohol.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EFS20
EFS30
B
Channel 2 (g)
EFS2
EFS5
EFS10
EFS20
EFS30
C
Channel 3 (g)
α,β-MeATP
EFS2
EFS5
EFS10
EFS20
EFS30
D
Channel 3 (g)
PA
EFS2
EFS5
EFS10
EFS20
EFS30
E
Channel 5 (g)
MRS 2500
EFS2
EFS5
EFS10
EFS20
EFS30
F
Channel 5 (g)
MRS 2500
EFS2
EFS5
EFS10
EFS20
EFS30
G
Channel 3 (g)
PA
MRS 2500
EFS2
EFS5
EFS10
EFS20
EFS30
H
Control
IBS-D
α,β-MeATP
PA (IBS-D)
Tension (mg/1 mg tissue)
FFS (Hz)
I
MRS 2500 (IBS-D)
PA + MRS 2500 (IBS-D)
Tension (mg/1 mg tissue)
EFS (Hz)
J
Control
MRS2500 (control)
IBS-D
MRS2500 (IBS-D)
Tension (mg/1 mg tissue)
EFS (Hz)

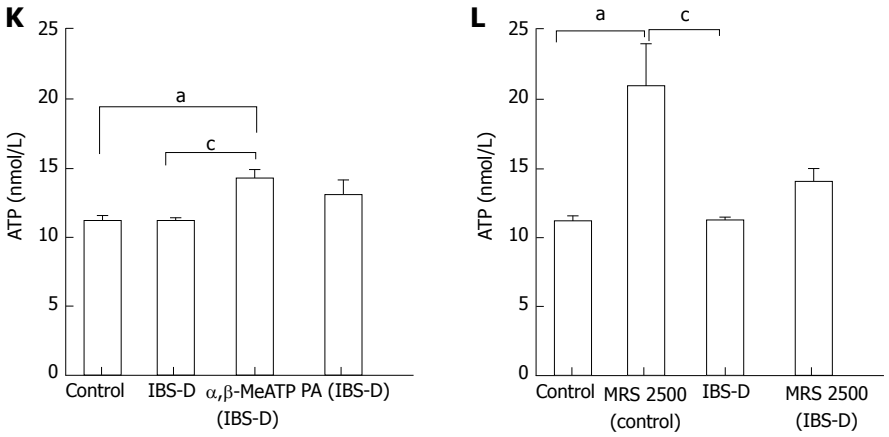

Figure 6 Effect of patchouli alcohol on a $P2Y_1$ receptor antagonist in EFS-induced contraction and ATP level of isolated colonic longitudinal smooth muscle. A-G: Mechanical recording, line chart, and histogram of the mechanism and effect of PA (100 μmol/L) on nerve-mediated rat colon contraction; H: Relaxation effect of α,β-MeATP and PA on EFS-induced contraction in IBS-D rats [control group (A): n = 32, IBS-D group (B): n = 31, α,β-MeATP-treated IBS-D group (C): n = 16, PA-treated IBS-D group (D): n = 8. $^aP < 0.05$ *vs* control group, $^bP < 0.01$ *vs* control group, $^dP < 0.01$ *vs* group IBS-D; ANOVA]; I: Relaxation response of PA to MRS 2500-treated IBS-D colon [MRS 2500-treated IBS-D group (F): n = 13, PA + MRS 2500-treated IBS-D group (G), n = 15. $^aP < 0.05$ *vs* MRS 2500-treated IBS-D group; unpaired t-test]; J: Effect of MRS 2500 on control and IBS-D rat colons [control group: n = 32, IBS-D group: n = 31, MRS 2500-treated control group (E): n = 13, MRS 2500-treated IBS-D group: n = 13. $^bP < 0.01$ *vs* control group, $^dP < 0.01$ *vs* group IBS-D; ANOVA]; K: ATP level of Kreb's solution [control group: n = 23, IBS-D group: n = 17, α,β-MeATP-treated IBS-D group: n = 21, PA-treated IBS-D group: n = 8. $^aP < 0.05$ *vs* control group, $^cP < 0.05$ *vs* group IBS-D; ANOVA]; L: [Control group: n = 23, MRS 2500 treated control group: n = 12, IBS-D group: n = 17, MRS 2500-treated IBS-D group: n = 16. $^aP < 0.05$ *vs* control group, $^cP < 0.05$ *vs* group IBS-D; ANOVA], EFS: Electrical field stimulation; IBS-D: Irritable bowel syndrome; PA: Patchouli alcohol.

0.05). To observe the effect of PA on the isolated colonic segment contraction induced by high extracellular KCl level, we tested the peak contractile responses of the tissues that were pretreated with PA (100 μmol/L, 1 min) before KCl administration (120 mmol/L) at the end of each experiment. KCl- and PA + KCl-treated control groups showed no significant differences ($P > 0.05$). However, in comparison with the KCl-treated IBS-D group, the contractile responses (mg contractions) of the PA + KCl-treated IBS-D group (11.87 ± 3.34) showed significant decrease in the peak tension ($P < 0.01$) (Figure 3).

Inhibitory effects of PA on cholinergic contractile responses of the isolated IBS-D rat colonic longitudinal Smooth muscle

Cholinergic neurotransmission produced an inverse alteration in the colon and jejunum of IBS-D rats. The contractile force of the colon smooth muscle was significantly higher in the IBS-D group than in the control group ($P < 0.05$). EC_{50} in the IBS-D group (EC_{50} = 0.94 μmol/L) that was obtained in the presence of CCh was lower than that in the control group (EC_{50} = 2.1 μmol/L). Compared with the control group, the contractile force of the jejunal smooth muscle was significantly lower in the IBS-D group ($P < 0.05$). EC_{50} in the IBS-D group (EC_{50} = 1.82 μmol/L) that was obtained in the presence of CCh was higher than that in the control group (EC_{50} = 0.78 μmol/L). The CCh-induced contraction in the colon was abolished by the pretreatment with a single concentration of PA (100 μmol/L). The IBS-D colon pretreated at a single PA concentration of 100 μmol/L significantly decreased the tension of CCh-induced (1×10^{-9} mol/L to 3×10^{-7} mol/L) contraction ($P < 0.01$) than that in the IBS-D group. Compared with the control group, the PA-pretreated control colon presented significantly decreased tension during the CCh-induced contraction (1×10^{-7} mol/L to 3×10^{-6} mol/L) ($P < 0.01$). However, at higher CCh concentrations, PA (100 μmol/L) treatment exhibited nonsignificant blocking effect on the colonic CCh-induced contractions, compared with the control group ($P > 0.05$). Similarly, compared with the control group, the pretreatment of IBS-D colon in 100 μmol/L of PA significantly decreased the tension of the CCh-induced contraction (1×10^{-9} mol/L to 3×10^{-8} mol/L) ($P < 0.05$); it also showed no significant difference at higher CCh concentrations of 1×10^{-7} mol/L to 1×10^{-5} mol/L ($P > 0.05$) (Figure 4).

Inhibitory effects of PA on the EFS-induced nitrergic contractile responses of the isolated colonic longitudinal smooth muscle in IBS-D rats

Compared with the control group, the EFS-induced contraction significantly increased in the colonic longitudinal smooth muscle in the IBS-D group at 2 Hz to 30 Hz ($P < 0.05$). Compared with IBS-D group, the EFS-induced contraction in the L-NAME-treated (10 μmol/L) control group presented no significant difference ($P > 0.05$). This result indicated higher tone in the L-NAME-induced contraction of IBS-D rat colon than in the untreated colon. Moreover, the L-NAME-mediated effect on EFS was abolished by the pretreatment with PA in both the control and IBS-D colon specimens. The EFS-induced contractions were significantly decreased by the coapplication of PA

(100 μmol/L) and L-NAME in the IBS-D colon at 5 Hz to 30 Hz, compared with that in the L-NAME-treated IBS-D group ($P < 0.05$). Similarly, the EFS-induced contractions were significantly decreased by the co-application of PA (100 μmol/L) and L-NAME in the control colon at 5 Hz to 30 Hz, compared with that in the L-NAME-treated control group ($P < 0.05$) (Figure 5).

Effects of PA on the P2Y$_1$ receptor antagonist during the EFS-induced contraction and ATP level of the isolated colonic longitudinal smooth muscle from IBS-D rats

EFS-induced contractions of the colonic longitudinal smooth muscle were significantly decreased by the P2-purinoceptor agonist α,β-MeATP (100 μmol/L) in the IBS-D colon at 2 Hz to 30 Hz compared with that in the untreated IBS-D group ($P < 0.01$). Meanwhile, no significant difference was observed in the EFS-induced contractions between the PA-treated (100 μmol/L) IBS-D and α,β-MeATP-treated IBS-D groups ($P > 0.05$). The EFS-induced contractions were significantly decreased by the coapplication of PA (100 μmol/L) and P2Y$_1$ receptor antagonist MRS 2500 in the IBS-D colon at 20 and 30 Hz compared with that in the IBS-D group treated with MRS 2500 only (1 μmol/L) ($P < 0.05$). The EFS-induced contraction was significantly decreased by MRS 2500 relative to that in IBS-D group (1 μmol/L) ($P < 0.01$). The ATP assay yielded the following results. No significant difference was detected in the ATP levels between the IBS-D and control groups ($P > 0.05$). However, α,β-MeATP significantly increased the ATP level of the IBS-D rat colon compared with those of the control ($P < 0.05$) and IBS-D groups ($P < 0.05$). MRS 2500 significantly increased the ATP level of the control rat colon compared with that in the control ($P < 0.05$) and IBS-D groups ($P < 0.05$). The ATP level did not show significant difference between the MRS 2500-treated and untreated IBS-D groups ($P > 0.05$) (Figure 6).

DISCUSSION

Due to the lack of standard treatment algorithm for IBS-D and the limited drugs used to alleviate the symptoms instead of the treatment of disease, alternative therapeutic medications for patients with IBS-D are urgently needed. Pogostemonis Herba is a vital aromatic damp-resolving agent that is often prescribed to treat vomiting and diarrhea. Previous studies[16,17] have demonstrated the antidiarrheal effects of Pogostemonis Herba. PA is a major active ingredient of this herbal medicine, which exhibits notable bioactivities and involves wide applications. The present study aimed to investigate the neurogenic response of PA on an isolated stress-induced IBS-D rat model to identity the potential mechanisms of PA in IBS-D treatment. Given that IBS-D is closely related to motility disorders of the colon rather than that of the small intestine[18,19], The present study focused on the colonic contractile responses.

ENS coordinates the movement patterns in the gastrointestinal tract[20]. The receptors on the neurons and neuroglia in ENS mediate the essential gastrointestinal functions[21,22], including gastrointestinal muscle control. Our study clearly demonstrated that PA (3×10^{-7} mol/L to 1×10^{-4} mol/L) causes concentration-dependent relaxation of the spontaneous contraction of the colonic longitudinal smooth muscles *in vitro* at EC_{50} of 41.9 μmol/L. This study is the first to directly demonstrate the inhibition of PA in rat colon motility. However, the mechanism of this acute response to PA remains unknown. Thus, we further investigated whether this acute response to PA was due to the alteration of the neurotransmissions in ENS using the neuropharmacological and electrophysiological technologies to contract the isolated longitudinal smooth muscles of rat colons. We also determined whether this property of PA could be applied to regulate the neurotransmission disorders in IBS-D rats.

The voltage-gated potassium channel (VGKC) function is determined by the membrane potential. The increased extracellular potassium levels result in the depolarization of the intestinal smooth muscle cells through decreasing the resting membrane potential, followed by muscle contractions at the suprathreshold stimulus. VGKCs are primarily regulated by extracellular drugs and neurotransmitters[23]. Previous studies have shown that the release of neurotransmitters, such as nitric oxide (NO) or acetylcholine, may be induced by KCl at concentrations higher than 60 mmol/L[24]. High sensitivity to KCl-induced contractions was noted in our IBS-D rat colons, which is consistent with a previous study[25]. In the present study, the contractile responses induced by high extracellular KCl levels (120 mmol/L) were abolished by PA in the IBS-D rat colon segments. This result suggests that PA may have functions similar to potassium channel blockers. Although further study is required to confirm this assumption, the involvement of the PA-mediated hyperpolarization on the colonic relaxation of IBS-D rat colons has been shown.

Under normal conditions, the enteric smooth muscle, which is innervated by the autonomic nervous system, contracts and relaxes rhythmically[26]. Previous studies confirmed that the colon motility dysfunction and abnormal neurotransmission changes[27] are important mechanisms underlying the IBS-D development. Neuropathic changes in ENS most possibly generated the symptomatic bowel diseases, including IBS-D[28]. In ENS, the cholinergic excitatory motor neurons exert excitatory effects on the gastrointestinal tract. PA inhibited the CCh-induced contractions; thus, PA may regulate acetylcholine transmission by antagonizing the muscarinic receptor or nicotinic acetylcholine receptor. Cholinergic nerves were excited in colons of stress-induced IBS-D rats, which is consistent with the published data[29]. Pretreatment with a single PA concentration strongly inhibited the CCh-induced (1 ×

10^{-9} mol/L to 3×10^{-7} mol/L) contractions in the IBS-D rat colon *in vitro*. Our study is the first to demonstrate that PA exerts an inhibitory effect on the cholinergic nerves of the IBS-D rat colons. However, whether this inhibitory effect against the acetylcholine operates on the muscarinic receptors, nicotinic acetylcholine receptors, or both, needs further research. In contrast, the isolated jejunum from IBS-D rats exhibited inhibitory effect on the cholinergic nerves. The wrap-restraint stress results in decreased small intestinal transit, whereas it resulted in increased large intestinal transit in rats[30]. Thus, these acetylcholine disorders may result from the wrap-restraint stress. However, further results are needed to confirm this finding. IBS-D treatment may also contribute to the cholinergic receptor-blocking effect of PA on the isolated colon but not the jejunum.

NO is a vital non-adrenergic, non-cholinergic inhibitory transmitter in the gastrointestinal tract. NO is involved in both the colonic relaxation and contractions[31]; however, it typically participates in the nitrergic relaxation. Subsequently, mechanisms of the nitrergic nerves in the gut remain unclear, and the modulation of the nitrergic and cholinergic transmission in the nerve-muscle pathway[32,33] requires further study. NO synthases utilize L-arginine and molecular oxygen to generate NO and L-citrulline. NO synthase inhibitor L-NAME significantly increases the basal tone and enhances the phasic contractions in the rat proximal colon[34]. Our results showed that the increased tone in the IBS-D rat colonic longitudinal smooth muscle in response to L-NAME during the EFS-induced contraction could be inhibited by PA. This observation suggests the lack of NO in the stress-induced IBS-D rat colon. In EFS, we observed that PA contributed to inhibit the contractions induced by the NO synthase inhibitor L-NAME (10 μmol/L) in the isolated rat colon tissues. This result indicates that PA may increase the synthesis of NO in rat colon. In the present study, we found that PA exerts a relaxation effect on the nitrergic nerves of the colonic longitudinal smooth muscle of IBS-D rats *in vitro*. In addition, PA may be a NO donor.

ATP is a purine inhibitory neurotransmitter that mediates the non-adrenergic, non-cholinergic relaxation in the gastrointestinal smooth muscles[35,36]. Studies showed that in wrap-restraint stress rat model, ATP participates in the intestinal motility[37]. However, the effect and mechanism of ATP in the IBS-D rat colon still need clarification. On the other hand, α,β-MeATP is a P2-purinoreceptor agonist. Our results demonstrated that excess contraction of the IBS-D rat colon can be inhibited by α,β-MeATP. Under EFS conditions, PA produced an inhibitory effect on the coadministration of MRS 2500 (1 μmol/L) in the IBS-D rat colon. PA affected the muscle tone and caused ATP-induced relaxation, indicating that PA may be involved in the activating effect of ATP on the IBS-D rat colon. However, this observation requires further investigation. $P2Y_1$ receptor is involved in ENS control and coordination of intestinal motility[38]. $P2Y_1$ receptor functions as a receptor for extracellular ATP, which can be activated by the endogenous ligands of ATP and ADP. Recent studies suggested that highly potent and selective $P2Y_1$ receptor antagonist MRS 2500 inhibited the ATP-induced relaxation. However, when we further tested the effect of MRS 2500 on the rat longitudinal smooth muscle, 1 μmol/L of MRS 2500 produced a significant relaxation effect in the control rats *in vitro* but not in the stress-induced IBS-D rats. A previous work[39] reported an unexplained inhibitory effect of MRS 2500 (1 μmol/L) on spontaneous motility *in vitro*. Thus, ATP may play a complementary role in regulating colonic motility of IBS-D rats. In our continuing study, ATP assay results agreed with the conclusions mentioned above. Subsequently, we cannot explain the MRS 2500-induced relaxation of the nerve-mediated contraction elicited by EFS *in vitro*. Thus, additional *in vivo* and *in vitro* studies are necessary to clarify this phenomenon.

Electrophysiological methods were used for our neuropharmacological study. This method shows the effects of PA on the structural and physiological components of the biopsychosocial model of IBS-D; the latter may enable the development for clinical research and application. However, the following limitations must be considered. First, we cannot identify the pathway dominating the IBS-D colon response to PA treatment. These inhibitory effects by PA may be related to the changes in one upstream switch. Nevertheless, our results strongly confirmed the inhibitory effects of PA on the spontaneous, CCh-induced, and EFS-induced colonic contractions. Second, animal models and mechanisms of different IBS-D models vary. The wrap-restraint stress model mimics but cannot fully represent the physiological and pathological conditions of patients with IBS-D. Thus, whether the PA treatment is appropriate for other IBS-D models or patients requires further study. Third, the role of ATP in the wrap-restraint stress IBS-D model or the involvement of ATP in mechanism of PA treatment of IBS-D rats is unclear. Fourth, the nature of our study limits our conclusion because a direct correlation between colonic contractions and electrical signaling cannot be completely confirmed. However, the present study provides evidence regarding the involvement of PA in neuromediated relaxation of the longitudinal smooth muscle in rat colon.

In conclusion, PA exerts inhibitory effects on the IBS-D rat colon, which supports our hypothesis. In addition, related responses possibly involve cholinergic, nitrergic, and K^+ channel pathways. ATP may not be the dominant pathway for participation of PA in the colonic relaxation of the stress-induced IBS-D rats. PA is a potential new candidate to effectively treat IBS-D. The findings in this study may help extend the pharmacological applications of PA. PA may be responsible for the antidiarrheal effect of Pogostemonis

Herba. Additional *in vivo* and *in vitro* investigations on the effect of PA are needed, and potential pharmacological target protein for PA in treatment of IBS-D rat colon needs to be studied.

ARTICLE HIGHLIGHTS

Research background

Irritable bowel syndrome (IBS) is a prevalent functional bowel disorder that inflicts a significant socioeconomic burden and decreases the patient quality of life. In China, the percentage of patients with diarrhea-predominant IBS (IBS-D) is 74.1%. Therefore, the neuropsychological factors have gained much attention in the clinical and basic studies on IBS-D. However, a standard treatment algorithm has not been established for this condition. Pogostemonis Herba is used in Asian countries to treat functional gastrointestinal disorders; patchouli alcohol is the major active ingredient of Pogostemonis Herba. Previous studies have indicated that patchouli alcohol (PA) may participate in the neurotransmission regulation of the smooth muscles in the digestive system. However, no research on the pharmacological effects of PA in the neurotransmission regulation has been published.

Research motivation

This study aimed to investigate the effects of PA on the isolated IBS-D rat colon and its related mechanisms. The findings in this work can help extend the pharmacological applications of PA.

Research objectives

The main objective of this study was to test our hypothesis that the mechanism of PA in treatment of IBS-D is related to the drug regulation of the neural pathways in the enteric nervous system *via* its influence on the neurotransmitter release with focus on PA research. Additional *in vivo* and *in vitro* investigations on the effect of PA and the identification of the potential pharmacological target protein in PA to treat IBS-D rat colon are needed.

Research methods

In this *in vitro* study, the effect of PA on colonic spontaneous motility was studied using the cumulative log concentration (3×10^{-7} mol/L to 1×10^{-4} mol/L). Responses to CCh (10^{-9} mol/L to 10^{-5} mol/L) and neurotransmitter antagonists, including L-NAME (10 μmol/L), MRS 2500 (1 μmol/L), agonist α,β-methyleneadenosine 5'-triphosphate trisodium salt (100 μmol/L), and single KCl doses (120 mmol/L), were obtained from the proximal and distal colon segments of rats. Effects of blockers against the antagonistic responses by pretreatment with PA of 100 μmol/L for 1 min were also assessed. enteric nervous system (40 V, 2 Hz to 30 Hz, 0.5 ms pulse duration, and 10 s) was performed to observe the nonadrenergic, noncholinergic neurotransmitter release in the IBS-D rat colon. Moreover, the ATP level of Kreb's solution was also determined.

Research results

In this study, PA exerted a concentration-dependent inhibitory effect on the spontaneous contraction of the colonic longitudinal smooth muscle. Pretreatment of PA could inhibit the peak tension of high extracellular concentration of the KCl-induced contraction of the IBS-D rat colon. The cholinergic contractile response in the colonic smooth muscle of IBS-D rat, which was induced by CCh, was reduced by the pretreatment of PA. Lack of nitrergic neurotransmitter, which was released in the stress-induced IBS-D rat, showed contraction effects on the colonic smooth muscle. Pretreatment of PA resulted in the relaxant effects on the L-NAME-induced contraction. Thus, ATP may not be the main neurotransmitter involved in the inhibitory effects of PA in the colonic relaxation of the stress-induced IBS-D rats. Therefore, these results indicated that PA played a role in the neurotransmission in ENS of colon, which may help extend the pharmacological applications of PA.

Research conclusions

PA exerts inhibitory effects on the IBS-D rat colon, which supports our hypothesis. In addition, related responses possibly involve cholinergic, nitrergic, and K^+ channel pathways. ATP may not be the dominant pathway for participation of PA in the colonic relaxation of the stress-induced IBS-D rats. PA is a potential new candidate to effectively treat IBS-D. The findings in this study may help extend the pharmacological applications of PA. PA may be responsible for the antidiarrheal effect of Pogostemonis Herba. Additional *in vivo* and *in vitro* investigations on the effect of PA are needed, and potential pharmacological target protein for PA in treatment of IBS-D rat colon needs to be studied.

Research perspectives

Our results strongly confirmed the inhibitory effects of PA on the spontaneous, CCh-induced, and EFS-induced colonic *in vitro* contractions. PA acts as a neurotransmitter agent in ENS, and is thus considered to be a new treatment option for IBS-D. However, more questions will be addressed in the future studies. For instance, the pathways dominating the IBS-D colon in response to PA treatment; and the structural and functional changes in the potential target proteins under the effect of PA. The relaxation effects of PA may be related to changes in one upstream switch. Our further studies will focus on the effect of PA on the potential target proteins in IBS-D rats both *in vivo* and *in vitro*. Patch-clamp methods will be used to measure the K^+ current, and immunofluorescence will be used to investigate the expression and colocalization of the target proteins. Furthermore, Western blot and qPCR will be performed to evaluate the expression of the target proteins.

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ORIGINAL ARTICLE

Basic Study

Recombinant expressed vasoactive intestinal peptide analogue ameliorates TNBS-induced colitis in rats

Chun-Lan Xu, Yu Guo, Lei Qiao, Li Ma, Yi-Yi Cheng

Chun-Lan Xu, Yu Guo, Lei Qiao, Li Ma, Yi-Yi Cheng, The Key Laboratory for Space Bioscience and Biotechnology, School of Life Sciences, Northwestern Polytechnical University, Xi'an 710072, Shaanxi Province, China

ORCID number: Chun-Lan Xu (0000-0002-0139-5046); Yu Guo (0000-0002-1652-5739); Lei Qiao (0000-0003-2907-7629); Li Ma (0000-0002-0192-6847); Yi-Yi Cheng (0000-0003-2684-0588).

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Correspondence to: Chun-Lan Xu, PhD, Associate Professor, The Key Laboratory for Space Bioscience and Biotechnology, School of Life Sciences, Northwestern Polytechnical University, 127 Youyixi Road, Xi'an 710072, Shaanxi Province, China. clxu@nwpu.edu.cn
Telephone: +86-29-88460543
Fax: +86-29-88460332

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Abstract

AIM

To investigate the modulatory effect of recombinant-expressed vasoactive intestinal peptide (VIP) analogue (rVIPa) on trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats.

METHODS

Forty-eight rats were randomized into six groups: normal control group (Control), model control group (TNBS), ethanol treatment group (ETOH), and VIP treatment groups with different dosage ($rVIPa_{1nmol}$, $rVIPa_{2nmol}$, $rVIPa_{4nmol}$). Diarrhea and bloody stool were observed. Colonic damage was evaluated histologically. The levels of tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), myeloperoxidase (MPO) and endotoxin in colonic tissue and serum were determined by enzyme-linked immunosorbent assay (ELISA). The expression of occludin, ZO-1, Toll-like receptor 4 (TLR4),

and nuclear factor-kappa B p65 (NF-κB p65), IκBα, and p-IκBα were detected by Western blot.

RESULTS

Administration with 2 nmol rVIPa prevented TNBS-induced necrosis, hyperemia, swelling, inflammation, *etc.*, pathologic changes observed in the inner surface of colon in experimental rats. Moreover, rVIPa significantly decreased colonic TNF-α level ($P < 0.001$), MPO activity ($P < 0.001$) and serum endotoxin level ($P < 0.01$), and remarkably increased colonic IL-10 content ($P < 0.001$) in rats with TNBS-induced colitis. Furthermore, compared to the TNBS-induced colitis group, 2 nmol rVIPa treatment up-regulated the levels of occludin ($P < 0.05$) and ZO-1 ($P < 0.05$), NF-κB p65 ($P < 0.01$) and IκBα ($P < 0.001$), and down-regulated the levels of TLR4.

CONCLUSION

rVIPa ameliorates TNBS-induced colonic injury and inflammation and effectively protected the intestinal mucosal barrier function in rats. The mechanism may be related to TLR4/NF-κB-mediated signaling pathway. rVIPa could be used as a new alternative therapy for intestinal inflammatory disorders.

Key words: Vasoactive intestinal peptide; Intestinal mucosal barrier; Tight junction; Toll-like receptors; Recombinant expression

Core tip: Vasoactive intestinal peptide (VIP) is a neuropeptide with potent anti-inflammatory activities. Recombinant VIP analogue (rVIPa with amino acid sequence "HSKAVFTKNYTRLRKQMAVKKYLNSILN") with higher antimicrobial activity and stability than natural peptide was produced by an effective and low-cost production method. The current study first indicated that rVIPa could alleviated TNBS-induced colitis *via* TLR4/NF-κB-mediated signaling pathway. In summary, these results suggest a protective role and anti-inflammatory effect of rVIPa in inflammatory bowel disease and indicate that rVIPa has therapeutic potential for intestinal inflammatory disorders. The study contributes to identify and produce novel anti-inflammatory agents from human innate host defense mechanisms in the process of biological evolution.

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INTRODUCTION

Crohn's disease (CD) belonging to a type of inflammatory bowel disease (IBD), is a group of chronic and relapsing intestinal inflammatory disorders[1]. A proportion of patients with CD given the currently available medical therapies remains incurable[2]. CD causes transmural inflammation of the gastrointestinal wall. Furthermore, prolonged inflammation of the intestinal tract affects the patients' quality of life and increases the risk of colorectal cancer development[3]. Rapid increase of multidrug-resistant pathogenic microorganisms makes it urgent to research and develop promising and alternative anti-inflammatory molecules. Endogenous bioactive peptides with remarkably structural and functional diversity represent an emerging and potential anti-inflammatory agents[3,4].

Vasoactive intestinal peptide (VIP) is considered a paradigm as an endogenous neuroendocrine-immune mediator with therapeutic potential for a variety of inflammatory disorders such as IBD[5-7]. Previous studies indicated that VIP exerts a lines of important modulatory effects in intestinal pathophysiology, including CD[8-10]. VIP can inhibit the neurodegeneration induced by the loss of neurons, which may be mediated by glial cells through the production of neurotrophic factors and the inhibition of proinflammatory mediators[11]. VIP is also recognized to belong to host defense peptides[12,13]. Furthermore, VIP is a small and linear peptide. VIP as an endogenous hormone, can exhibit efficient adjustment effect for a low dose. These advantages suggest that VIP possesses a promising potential for developing as a novel and safe anti-inflammatory agent.

However, due to several shortcomings such as instability, low efficient and expensive production methods[14,15], VIP has not obtained widely application at present. Therefore, in order to promote the development and application of VIP, choosing the VIP analogue with high stability and anti-inflammatory activity and establishing an effective, low-cost production strategy are very urgent. Previous research indicate that molecular modification of VIP enhances its antimicrobial activity and stability compared with the nature VIP[16]. For the effective and low-cost production method, recently, different recombinant expression strategies have been conducted to produce unique antimicrobial peptides such as fusion partner-mediated *Escherichia coli* expression system[17-19].

In our previous study, we obtained a kind of VIP analogue with an enhanced antimicrobial potency, no-hemolysis, and high stability through the rational design and scanning, and established an effective and low-cost genetic engineering production technology. However, it remains to be elucidated whether molecular modification and recombinant expression affect its natural anti-inflammatory activities or not. Therefore, the current study was conducted to investigate its anti-inflammatory activity, and the possible mechanism of VIP analogue produced by recombinant expression after rational design. The study contributes to better understanding of colitis and leads to advancement in novel treatment design.

MATERIALS AND METHODS

rVIPa and other reagents

rVIPa with amino acid sequence "HSKAVFTK-NYTRLRKQMAVKKYLNSILN" was prepared in our laboratory based on the recombinant expression and molecular modification of natural VIP. Moreover, its quality and purity were identified by high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS). The 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) was purchased from Sigma Chemical Co. (St.Louis, MO, United States). ELISA kits for tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), and myeloperoxidase (MPO) were purchased from R&D Systems (Minneapolis, MN, United States). Primary antibodies against ZO-1, occludin, TLR4, p-IκBα, IκBα, NF-κB p65 and β-actin, and peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, United States). Total superoxide dismutase (T-SOD) activity assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Bicinchoninic acid (BCA) protein assay kit was from Solarbio Life Sciences Co. (Beijing, China).

Experimental Animals

This experiment was approved by the Institutional Animal Care and Use Committee of the Northwestern Polytechnical University (permit Number: 2016014) and was conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals. Forty-eight adult male Sprague-Dawley (SD) rats (200 ± 20 g) were purchased from the Experimental Animal Center of Xi'an Jiaotong University. During the whole experimental period, rats were maintained in the Animal Experimental Center of Northwestern Polytechnical University at room temperature of 25 ℃, relative humidity of 50% and a 12 h light and dark cycle.

Induction of experimental colitis and administration of rVIPa

In the experiment, healthy male SD rats were assigned randomly to six groups: Normal control group saline and phosphate buffer solution (PBS), model group TNBS and PBS, positive control group (50% ETOH and PBS), and rVIPa groups (TNBS and rVIPa of 1 nmol, 2 nmol, and 4 nmol), with eight rats in each group. Colon colitis was induced rectally by TNBS, as in the following description[20]. First, TNBS was dissolved in 50% ethanol while all experimental rats fasted for 24 h. Then, SD rats from the TNBS-induced model groups were anesthetized and administered with 20 mg (in 250 μL of 50% ethanol) of TNBS *via* a 15-cm (diameter 2.0 mm) length of rubber infusion tube advanced 8 cm in the anus. The rats were seized by the tail for duration of 30s to ensure uniform contact with colonic mucosa. Following the same protocol, positive control animals were given 250 μL 50% ethanol. Controls were given the same volume of normal saline. Rats from the rVIPa treatment groups were given 500 μL rVIPa with concentration of 1 nmol, 2 nmol, or 4 nmol intraperitoneally (*i.p.*) every other day after TNBS induction of colitis until the end. Non-rVIPa-treated rats were administered the same volume of PBS. The period of animal experiment lasted 9 d. The animals' weight, diarrhea, and rectal bleeding were observed and recorded daily.

Histological assessment of colon pathomorphology damage

The rats were anesthetized with ether and the abdomen was opened. The entire colon was immediately removed and the total length was measured. Then the colon was split longitudinally, and gently washed with normal saline. Histologicals assessment of colonic damage was according to the following scoring criteria: 0, no ulcer and no inflammation; 1, no ulcer and local hyperemia; 2, ulcer and no inflammation; 3, ulceration and inflammation at only one site; 4, ulceration and inflammation at two or more sites; 5, area of ulceration extending more than 0.5 cm.

Measurement of MPO and T-SOD activity in serum and colon tissue

After the experimental rats were anesthetized with isoflurane, blood samples were collected by cardiac puncture and used for preparing serum according to general method. Then, 20 mg colon tissue was homogenized in 200 μL ice-cold RIPA lysate with 2 μL PMSF with a tissue homogenizer. The supernatant of homogenates was collected by centrifuging with 10000 *g* at 4 ℃ for 5 min. All samples of serum and supernatant of colon homogenates were stored at -80 ℃ until further analysis. MPO and SOD activities were determined by corresponding assay kits. The absorbance was measured by using a microplate reader (Synergy2, BioTek Instruments, Inc., Winooski, VT, United States) at 450 nm.

Determination of Cytokine level and endotoxin in serum and colon tissue

TNF-α, IL-10, and endotoxin level in serum and colon tissue were measured by specific rat ELISA assay kits according to the manufacturer's instructions, respectively.

Expression of occludin, TLRs and NF-κB analyzed by Western blot

Supernatant of colon was prepared according to the following method. Protein concentration in the supernatant was measured by BCA kit. Approximately 40 μg protein from each sample was loaded to the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. After being blocked for 2 h in Tris-buffered saline/Tween 20 (TBST,

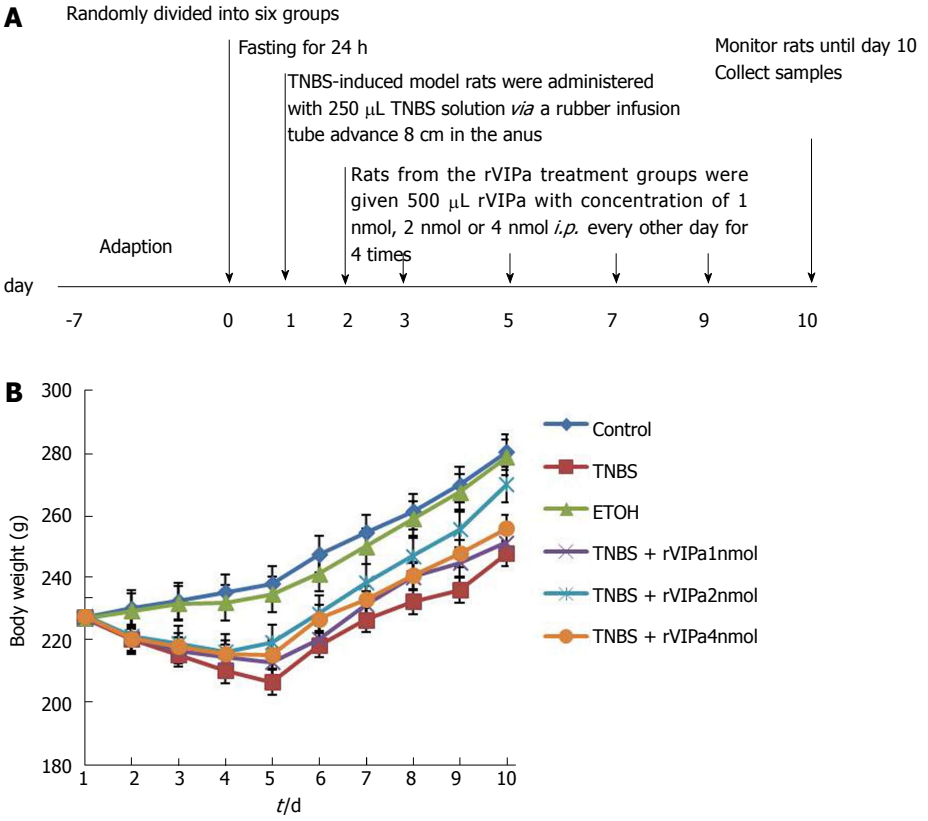

Figure 1 Administration of rVIPa inhibited the pathogenic effects and body weight loss caused by TNBS. A: The experimental scheme of induction of colitis and administration of rVIPa; B: Body weight of rats between days 1-10. rVIPa: Recombinant expressed vasoactive intestinal peptide analogue; TNBS: Trinitrobenzene sulfonic.

2 mmol/L Tris-HCl, pH 7.6, 13.7 mmol/L NaCl, and 0.1% Tween 20) containing 5% skimmed milk, membrane were incubated with primary antibodies TLR4 (1:500), ZO-1 (1:1000), occludin (1:1000), p-IκBα (1:1000), NF-κB p65 (1:1000), IκBα (1:1000), and β-actin (1:500) at 4 ℃ overnight. Subsequently, the membrane was incubated with the secondary antibody. The immunoreactive protein bands were visualized using a Clarity Western ECL substrate kit (BioRad, CA, United States) with HRP-conjugated secondary antibody for film-based imaging. Denditometry analysis of protein bands from scanned-ray films were performed using Scion Image software (Scion Corp., Frederick, MD, United States). The values were normalized against the intensity of β-actin.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and by using Student's *t* test (SPSS 19.0, Chicago, IL, United States). All data are shown as mean ± standard error of mean (SEM), and differences were considered to be significant at $P < 0.05$.

RESULTS

Body weight and other symptoms of experimental rats

The experimental scheme of induction of colitis and administration of rVIPa is shown in Figure 1A. During the whole period, the activity, diet, stool traits, and body weight of each group of rats were observed. TNBS-treated-alone colitis rats exhibited bradykinesia, reduced food intake, profound and sustained body weight loss, and diarrhea. Moreover, compared to the normal control and vehicle-treated rats, the feces from TNBS-treat-colitis rats contained blood and mucus. Conversely, moderate concentration of rVIPa (2 nmol) significantly ameliorated the TNBS-induced physiopathological effects and improved the TNBS-induced body weight loss in contrast with the untreated group or other concentrations of rVIPa-treated groups (as shown in Figure 1B).

Histological assessments

As shown in Figure 2A, histological examination demonstrated a regular morphology on normal rats. Compared to the normal control or ethanol-treated controls, the macroscopic assessment of colon tissue exhibited superficial bleeding and erosion, necrosis, hyperemia, and inflammation in the mucosa. Treatment with 2 nmol or 4 nmol rVIPa effectively relieved the pathological symptoms and prevented the hyperemia and inflammation in the experimental rats'colon (Figure 2B). Moreover, 2 nmol rVIPa inhibited TNBS-induced decrease of colon length (Figure 2C).

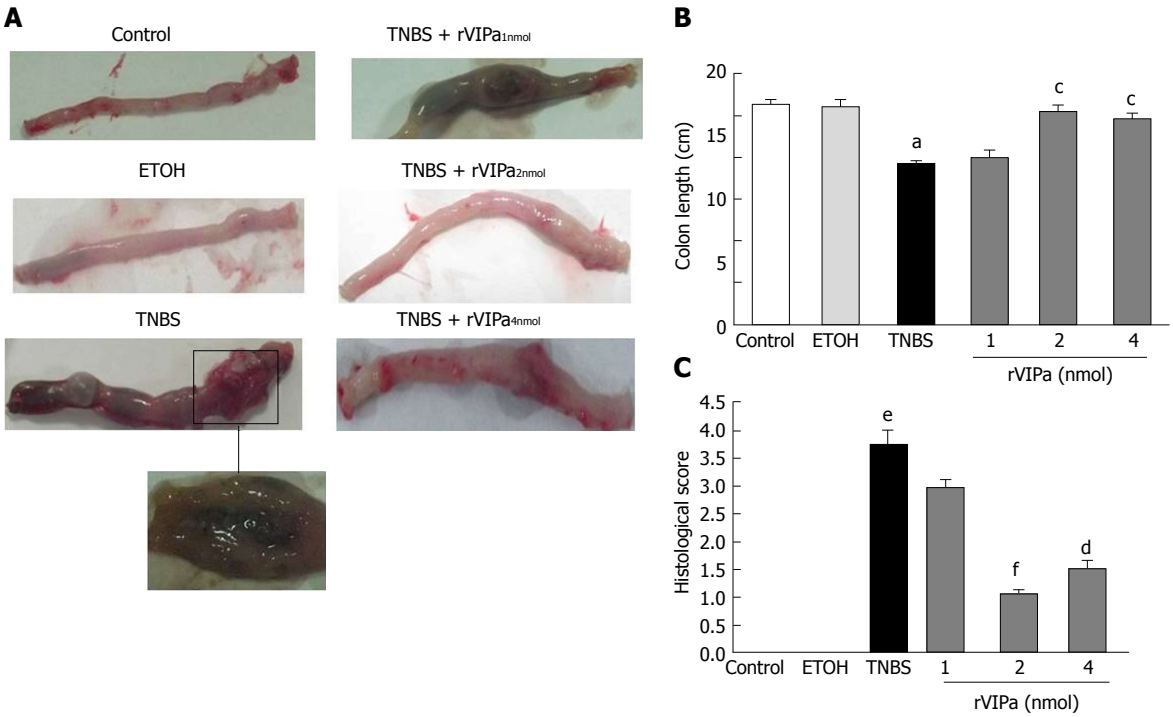

Figure 2 Histological observation and evaluation of colon tissue. A: Visual inspection of TNBS-induced colitis of colon tissue in rats and effect of rVIPa with different concentrations; B: Histologic score; C: Colon length. Data represent mean ± SEM (n = 8). [a]P < 0.05, [e]P < 0.001 *vs* control rats; [c]P < 0.05, [d]P < 0.01, [f]P < 0.001 *vs* TNBS-induced model rats.

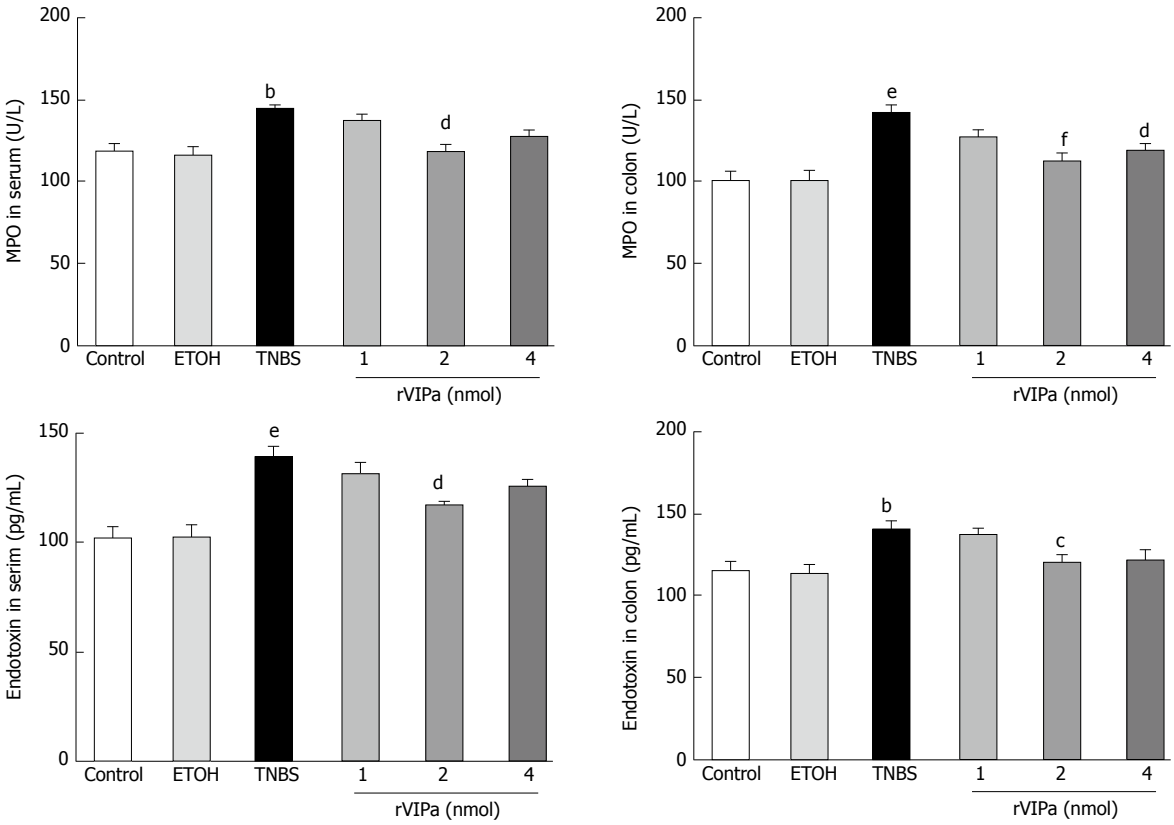

Figure 3 Myeloperoxidase and total superoxide dismutase activities in serum and colon tissue. Data represent mean ± SEM (n = 8). [b]P < 0.01, [e]P < 0.001 *vs* control rats; [d]P < 0.01, [f]P < 0.001 *vs* TNBS-induced model rats. MPO: Myeloperoxidase; T-SOD: Total superoxide dismutase; rVIPa: Recombinant expressed vasoactive intestinal peptide analogue; TNBS: Trinitrobenzene sulfonic.

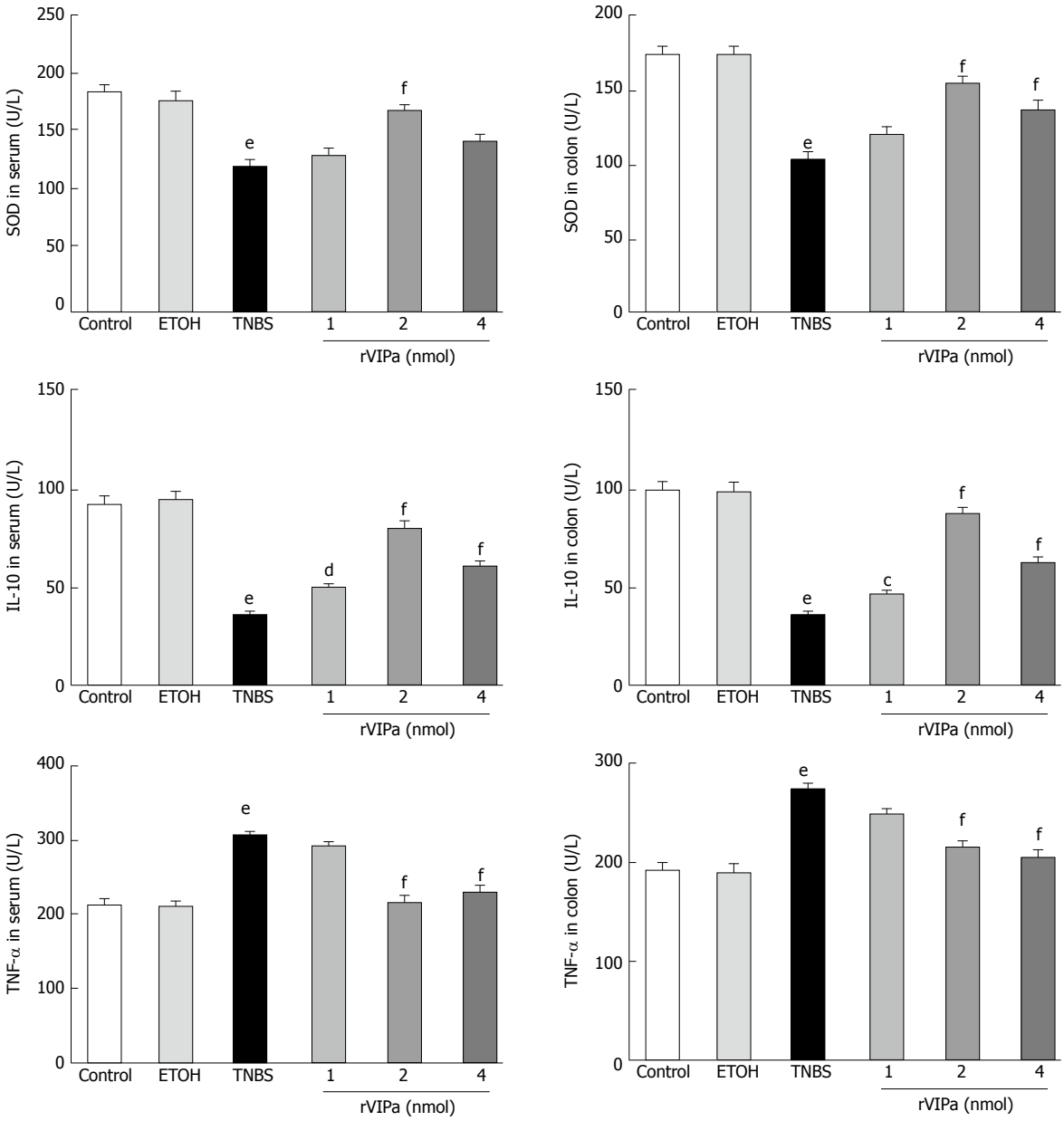

Figure 4 TNF-α, IL-10, and endotoxin level in serum and colon tissue. Data represent mean ± SEM (n = 8). $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* Control rats; $^{c}P < 0.05$, $^{d}P < 0.01$, and $^{f}P < 0.001$ *vs* TNBS-induced model rats. TNF-α: Tumor necrosis factor; IL-10: Interleukin-10; rVIPa: Recombinant expressed vasoactive intestinal peptide analogue; TNBS: Trinitrobenzene sulfonic.

MPO and T-SOD activity in serum and colon tissue

As shown in Figure 3, the current results showed that compared with the normal control and ethanol-treated control rats, colon TNBS treatment significantly increased the activity of MPO. However, 2 nmol rVIPa treatment significantly inhibited the TNBS-induced increase of MPO activity in colon tissue. At the same time, we examined the T-SOD activity in serum and colon tissue. The results showed that compared with the normal control and ethanol-treated rats, administration with TNBS significantly reduced the T-SOD activity in serum and colon tissue. In contrast, 2 nmol rVIPa treatment obviously relieved the TNBS-induced increase of the T-SOD activity in serum and colon tissue.

TNF-α, IL-10, and endotoxin level in serum and colon tissue

As shown in Figure 4, the levels of TNF-α and endotoxin in the serum and colon of TNBS-treated group were apparently higher than those in the normal control and vehicle groups. However, the level of anti-inflammatory factor IL-10 in the serum and colon of TNBS-treated model rats was significantly lower than that in the normal control and vehicle groups. Conversely, compared with the TNBS-treated group, 2 nmol and 4 nmol rVIPa treatment reduced the level of TNF-α and endotoxin, and increased the level of IL-10 in the serum and colon.

The expression levels of tight junction protein

The results of intestinal tight junction (TJ) protein-

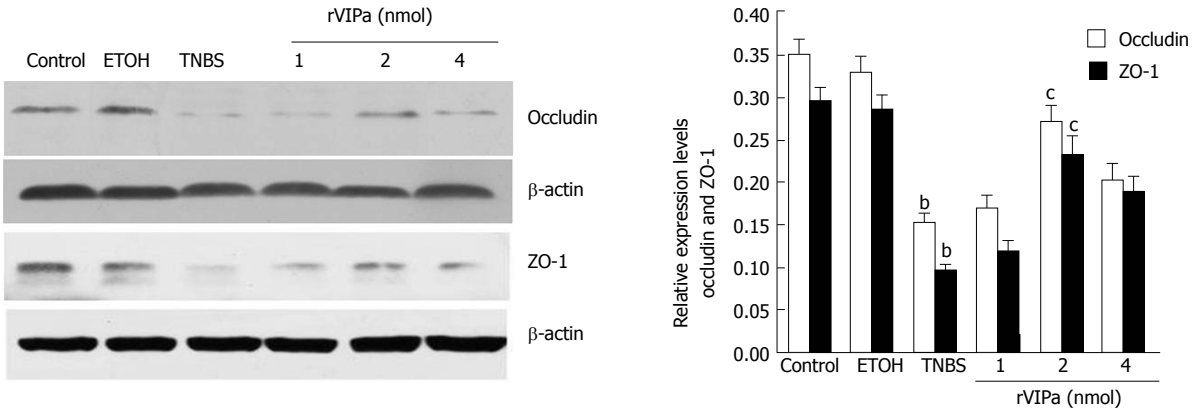

Figure 5 Protein expression level of occludin and ZO-1 in colon. Data represent mean ± SEM (n = 8). $^{b}P < 0.01$ *vs* control rats; $^{c}P < 0.05$ *vs* TNBS-induced model rats. rVIPa: Recombinant expressed vasoactive intestinal peptide analogue; TNBS: Trinitrobenzene sulfonic.

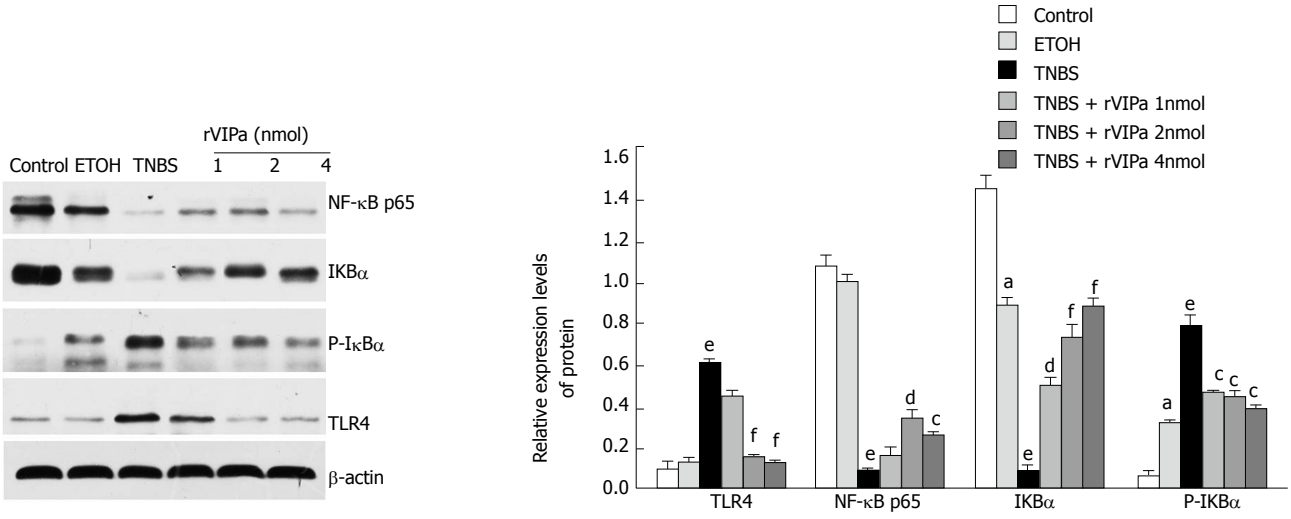

Figure 6 Effects of rVIPa with different concentration on TLR4/NF-κB signaling proteins expression level in colon tissue. Data represent mean ± SEM (n = 8). $^{b}P < 0.01$ *vs* control rats; $^{c}P < 0.05$ *vs* TNBS-induced model rats. rVIPa: Recombinant expressed vasoactive intestinal peptide analogue; TLR4: Toll-like receptor 4; NF-κB: Nuclear factor of kappa B; rVIPa: Recombinant expressed vasoactive intestinal peptide analogue; TNBS: Trinitrobenzene sulfonic.

occludin and ZO-1 expression levels determined by Western blot analysis were shown in Figure 5 and demonstrated that compared with the normal control rats, occludin and ZO-1 expression were decreased in the TNBS-treated rats. Conversely, 2 nnol rVIPa inhibited the above effect. However, relatively low and high concentrations of rVIPa did not exhibit protective effects on the intestinal barrier function damage.

Expression of TLRs/NF-κB signaling pathway

As shown in Figure 6, compared with the control rats, the protein level of TLR4 in TNBS-induced colitis was increased. However, 2 nmol rVIPa treatment significantly down-regulated the level of TLR4 during the TNBS-induced colitis. NF-κB is an important regulator of inflammation-related gene expression. Moreover, compared with the normal control, TNBS treatment significantly down-regulated NF-κB p65 and IκBα expression levels, and up-regulated the expression level of p-IκBα in colon tissue. NF-κB p65 and IκBα expression levels in the rVIPa treatment groups were higher than those in the model group, which suggested that rVIPs may exhibit anti-inflammatory efficiency *via* TLRs/ NF-κB signaling pathway.

DISCUSSION

IBD mainly includes CD and ulcerative colitis (UC), which affect quality of life for patients, and even give rise to the risk of enteric cancers. The lack of effective prevention and therapeutic measures are largely due to the pathogenesis of IBD, which is poorly understood. VIP is a neuroimmunopeptide concerned with homeostasis and health. Considerable lines of data suggested that VIP may be a potential candidate which can be developed to treat inflammatory diseases including CD[5-7]. Moreover, the VIP-receptor plays a key role in the modulatory effect of VIP on IBD[5].

As a neuroendocrine mediator, VIP possesses a wide range of biological functions. However, some disadvantages limit its practical application. Therefore, in our previous research, we designed a VIP analogue,

via amino residue substitution ($Asp^3 \rightarrow Lys^3$, $Asp^8 \rightarrow Lys^8$), with safety, favorable stability and improved antimicrobial activities, and established a green, efficient and low-cost production technology *via E. coli*-recombinant expression system[21]. However, it remains to be elucidated whether the molecular modification and recombinant generation affect its anti-inflammatory activity or not. TNBS-induced colitis shows the similar pathological features to the human CD[22,23]. Therefore, an experimental acute CD model usually established by intrarectal administration of TNBS in mice or rats. In the current study, we investigated the role of rVIPa in TNBS-induced colitis. The results indicated that 2 nmol rVIPa effectively relieved body weight loss, pathological symptoms and prevented hyperemia and inflammation in the colon tissue in the experimental rat model of colitis. This study first verified the the beneficial effect of rVIPa on colitis *via in vivo* experiment. Notably, high concentration of rVIPa exhibited reduced anti-inflammatory effects. Therefore, as for rVIPa derived from an endogenous neurotransmitter belonging to the hormone family, dose is very crucial. In the treatment of IBD, the benefical effect of rVIPa on the damaged colon may be associated with its anti-inflammatory, antibacterial and immune-modulatory activities. In the TNBS-induced colitis rats, increase of TNF-α and endotoxin level and MPO activity, and decrease of IL-10 level and T-SOD activity were observed in the serum and colon tissue. Conversely, treatment with proper dose of rVIPa significantly suppressed the UC development and inflammatory response. VIP restores immune tolerance by down-regulation of the inflammatory response and by induction of regulatory T cells[15]. VIP protects against bacterial pathogen EPEC-induced epithelial barrier disruption and colitis, which may be associated with the inhibition of PKCε activation by VIP[24]. Previous research indicate that exogenous VIP alleviates symptoms of histopathology of TNBS-induced colitis, which may be related to the decrease of the inflammatory and T helper cell type 1 (Th1)-driven autoimmune components[25]. Impaired goblet cell development and obviously intestinal barrier dysfunction were observed in VIP knockout (KO) (VIP KO) mice[26]. Moreover, VIPKO enhanced the susceptibility of mice to DNBS and DSS-induced colitis. However, VIP-treated VIPKO mice restores the phenotype and effectively protects against DSS-colitis[7]. However, other research suggests that deficient in VIP reduces the pathology caused by TNBS-induced colitis in mice[27]. The role of VIP in colitis need further investigate.

Intestinal epithelial cells, as the important component of intestinal physical barrier, play an key role in resisting harmful substances such as pathogenic microorganism and toxin, *etc.*, and maintaining the intestinal homeostasis[28,29]. The intestinal epithelium barrier can be easily disrupted during gut inflammation such as UC or CD. Recent evidence suggests that it is the crucial pathological feature of IBD that colonic epithelial barrier dysfunction heightens bacterial translocation, and followed by stimulating inflammatory response in the colonic mucosa[30,31]. Moreover, impaired intestinal epithelium permeability contributes to ongoing bowel symptoms in patients with IBD and mucosal healing[32]. In the current study, we found that treatment with rVIPa increased the expression of occludin. Future studies may further reveal the key roles of the intestinal barrier function in the pathophysiology of IBD, which will contribute to explore alternative and targeted therapy methods[33].

Inflammatory molecules s have been considered as elementary inducing factors involved in the disruption of the intestinal TJs, and gut permeability and bacterial translocation[34]. TNF-α is recognized as one of the important proinflammatory cytokines. Treatment with rVIPa reduced the production of systemic and mucosal inflammatory factors and rescued the T colon injuries caused by TNBS and improved the expression of TJsi. TNF-α triggers the increase of intestinal epithelial permeability by disrupting TJs which is related to the MLCK up-regulation[35]. Moreover, NF-κB is involved in positively regulating MLCK as the positive upstream regulator[35]. Activation of NF-κB plays an important role in the intestinal inflammation[36], which is usually recognized as a central "switch" in the key initial steps of inflammation response. The present study also confirmed the idea. Compared with that of normal rats, up-regulation of NF-κB p65 and p-IκBα, and down-regulation of IκBα in inflammatory colonic tissue. Administration of rVIPa decreased the expression of NF-κB p65 and inhibited the degradation of IκBα.

VIP regulates the immune response *via* TLRs signaling pathway. VIP modulating TLR has been tested in the mucosal immune system using TNBS-induced colitis[37]. VIP exerts an important role in regulating the balance of proinflammatory and anti-inflammatory factors not only in the colon but also in mesenteric lymph nodes. TLRs mediates the expression regulation of inflammation-related genes in a cascade style[38]. The current study shows that rVIPa treatment inhibites TNBS-induced the up-regulation of TLR4, which suggests that rVIPa could regulate the inflammatory response by modulating TLR expression. The VIP/pituitary adenylate cyclase-activating peptide (PACAP) system is considered a potential candidate applicated to anti-inflamatory therapy as a neuroendocrine-immune mediator. Most data validate the therapeutic effect through the modulation of TLR2 and 4[39]. The protective effect of rVIPa is mediated by the down-regulation of inflammatory cytokines, which may be involved in TLRs/ NF-κB /VIP system.

In summary, the study indicated that the VIP analogue produced by recombinant expression significantly inhibited the progression of colitis, exhibiting a protective effect on the colon, which may be related to the inhibition of proinflammatory cytokines, up-regulated TJ expression, and promotion of anti-inflammatory cytokines *via* TLRs/NF-κB signaling

pathway. By modifying the structure of VIP, its anti-inflammatory activity could be further improved. This study provides a simple, low-cost strategy for identifying and producing a novel anti-inflammatory agent from human innate host defense mechanisms in the process of biological evolution.

ARTICLE HIGHLIGHTS

Research background

Crohn's disease (CD) is a type of inflammatory bowel disease (IBD) Prolonged inflammation of the intestinal tract affects the patients' quality of life and increases the risk of colorectal cancer development. Endogenous antimicrobial and immunoregulatory peptides represent an emerging category of therapeutic agents, are gaining considerable interest in the scientific community. VIP is a neuropeptide with potent anti-inflammatory activities. Recombinant expressed VIP analogue with higher antimicrobial activity and stability than natural peptide was produced by an effective and low-cost production method. The results indicated that rVIPa alleviated TNBS-induced colitis *via* TLR4/NF-κB-mediated signaling pathway. rVIPa could be used as a new alternative therapy for intestinal inflammatory disorders. The study contributes to advancement in novel treatment design.

Research motivation

At present, a proportion of patients with Crohn's disease could not obtain high efficacy of the available medical therapies. Therefore, it is urgent to develop new anti-inflammatory agents with high efficacy, safety and low cost. The natural biological peptides and their analogues will provide important and significant resources for the development of molecules that can block inflammatory pathways.

Research objectives

rVIPa with high antimicrobial activity and stability was produced by an effective and low-cost biotechnology. The current study was conducted to investigate the modulatory effect of rVIPa on colon in rats with TNBS-induced colitis. The study contributes to the development of a kind of new and novel therapeutic agent from endogenous bioactive peptides for IBD.

Research methods

The current study investigated the anti-inflammatory activity, and the possible mechanism of rVIPa through establishing acute colitis model in rats administrated of TNBS intrarectally. In addition to the body weight change, histological assessment, MPO and endotoxin analyzed by ELISA, and tight junction proteins levels analyzed by Western Blot, TLR4/NF-κB-mediated signaling pathway were also investigated.

Research results

The current study first find that VIP analogue produced by recombinant expression significantly ameliorates the colon injury and inflammation caused by TNBS in rats, and exhibits a protective effect on colitis. Moreover, administration with rVIPa inhibited proinflammatory cytokines, up-regulated tight junction proteins expression, and promotion of anti-inflammatory cytokines *via* TLRs/NF-κB signaling pathway.

Research conclusions

rVIPa alleviated TNBS-induced inflammation and effectively protected the intestinal mucosal barrier function in rats, which may be related to TLR4/NF-κB-mediated signaling pathway. These results suggested that rVIPa could be explored to a new alternative therapy for intestinal inflammatory disorders.

Research perspectives

By rational design and molecular modification, the antimicrobial and anti-inflammatory activity, and stability for endogenous bioactive peptides could be further improved. This study provides a simple, low-cost strategy for identifying and producing a novel anti-inflammatory agent from human innate host defense mechanisms in the process of biological evolution.

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ORIGINAL ARTICLE

Retrospective Study

Split-dose bowel preparation improves adequacy of bowel preparation and gastroenterologists' adherence to National Colorectal Cancer Screening and Surveillance Guidelines

Stacy Bartnik Menees, H Myra Kim, Philip Schoenfeld

Stacy Bartnik Menees, Division of Gastroenterology, University of Michigan Health System, Ann Arbor, MI 48105, United States

Stacy Bartnik Menees, Division of Gastroenterology, Ann Arbor Veterans' Administration Hospital, Ann Arbor, MI 48105, United States

H Myra Kim, Center for Statistical Consultation and Research, University of Michigan, Ann Arbor, MI 48105, United States

H Myra Kim, Department of Biostatistics, University of Michigan, Ann Arbor, MI 48105, United States

Philip Schoenfeld, Division of Gastroenterology, John D. Dingell VA Medical Center, Detroit, MI 48201, United States

ORCID number: Stacy Bartnik Menees (0000-0002-5891-4118); H Myra Kim (0000-0002-0604-8027); Philip Schoenfeld (0000-0002-7620-2878).

Author contributions: Menees SB conducted the data collection, analysis, and interpretation, and wrote and revised the manuscript; Schoenfeld P designed the research; Menees SB performed the research; Kim HM analyzed the data; Menees SB, Kim HM, and Schoenfeld P both contributed to draft revision.

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Data sharing statement: No additional data are available.

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Correspondence to: Stacy Bartnik Menees, MD, MSc, Division of Gastroenterology, University of Michigan Health System, 3912 Taubman Center, SPC 5362, Ann Arbor, MI 48105, United States. sbartnik@med.umich.edu
Telephone: +1-734-2323739

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Abstract

AIM

To quantify the impact of split-dose regimen on endoscopists' compliance with guideline recommendations for timing of repeat colonoscopy in patients with normal colonoscopy or 1-2 small polyps (< 10 mm).

METHODS

A retrospective chart review of all endoscopy reports was undertaken in average-risk individuals > 50 years old with a normal screening colonoscopy and 1-2 small polyps. Data were abstracted from two time periods, pre and post-split-dose bowel preparation institution. Main outcome measurements were recommendation for timing of repeat colonoscopy and bowel preparation quality. Bivariate analysis by χ^2 tests and Student's

t-tests were performed to assess differences between the two cohorts. Multivariable logistic regression was used with guideline consistent recommendations as the dependent variables and an indicator for 2011 cohort as the primary predictor.

RESULTS

Four thousand two hundred and twenty-five patients were included in the study; 47.0% (1987) prior to the institution of split dose bowel preparation, and 53.0% (2238) after the institution of split dose bowel preparation. Overall, 82.2% (n = 3472) of the colonoscopies were compliant with guideline recommendations, with a small but significantly increased compliance rate in year 2011 (83.7%) compared to year 2009 (80.4%, P = 0.005), corresponding to an unadjusted odds ratio of 1.25 (95%CI: 1.07-1.47; P = 0.005). Colonoscopies with either "Adequate" or "Excellent" had increased from 30.6% in year 2009 to 39.6% in year 2011 (P < 0.001). However, there was no significant difference in poor/inadequate category of bowel preparation as there was a mild increase from 4.6% in year 2009 to 5.1% in year 2011 (P = 0.50).

CONCLUSION

Split-dose bowel regimen increases endoscopists' compliance to guidelines in average-risk patients with normal colonoscopy or 1-2 small polyps.

Key words: Colorectal cancer screening; Bowel preparation; Colonoscopy; Average-risk

Core tip: We evaluated the impact of split-dose regimen on endoscopists' compliance with guideline recommendations for timing of repeat colonoscopy in patients with normal colonoscopy or 1-2 small polyps (< 10 mm). We retrospectively evaluated 4255 patients who underwent colonoscopy during two time periods, pre and post the institution of split-dose bowel preparation. We found that split-dose bowel regimen increased endoscopists' compliance to guidelines in average risk patients with normal colonoscopy or 1-2 small polyps. Additionally, bowel preparation quality with either "Adequate" or "Excellent" had increased between the two time periods.

Menees SB, Kim HM, Schoenfeld P. Split-dose bowel preparation improves adequacy of bowel preparation and gastroenterologists' adherence to National Colorectal Cancer Screening and Surveillance Guidelines. *World J Gastroenterol* 2018; 24(6): 716-724 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i6/716.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i6.716

INTRODUCTION

National guidelines state that average-risk 50+-year-old individuals who have normal screening colonoscopy should get a repeat colonoscopy in 10 years. However, physician recommendations do not always comply with guidelines. Krist *et al*[1] reviewed whether endoscopists' recommendations for patients undergoing colonoscopy for all indications adhered to published guidelines for follow-up recommendations. In only 64.9% of all reports, the endoscopist specified when retesting should occur. Recommendations were consistent with current guidelines in 36.7% of cases. However, Krist *et al*[1] did not account for patient bowel preparation at the time of the procedure in determining guideline consistent recommendation. Based on our previous research, bowel preparation was the single most important factor determining compliance by endoscopists for follow-up colonoscopies[2]. Patients with fair bowel preparation were 18.0 times (95%CI: 12.0-28.0) more likely to have recommendations inconsistent with guidelines compared to patients with excellent/good preparations.

In addition to association with guideline inconsistent recommendations, suboptimal colonoscopy preparation reduces adenoma detection rates (ADRs) and is a risk factor for incomplete colonoscopy[3-5]. To reduce the incidence of suboptimal bowel preparation, research has focused on the timing of the bowel preparation dosing in relation to the colonoscopy. The split-dosing regimen, where patients take a portion of the laxative the evening prior to colonoscopy and the other half on the day of colonoscopy, improves the bowel preparation quality. Studies have consistently shown that split-dose regimen is superior to administration of preparation on the day or night before the colonoscopy[6]. As early as 2009, the American College of Gastroenterology colorectal cancer (CRC) screening guidelines recommended institution of split-dose bowel preparation[7]. However, adoption of the split-dose bowel preparation has lagged due to providers' concern of patient compliance[8,9].

New quality measures assessing physician adherence to guidelines have been instituted by the Centers for Medicare and Medicaid Services (CMS) through the Physician Quality Reporting System (PQRS). For the PQRS, participating endoscopists will report the frequency of recommending repeat colonoscopy in 10 years after a normal colonoscopy in an average-risk patient. For participation, endoscopists will receive a small bonus in Medicare payments. In 2014, failure to report this resulted in a reduction in Medicare payments. Beside this economic factor, it is essential to guide CMS on what is an acceptable compliance rate for this quality measure (the frequency of recommending repeat colonoscopy in 10 years after a normal colonoscopy in an average-risk patient) and continue to assess the impact of bowel preparation on physician recommendation, particularly with split-

Table 1 Utilized colonoscopy preparations

Bowel preparation	Diet and fluid instructions	Traditional dosing (night before)	Split dose-bowel preparation
PEG, HalfLytely, NuLYTELY, or TriLyte,	Clear liquid diet for lunch and dinner; Ingest other clear liquids between doses of laxative.	Take 8 oz. every 15 min until it is gone between 12 pm and 6 pm.	Between 5 pm and 6 pm the night before colonoscopy, drink one (8 oz.) glass of laxative and continue drinking one (8 oz). glass every 15 min until 2l (64 oz.) of the preparation solution is gone; Drink the final 2l (64 oz.) of prep solution 5 h before the patient needs to leave for the procedure.
MoviPrep,	Clear liquid diet for lunch and dinner; Ingest other clear liquids between doses of laxative.	Take 8 oz. every 15 min until it is gone between 12 pm and 6 pm.	Between 5 pm and 6 pm, begin drinking the preparation; The MoviPrep container is divided by 4 marks; Every 15 min drink the solution down to the next mark (about 8 oz.), until the full liter has been consumed; Over the course of the evening, drink an additional 0.5 L of clear liquids; The next day, drink the final liter (32 oz.) of preparation solution 5 h before the patient needs to leave for the procedure.
MiraLAX (PEG 3350) /Gatorade	Clear liquid diet for lunch and dinner; Ingest other clear liquids between doses of laxative.	Take 2 tablets of bisacodyl between 12 pm and 6 pm, 4 h later take 8 oz. of the MiraLAX/Gatrade mixed in 2 liters of Gatorade every 15 min until gone.	At 12 noon, take 2 Dulcolax tablets; Between 5 pm and 6 pm, drink one (8 oz.) glass of the Miralax/Gatorade solution and continue drinking one (8 oz.) glass every 15 min thereafter until half the mixture (32 oz.) is gone. The next day, drink the final liter (32 oz.) of preparation solution 5 h before the patient needs to leave for the procedure.
OsmoPrep	Clear liquid diet for lunch and dinner; Ingest other clear liquids between doses of laxative.	Take first set of 20 tablets between 12 noon and 6 pm; Take a dose of 4 tablets every 15 min with at least 8 ounces of clear liquid; The second set of 12 tablets was to be taken 10-16 h after in the same manner as described above.	Between 5 and 6 pm take 4 tablets with 8 oz. of any clear liquid every 15 min; The patient will take a total of 20 tablets and drink 40 oz. of clear liquids over a 1 h period. The next day, 5 h before the patient needs to leave for your procedure, take 4 tablets with 8 oz. of any clear liquid every 15 min; The patient will take a total of 12 tablets and drink 24 oz. of clear liquid over a 30-min period.

dose bowel preparation. We hypothesized that the institution of split-dosing bowel preparation will reduce recommendations inconsistent with guidelines for follow-up colonoscopies in patients with normal colonoscopy or 1-2 small polyps (< 10 mm). Therefore, the objective of this research was to quantify the impact of split-dose regimen on endoscopists' compliance with guideline recommendations for timing of repeat colonoscopy in patients with normal colonoscopy or 1-2 small polyps (< 10 mm).

MATERIALS AND METHODS

Study design

With Institutional Review Board approval, a retrospective comparative review of medical records from two time periods (pre and post institution of split-dose bowel regimen) was performed. Medical records of consecutive average-risk patients aged ≥ 50 years and undergoing colonoscopy for CRC screening in the outpatient setting between January 1, 2009 and December 31, 2009 (pre-implementation of split-dose bowel regimen) and between January 1, 2011 and December 31, 2011 (post-implementation of split-dose bowel regimen) were reviewed. Inclusion criteria were average-risk patients referred for CRC screening colonoscopy with none, 1, or 2 identified polyps. Subjects were excluded for the following reasons: concurrent gastrointestinal symptoms (*e.g.*, anemia, overt or obscure gastrointestinal (GI) blood loss, abdominal pain, diarrhea, unexplained weight loss); family history of CRC; personal history of CRC, colon polyps, hereditary CRC syndrome, or inflammatory bowel disease; detection of any colonic polyps, or incomplete colonoscopies (*i.e.*, failure to visualize the appendiceal orifice and cecum). Only colonoscopies performed by gastroenterologists that were present in both calendar years were included. Patients with follow-up recommendations for "Barium enema" or "Discontinue due to age" were also excluded.

Protocol for bowel preparation and definition of bowel preparation quality

Bowel preparation details are included in Table 1 for same day bowel preparation utilized in 2009 and split-dose bowel preparation utilized in 2011. Bowel preparation quality and other endoscopic data were reported *via* the ProVation Medical Systems v.42 and University of Michigan endoscopy sites, respectively. Endoscopists rated bowel preparation quality according to the percentage of colonic mucosa visualized during the colonoscopy based on the Aronchick score which

Table 2 Patient and procedure characteristics by study year: 2009 is prior to split dosing, and 2011 is after split dosing

Characteristic	2009 (n = 1987)	2011 (n = 2238)	Total (n = 4225)	P value[1]
Age, yr				
≤ 55	1096 (55.2)	1311 (58.6)	2407	< 0.001
55-65	606 (30.5)	674 (30.1)	1,280	
65-75	233 (11.7)	253 (11.3)	486	
≥ 75	52 (2.6)	0 (0)	52	
Female	1099 (55.3)	1182 (52.8)	2281	0.100
Race/ethnicity				
White or Hispanic	1652 (83.1)	1846 (82.5)	3498	< 0.001
Black	131 (6.6)	160 (7.1)	291	
Asian	128 (6.4)	129 (5.8)	257	
Other	20 (1.0)	100 (4.5)	120	
BMI (kg/m^2), mean (SD)	1871 (94.2)	2213 (98.9)	4084	0.610
Current narcotics[2] use				
Yes	131 (6.6)	163 (7.3)	294	0.360
No	1856 (93.4)	2066 (92.3)	3922	
Current TCA use				
Yes	47 (2.4)	31 (1.4)	78	0.020
No	1940 (97.6)	2198 (98.2)	4138	
Type II diabetes				
Yes	164 (8.3)	201 (9.0)	365	0.400
No	1823 (91.7)	2037 (91.0)	3860	
Bowel preparation quality				
Adequate or excellent	592 (30.6)	886 (39.6)	1478	< 0.001
Good	1044 (54.0)	1048 (46.9)	2092	
Fair	209 (10.8)	189 (8.5)	398	
Inadequate or poor	89 (4.6)	113 (5.1)	202	
Polyp presence				
Yes	745 (37.5)	807 (36.1)	1552	0.330
No	1242 (62.5)	1431 (63.9)	2673	
Preparation type				
8L PEG	5 (0.3)	17 (0.8)	22	< 0.001
MiraLAX/Gatorade	541 (28.3)	1228 (57.1)	1769	
4L PEG, GoLYTELY, NuLYTELY, Colyte, TriLyte	1015 (53.1)	771 (35.9)	1786	
Half-Lytely,Osmoprep, Moviprep, and others	352 (18.4)	133 (6.2)	485	
Preparation type				
MiraLAX/Gatorade	541 (28.3)	1228 (57.1)	1769	< 0.001
4L PEG, GoLYTELY, NuLYTELY, Colyte, TriLyte	1015 (53.1)	771 (35.9)	1786	
Half-Lytely, Osmoprep, Moviprep, and others	357 (18.7)	150 (7.0)	507	
GI fellow presence				
Yes	187 (9.4)	303 (13.5)	490	< 0.001
No	1800 (90.6)	1935 (86.5)	3735	

All values are n (%), unless otherwise specified. The total number of patients for each characteristic may not add to total (n = 4225) due to missing data. [1]From testing differences in the distribution of characteristics between years, based on t-test for continuous variables and χ^2 test for categorical variables; [2]Narcotics are opioids and their derivatives. Common narcotics include morphine, heroin, hydrocodone, oxycodone, methadone, and clonitazene. BMI: Body mass index; TCA: Tricyclic antidepressant.

has been previously validated: "Excellent": greater than 95% of mucosa visualized; "Good": 90%-95% of mucosa visualized; "Fair": 80%-90% of mucosa visualized; and "Poor": less than 80% of mucosal visualization. An endoscopist could also report bowel preparation quality as "Adequate" or "Inadequate" if they felt the preparation did or did not, respectively, allow for the detection of polyps 5 mm or larger[10].

Endoscopists' recommendation intervals

Data were abstracted from patient colonoscopy report forms, pathology report, and follow-up pathology letter for the endoscopists' recommendation for follow-up screening colonoscopy. Follow-up recommendations were determined by adherence to the American College of Gastroenterology 2009 guidelines[7]. Recommendations consistent with guidelines included follow-up in 10 years, follow-up in 5 to 10 years for 1-2 small adenomas (as determined by pathologists), or ⩽ 1 year if bowel preparation quality was rated poor or inadequate regardless of the number of polyps. Any deviations from these recommendations were considered inconsistent with guidelines. If no recommendation was given by the endoscopist, it was classified as inconsistent with guidelines.

Subject and procedure data

Data were collected from medical notes on subject demographic, clinical, and procedural factors. Demographics included age, gender, and race/ethnicity; clinical factors comprised body mass index (BMI), concurrent narcotics and tricyclic antidepressant (TCA)

Table 3 Adjusted odds ratios of guideline consistent recommendations in average-risk patients (n = 4023)

Characteristic	OR	*P* value	95%CI
Year 2011	1.25	0.010	(1.05, 1.47)
Age, yr			
⩽ 55	1.00		
55-65	0.80	0.020	(0.67, 0.97)
65-75	0.64	< 0.001	(0.50, 0.82)
⩾ 75	0.40	0.004	(0.21, 0.74)
Male	0.94	0.440	(0.79, 1.11)
Race/ethnicity			
White or Hispanic	1.00		
Black	0.90	0.520	(0.66, 1.24)
Asian	1.09	0.630	(0.76, 1.56)
Others	1.00	0.990	(0.61, 1.64)
BMI (kg/m^2)	0.99	0.100	(0.97, 1.00)
Current Narcotics[1] use	0.79	0.140	(0.59, 1.08)
Current TCA use	0.89	0.710	(0.50, 1.60)
Type II diabetes	0.68	0.006	(0.52, 0.89)
Polyp presence	2.29	< 0.001	(1.89, 2.77)
GI fellow presence	1.48	0.020	(1.06, 2.08)

[1]Narcotics are opioids and their derivatives; Common narcotics include morphine, heroin, hydrocodone, oxycodone, methadone, and clonitazene. BMI: Body mass index; TCA: Tricyclic antidepressant.

usage, and diabetic status. Colonoscopy procedure data were collected on type of bowel preparation agent used, whether a GI fellow participated, and procedure completion status. Specific endoscopist characteristics were not collected due to Institutional Review Board concerns of the ability to identify specific gastroenterologists with the collected information.

Statistical analysis

Recommendation appropriateness was determined as either consistent or inconsistent with guidelines as described above under Endoscopist Recommendation Intervals. Primary exposure variable of interest was the institution of split-dose bowel preparation, and thus was the year 2011 *vs* 2009. χ^2 tests and Student's *t*-tests were used to assess differences in various demographic and procedure characteristics between 2009 *vs* 2011 cohort. To test if the institution of split-dosing bowel preparation reduced the percent of follow-up recommendations consistent with guidelines in patients with normal colonoscopy, logistic regression was used with guideline consistent recommendations as the dependent variables and an indicator for 2011 cohort as the primary predictor. Other independent predictors of guideline consistent recommendations included age, sex, race, BMI, narcotics use, TCA use, diabetes, and procedure characteristics. Odds ratios (ORs) and 95% confidence intervals (CIs) were obtained from the logistic regression model parameter estimates. Statistical analyses were performed using Stata 13.1 (StataCorp LP, College Station, TX, United States)

RESULTS

A total of 4225 patients were included in the study, with 47.0% (1987) from year 2009 prior to the institution of split-dose bowel preparation, and 53.0% (2238) from year 2011 after the institution of split dose bowel preparation. Overall, 82.2% (n = 3472) of the colonoscopies were compliant with guideline recommendations, with a significantly higher compliance rate in year 2011 (83.7%) than in year 2009 (80.4%, P = 0.005), corresponding to an unadjusted OR of 1.25 (95%CI: 1.07-1.47; P = 0.005).

Patient and procedure characteristics are summarized by study year in Table 2. Patients from year 2011 tended to be younger than those from year 2009 (means of 55.5 *vs* 56.1, respectively), but the difference was significant because 0% of year 2011 patients were 75 year or older, while 2.6% (52 of 1987) of year 2009 patients were 75 years or older. Patients from the two years were also different with respect to other characteristics including race and tricyclic antidepressant use. Although the distribution of bowel preparation quality was significantly different between the two years, the difference was not in "Poor" or "Inadequate" bowl quality. Specifically, in colonoscopies with bowel preparation quality ratings noted using four-level quality ratings only, colonoscopies rated as "Excellent" increased from 26.5% in year 2009 to 37.8% in year 2011, while those rated as "Poor" increased only slightly from 2.8% in 2009 to 3.1% in 2011. Similarly, when the binary rating of "Adequate" was combined with "Excellent" and "Inadequate" combined with "Poor" for colonoscopies without the four-level bowel preparation quality noted, the percentage of colonoscopies with "Excellent" or "Adequate" bowel preparation increased from 30.6% in year 2009 to 39.6% in year 2011 (P < 0.001, from comparing "Excellent" or "Adequate" *vs* other quality ratings), while the percentage of "Poor" or "Inadequate" quality increased from 4.6% in year 2009 to 5.1% in year 2011 (P = 0.50). Thus, the split-dose preparation appears to have resulted in a higher percentage of excellent/adequate preparation quality by reductions in mid-quality colonoscopies, but made no significant difference in poor/inadequate category of bowel preparation. Another important shift between the two years was the higher rate of MiraLAX/Gatorade use as the bowel preparation type in 2011 compared with 2009 (57.1% *vs* 28.3%).

Year 2011 cohort remained more likely to give guideline consistent recommendations (OR = 1.25, Table 3) even after adjusting for age, gender, race, BMI, current narcotics use, TCA use, type Ⅱ diabetes, site, and presence of polyp. Increasing age was associated with significantly lower odds of guideline consistent recommendations, and patients with type Ⅱ diabetes was associated with 0.68 times lower (P = 0.005)

Table 4 Adjusted[1] odds ratios of guideline consistent recommendations in average-risk patients obtained from logistic regression models fit separately by each year

	2009 Before split-dosing			2011 After split-dosing		
	OR	*P* value	95%CI	OR	*P* value	95%CI
Prep quality						
Adequate or excellent	1.00					
Good	0.55	0.001	(0.38, 0.78)	0.44	< 0.001	(0.32, 0.61)
Fair	0.04	< 0.001	(0.03, 0.06)	0.06	< 0.001	(0.04, 0.09)
Inadequate or Poor	0.17	< 0.001	(0.09, 0.30)	0.08	< 0.001	(0.05, 0.13)
Bowel prep type						
MiraLAX/Gatorade	1.00			1.00		
4L PEG, GoLYTELY, NuLYTELY, Colyte, TriLyte	0.65	0.013	(0.46, 0.91)	1.10	0.485	(0.84, 1.45)
Half-Lytely, Osmoprep, Moviprep, and others	0.62	0.032	(0.41, 0.96)	0.70	0.134	(0.44, 1.12)

[1]Adjusted also for all variables listed under Table 3.

odds of guideline consistent recommendations. On the other hand, the participation of GI fellows (P = 0.02) or having a colonoscopy with one or two polyps (as determined by pathology) were associated with a significantly higher likelihood (P < 0.001) of guideline consistent recommendations.

Between the two years, in addition to the implementation of split-dosing, the primary exposure of interest, we found that both bowel quality and bowel preparation distributions have changed significantly. To assess if the increased compliance between the two years can be explained by changes in preparation quality (which we expected to be associated with split dosing), changes in preparation type, or both, we further adjusted the model with the bowel preparation quality and bowel preparation type. After further adjusting for bowel preparation quality, we no longer found compliance difference between the two years (OR = 1.12, 95%CI: 0.93-1.34, P = 0.25). In addition, compared with "Excellent" or "Adequate" quality, all other preparation quality ratings were associated with lower odds of compliant recommendations: the adjusted OR of compliance for "Good" preparation was 0.48 (P < 0.001), "Fair" was 0.05 (P < 0.001), and "Poor" or "Inadequate" was 0.11 (P < 0.001). Similarly, when bowel preparation type categories were added to the model, the difference between the two years in compliance was no longer significant (OR = 1.16, P = 0.10), although bowel preparation type was not statistically significant, either. Lastly, compliance difference between the two years was not significant (OR = 1.03, P = 0.76) when both preparation quality and preparation type were included.

In summary, from year 2009 to year 2011, a significant increase in guideline consistent recommendations was seen, a significant increase in "Excellent" or "Adequate" colonoscopies was seen, and a significant increase in the use of MiraLAX/Gatorade was seen. However, the significant increase in guideline consistent recommendations from year 2009 to 2011 was no longer significant after controlling for either bowel preparation quality or bowel preparation type. To explore further whether the changes in preparation quality or preparation type led to increased compliance, we also fit logistic regression models separately by year (Table 4). In both 2009 and 2011 colonoscopies, we found preparation quality to be an independent predictor of compliance whether adjusted for preparation type or not; however, preparation type was a significant predictor of compliance only in 2009 colonoscopies, prior to split-dosing. These results suggested that split dosing likely reduced any differences in preparation quality associated with preparation type and hence resulted in less difference in compliance.

DISCUSSION

In our analysis of CRC screening in average-risk patients, implementation of split-dose bowel preparation led to an increase in guideline consistent recommendation, as indicated by increased percentage of guideline consistent recommendations from year 2009 to year 2011. Both the unadjusted and covariate adjusted ORs indicated a significant increase in guideline consistent recommendation from the year before to after the implementation of split-dose preparation. Additionally, we also found an increase in the percentage of bowel preparations rated as "Excellent" in quality between years 2009 and 2011, and it appeared that split-dose preparation led to a decrease in the percentage of "Good" and "Fair" preparation, but not in "Poor" preparation. Further analyses showed that an increase in guideline compliant recommendations from year 2009 to 2011 was explained by increased "Excellent" bowel preparation or decreased "Good" or "Fair" preparation. We reached this conclusion from the findings that: (1) a significant increase in guideline consistent recommendations was seen from year 2009 colonoscopies to year 2011 colonoscopies; (2) a significant increase in "Excellent" or "Adequate" colonoscopies was seen from year 2009 to year 2011; and (3) the significant increase in guideline consistent recommendations from year 2009 to 2011 was no longer significant after controlling for bowel

preparation quality. Our study adds further support for the use of split-dose bowel regimen as it is now uniformly recommended to optimize bowel preparation for colonoscopy[7,11-13].

This study is unique as it is the first to look at endoscopists' recommendations as an outcome pre- and post-introduction of split-dose bowel preparation. Studies of physician post-colonoscopy recommendations have shown varying compliance to guidelines. A retrospective review of screening and surveillance colonoscopies demonstrated endoscopists' compliance in 81% of subjects based on pathology[14]. However, recommendations were only provided in 74% of their cohort. This study removed bowel preparation as a factor as poor or fair bowel preparation or lack of bowel preparation data was the exclusion criteria. These findings are markedly better than those of Krist *et al*[1], where recommendations were only provided in 64.9% of reports. In studies including bowel preparation in their investigation, uniform bowel preparation quality impacts the likelihood of endoscopists' guideline compliance. Ransohoff *et al*[15] found that follow-up recommendations in bowel preparations less than excellent were associated with shorter surveillance intervals for those with no polyps, small or medium adenomas. Additionally, Rex *et al*[16] demonstrated that imperfect bowel preparation led to a higher likelihood of patients to be brought back earlier than suggested or required by current practice standards (20% *vs* 12.5%, $P = 0.04$). Our study contributes data for future benchmarks for endoscopist compliance of guidelines in the real world, split-bowel preparation setting, as the CMS requires reporting of quality indicators through the PQRS.

Significant patient and procedural characteristics were associated with both a higher and lower likelihood of guideline-inconsistent follow-up recommendations. Two patient characteristics associated with a higher likelihood of guideline-inconsistent recommendations included increasing age and the co-morbidity of diabetes mellitus. Both characteristics are associated with CRC. Increasing age is the strongest non-modifiable risk factor for the development of CRC[17]. The likelihood of CRC begins to increase after age 40 with a peak incidence between the ages of 65-79. For diabetes mellitus, Larsson *et al*[18] performed a meta-analysis of more than 2.5 million patients that demonstrated a 30% increased risk of CRC relative to non-diabetic individuals. This finding was constant even when controlling for BMI and physical activity. This literature may explain the association for early repeat colonoscopy recommendations in patients with these characteristics. However, two procedural characteristics, finding 1-2 polyps regardless of pathology and having a GI fellow participate in the colonoscopy, were less likely to have guideline-inconsistent follow-up recommendations. Surveys of gastroenterologists have shown an improvement in guideline knowledge and agreement for follow-up recommendations for colonoscopies[19,20]. Saini *et al*[19] assessed gastroenterologists' knowledge of the 2003 guidelines for management of various polyps. At that time, only 63.6% knew the correct interval for two small adenomas, but also 28.8% of gastroenterologists disagreed with the guideline. In 2010, Shah *et al*[20] surveyed Veterans Affairs gastroenterologists' similar questions about the 2006 polyp surveillance guidelines. Ninety-five percent of gastroenterologists identified the correct 5-10 years interval for one 8 mm adenoma. In this cohort of GI doctors, only 7% of those who knew guidelines correctly would deviate from clinical guidelines in their clinical practice. With the finding of any type of polyp, our endoscopists are compliant and know the guidelines. Additionally, gastroenterology fellows participating in the colonoscopy reduced the likelihood of inconsistent guidelines. The influence would have to be on the procedure itself as the attending physician is responsible for the follow-up pathology letter. In the literature, GI trainees have been noted to have positive impact on adenoma detection rate with the hypothesis that longer withdrawal times increase the likelihood of polyp detection or having an additional person involved in the colonoscopy allows optimal visualization[21-23]. For our cohort, the fellows' presence may have allowed better visualization (possible more patience with stool clearance or as a reminder for guideline compliance).

Our study has several potential limitations. As this study was retrospective in nature, the preparation type, preparation quality documentation, and endoscopist recommendations were limited to the medical records. Furthermore, patients who were prescribed spilt-dose bowel regimen during the second time period may not have actually taken it as recommended. Another limitation is the lack of use of quality assessment tool training, such as the Boston Bowel Preparation Scale; however, the grading scale in Provation is based on the Aronchick scale[10,24]. Additionally, there may be variability amongst physician reporting of bowel preparation quality that is not able to be captured by the retrospective nature of this study. The generalizability of the study may be limited since it involved only procedures performed by academic physicians, although the study was conducted at outpatient ambulatory surgery centers and in-hospital academic medical centers.

In conclusion, our study demonstrates that besides increasing bowel preparation quality, split-dose bowel preparation also increases guideline consistent recommendations in average-risk patients with normal colonoscopy or 1-2 small polyps. Our data adds further justification for the routine use of split-dose bowel preparation in daily practice. Education about recent guideline recommendations and the need for split-dose bowel preparation should be continued.

ARTICLE HIGHLIGHTS

Research background

Split-dose bowel regimen is considered standard of care for bowel preparation in national guidelines. Since it improves bowel preparation quality, we should

see an increase in endoscopists' compliance to guidelines.

Research motivation

Split-dose bowel regimen is recommended in national guidelines for colonoscopy bowel preparation. There is no data on how institution of split-dose bowel preparation can maximize the proportion of patients with an "excellent" bowel preparation and quantify the impact of "excellent" bowel preparation on increasing the likelihood of recommending an appropriate interval for repeat screening/surveillance colonoscopies.

Research objectives

To examine the impact of split-dose regimen on endoscopists' compliance with guideline recommendations for timing of repeat colonoscopy in patients with normal colonoscopy or 1-2 small polyps (< 10 mm).

Research methods

We conducted this retrospective study of colonoscopies performed in average-risk individuals aged 50 years or greater from two time periods, pre and post-split bowel preparation institution. Only patients with normal or 1-2 small polyps were included. Primary and secondary outcome measurements included: recommendation for timing of repeat colonoscopy and bowel preparation quality. Bivariate analysis and multivariable logistic regression analysis were utilized to assess the impact of split-dosing bowel preparation on both physician follow-up recommendation and bowel preparation quality.

Research results

After the institution of split-dose bowel regimen, there was a small, but significant increase in physician compliance to guideline recommendations in patients with normal colonoscopy and 1-2 small polyps. This correlated to the increase in both excellent and adequate bowel preparation. There was no measurable change in the amount of patients who had poor/inadequate bowel preparation.

Research conclusions

In this current study, our research supports the use of split-dose bowel regimen to help optimize bowel preparation. Improvement of bowel preparation quality increases the likelihood of physician compliance for follow-up colonoscopy in patients with normal colonoscopy and 1-2 small polyps.

Research perspectives

This study supports the use of split-dose bowel regimen for colonoscopy bowel preparation. Our study also acquired information on endoscopist compliance to CRC screening guidelines after the implementation of split-dose preparation in order to provide a new baseline for comparison. Improvement in endoscopist compliance can help make colonoscopy more cost-effective. It is crucial for endoscopists to abide by current guidelines, as recommending earlier colonoscopies not only exposes patients to excess procedural risk, but also drains limited resources that could be used for unscreened patients. This study provides pilot data for future endoscopist-based interventions.

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ORIGINAL ARTICLE

Retrospective Study

Clinical utility of hepatitis B surface antigen kinetics in treatment-naïve chronic hepatitis B patients during long-term entecavir therapy

Tien-Ching Lin, Yen-Cheng Chiu, Hung-Chih Chiu, Wen-Chun Liu, Pin-Nan Cheng, Chiung-Yu Chen, Ting-Tsung Chang, I-Chin Wu

Tien-Ching Lin, Yen-Cheng Chiu, Hung-Chih Chiu, Pin-Nan Cheng, Chiung-Yu Chen, Ting-Tsung Chang, I-Chin Wu, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan City, 70403, Taiwan

Wen-Chun Liu, Ting-Tsung Chang, I-Chin Wu, Infectious Disease and Signaling Research Center, National Cheng Kung University, Tainan 70403, Taiwan

ORCID number: Tien-Ching Lin (0000-0002-7708-5589); Yen-Cheng Chiu (0000-0003-0825-3769); Hung-Chih Chiu (0000-0001-9538-7188); Wen-Chun Liu (0000-0001-9048-6363); Pin-Nan Cheng (0000-0001-9331-9018); Chiung-Yu Chen (0000-0002-5418-8315); Ting-Tsung Chang (0000-0002-5073-2678); I-Chin Wu (0000-0001-8280-5133).

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Correspondence to: I-Chin Wu, MD, PhD, Assistant Professor, Department of Internal Medicine, National Cheng Kung University Hospital, No. 138 Sheng-Li Road, Tainan 70403, Taiwan. wichin@mail.ncku.edu.tw
Telephone: +886-6-2353535-3588
Fax: +886-6-2743166

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Abstract

AIM

To investigate the utility of hepatitis B surface antigen (HBsAg) kinetics in chronic hepatitis B patients during long-term entecavir treatment.

METHODS

This retrospective study included treatment-naïve chronic hepatitis B patients who received at least 2 years of consecutive entecavir treatment. Patients were followed up at three to six month intervals with liver biochemistry, hepatitis B virus DNA, and abdominal sonography. In hepatitis B e antigen (HBeAg)-positive patients, HBeAg levels were assessed every three to six month until results became negative. Serum

HBsAg levels were determined at the baseline, one-year and five-year time points. Liver cirrhosis was diagnosed through liver biopsy, imaging examinations, or clinical findings of portal hypertension. Hepatocellular carcinoma was diagnosed by histological examination or dynamic image studies.

RESULTS

A total of 211 patients were enrolled. The median treatment time was 5.24 (2.00-9.62) years. Multivariate analysis showed that lower baseline HBsAg levels were associated with an earlier virological response, earlier hepatitis B e antigen (HBeAg) seroconversion, and earlier biochemical response in HBeAg-positive patients (cut-off value: 4 log IU/mL) and an earlier virological response in HBeAg-negative non-cirrhotic patients (cut-off value: 2.4 log IU/mL). Although HBsAg levels decreased slowly during long-term entecavir treatment, higher HBsAg decrease rates were found in the first year for HBeAg-positive non-cirrhotic patients, and patients with higher baseline HBsAg levels. More favorable clinical outcomes were not observed by a rapid HBsAg decline *per se*, but depended on lower baseline HBsAg levels.

CONCLUSION

Baseline HBsAg can be used to predict treatment responses. HBsAg levels and decrease rates should be considered together according to disease status while interpreting HBsAg changes.

Key words: Chronic hepatitis B; Entecavir; Hepatitis B e antigen; Hepatitis B surface antigen; Kinetics

Core tip: Baseline hepatitis B surface antigen (HbsAg) levels could be used to predict virological, serological, and biochemical responses during entecavir treatment. HBeAg-positive non-cirrhotic patients had the highest HBsAg levels at the baseline and throughout entecavir treatment, and had the highest HBsAg decrease rates during the first year of entecavir treatment. HBsAg levels decrease slowly during the treatment. Therefore, HBsAg should be checked at a 1-year interval if hepatitis B virus DNA remains undetectable. A rapid HBsAg decline per se did not achieve better patient outcomes. In the interpretation of HBsAg changes, HBsAg levels and decrease rates should be considered together according to disease status.

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INTRODUCTION

Patients with chronic hepatitis B virus (HBV) infection are at risk of cirrhosis and hepatocellular carcinoma (HCC)[1]. Eradication of chronic HBV infection is difficult because of the presence of covalently closed circular DNA (cccDNA) in infected cells[2]. HBV cccDNA resides in the nucleus of infected cells as an episomal (*i.e.*, nonintegrated) plasmid-like molecule. The paucity of knowledge about cccDNA formation and degradation is a considerable obstacle to the development of anti-chronic HBV infection treatments[2].

Hepatitis B surface antigen (HBsAg) levels have been used to discriminate between different clinical phases[3], predict spontaneous HBsAg seroclearance[4,5], and identify inactive phases in hepatitis B e antigen (HBeAg)-negative patients[6]. HBsAg levels of < 100 IU/mL could predict HBsAg loss in HBeAg seroconverters[7] and identify HBeAg-negative patients with inactive virus[8].

HBsAg levels can also be used to guide pegylated interferon (PegIFN) treatment course. In HBeAg-positive patients with an HBsAg level > 20000 IU/mL after 24 wk of treatment, PegIFN discontinuation is suggested[9]. In HBeAg-negative patients without HBsAg level declines by week 12 and without HBV DNA level declines of > 2 log IU/mL, a sustained response to therapy is considered impossible[10]. PegIFN responders, compared with nonresponders, had greater declines in HBsAg and cccDNA levels. For PegIFN responders, mean HBsAg reduction levels were 2.5 ± 2.3 log IU/mL in HBeAg-positive patients and 2.5 ± 1.3 log IU/mL in HBeAg-negative patients after 48 wk of treatment[11].

HBsAg levels declined much less rapidly during nucleos(t)ide analogue (NA) treatment, compared with PegIFN treatment[12]. The declines in HBsAg levels from the baseline to week 48 during NA treatment were 0.3 to 0.5 log IU/mL in HBeAg-positive patients and -0.1 to 0.1 log IU/mL in HBeAg-negative patients[3]. For most patients, long-term NA treatment renders a consistent but slow reduction (0.084 log IU/year)[13].

For patients receiving NA treatment, HBsAg quantification may help to predict clinical outcomes. HBsAg levels of < 3000 IU/mL at the baseline combined with HBsAg declines of ⩾ 75% from the baseline could predict the eventual loss of HBsAg[14]. An HBsAg reduction of > 1 log IU/mL could reflect improved immune control[12,15], and a reduction of ⩾ 0.5 log IU/mL after 6 mo of treatment had a high negative predictive value for HBsAg seroclearance[16].

Serum HBsAg is closely related to serum HBV DNA and intrahepatic cccDNA in HBeAg-positive patients, but it is poorly correlated with serum HBV DNA and not correlated with intrahepatic cccDNA in HBeAg-negative patients[11,17]. HBsAg changes after NA treatment were also different between HBeAg-positive and HBeAg-negative patients[3]. Two studies reported that baseline HBsAg levels could help to predict HBsAg

decline or loss in HBeAg-negative patients[18,19]. On the contrary, some studies suggested that neither baseline HBsAg nor reduction in HBsAg could predict virological response in HBeAg-negative patients[20,21]. Therefore, these results are rather divergent in HBeAg-negative patients. The aim of the current study was to investigate the role of HBsAg levels in predicting treatment responses and the clinical significance of HBsAg kinetics for different disease statuses during long-term entecavir treatment.

MATERIALS AND METHODS

Patients

This retrospective study analyzed treatment-naïve chronic hepatitis B patients receiving at least two years of consecutive entecavir treatment at National Cheng Kung University Hospital. The exclusion criteria were (1) prior treatment history with NAs or interferon; (2) coinfection with hepatitis C virus or human immunodeficiency virus infection; (3) end-stage renal disease; (4) systemic chemotherapy due to active cancer; and (5) post-organ transplantation. During the study period, patients received entecavir as the only anti-HBV therapy. Indications for entecavir therapy followed the Asian Pacific Association for the Study of the Liver HBV treatment guideline[22]. Enrolled patients were started on entecavir between December 2007 and January 2015. This study was approved by the Institutional Review Board of National Cheng Kung University Hospital. We analyzed the medical charts and remaining serum samples of these patients in this study. The informed consents of remaining specimen usage were obtained from these patients at the request of the Institutional Review Board of National Cheng Kung University Hospital.

Monitoring

All enrolled patients underwent follow-up liver biochemistry testing, HBV DNA testing, and abdominal sonography at three to six month (twelve to twenty-four week) intervals. In HBeAg-positive patients, HBeAg levels were assessed every three to six month until results became negative. Serum HBsAg quantification was performed at the baseline, one-year (48 wk) time point, and five-year (240 wk) time point after treatment. Liver cirrhosis was diagnosed through liver biopsy, imaging examinations [abdominal sonography, computed tomography (CT), or magnetic resonance imaging (MRI)], or clinical findings of portal hypertension (esophageal or cardiac varices by esophagogastroduodenoscopy). HCC was diagnosed by histological examination (liver biopsy or surgery) or dynamic image studies (CT and MRI).

Virological response to treatment was defined as the point at which serum HBV DNA became undetectable (< 60 IU/mL) during treatment. HBeAg seroclearance was defined as a loss of HBeAg, whereas HBeAg seroconversion was defined as a loss of HBeAg and occurrence of anti-HBe, according to the Asian Pacific Association for the Study of the Liver HBV treatment guideline[22]. Because alanine aminotransferase (ALT) levels usually fluctuated and were affected by multiple factors during treatment, biochemical response was defined as ALT normalization [(≤ upper limit of normal (ULN)] for more than 6 mo during the study period and for the last 6 mo of the study period in patients with elevated baseline ALT levels. The ULN of ALT was 50 U/mL in male patients and 35 U/mL in female patients at National Cheng Kung University Hospital.

Laboratory measurements

Serum HBsAg levels were measured using the Architect HBsAg assay (Abbott, Chicago, IL, United States), with a linear range of 0.05 to 250 IU/mL. Samples with levels higher than 250 IU/mL were retested at a series of dilutions according to the manufacturer's instructions. Serum HBV DNA levels were determined using the Roche Cobas Amplicor [lower limit of detection (LLD): 60 IU/mL], the Roche Cobas TaqMan 48 analyzer (LLD: 29 IU/mL), the Roche Cobas AmpliPre/Cobas TagMan HBV Test, version 1.0 (LLD: 12 IU/mL), and the Roche Cobas AmpliPre/Cobas TagMan HBV Test, version 2.0 (LLD: 20 IU/mL). Baseline HBV DNA levels of serum samples collected from 22 patients (22/211, 10.4%) between December 2007 and October 2009 were measured by our in-house LightCycler real-time method, which was well correlated with results from the Roche Cobas Amplicor. HBV genotype was determined using melting curve analysis with LightCycler hybridization probes, as described previously[23].

Statistical analysis

Continuous variables are expressed as mean and standard deviation, except for treatment time, which is expressed as median and range. Categorical variables are expressed as numbers (percentages). Continuous variables were compared using Student's *t* test. The distributions of categorical variables were compared using the Chi-square test or Fisher's exact test when an expected value was less than 5. The cumulative incidence of treatment responses and clinical events with different variables were obtained using the Kaplan-Meier analysis, and the log-rank test was used to test for statistical difference. Multivariate analysis was performed using Cox proportional hazards regression to determine the factors that were independently associated with treatment responses and clinical events. A linear mixed model with a random intercept was used for analysis of longitudinal changes of HBsAg levels. In this model, groups and time points were treated as categorical variables and represented by dummy variables. Statistical analysis was performed using Stata 14.2 (Stata-Corp, Tx, United States). Results were

Table 1 Clinical characteristics of enrolled patients, categorized by HBeAg status *n* (%)

Characteristics	Total (*n* = 211)	HBeAg-positive (*n* = 62)	HBeAg-negative (*n* = 149)	*P* value[1]
Age (yr)	50.4 ± 11.9	43.8 ± 12.2	53.2 ± 10.6	< 0.0001
Male	147 (69.7)	39 (63.0)	108 (72.5)	0.170
Treatment time (yr)	5.24 (2.00-9.62)	4.39 (2.11-9.62)	5.35 (2.00-9.58)	0.590
Cirrhosis	66 (31.3)	12 (19.4)	54 (36.2)	0.016
HCC[2]	32 (15.2)	5 (8.1)	27 (18.1)	0.060
HBV genotype[3]	101:86:2 (53.4%:45.5%:1.1%)	21:39:01	80:47:01	0.001
B:C:B + C		(34.4%:63.9%:1.6%)	(62.5%:36.7%:0.8%)	
ALT (× ULN)	4.12 ± 5.88	4.42 ± 6.62	3.99 ± 5.56	0.630
HBV DNA (log IU/mL)	5.84 ± 1.70	7.24 ± 1.39	5.26 ± 1.50	< 0.0001
HBsAg (log IU/mL)	3.15 ± 0.80	3.80 ± 0.71	2.89 ± 0.67	< 0.0001

Continuous variables are expressed as mean ± SD, except treatment time, which is expressed as median (range). Categorical variables are expressed as numbers (percentages). [1]*P* value represents HBeAg-positive patients compared with HBeAg-negative patients; [2]HCC diagnosed before or within half a year of entecavir therapy; [3]HBV genotype could not be determined in 1 HBeAg-positive patient and 21 HBeAg-negative patients because of low HBV viral loads in these patients. ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; ULN: Upper limit of normal.

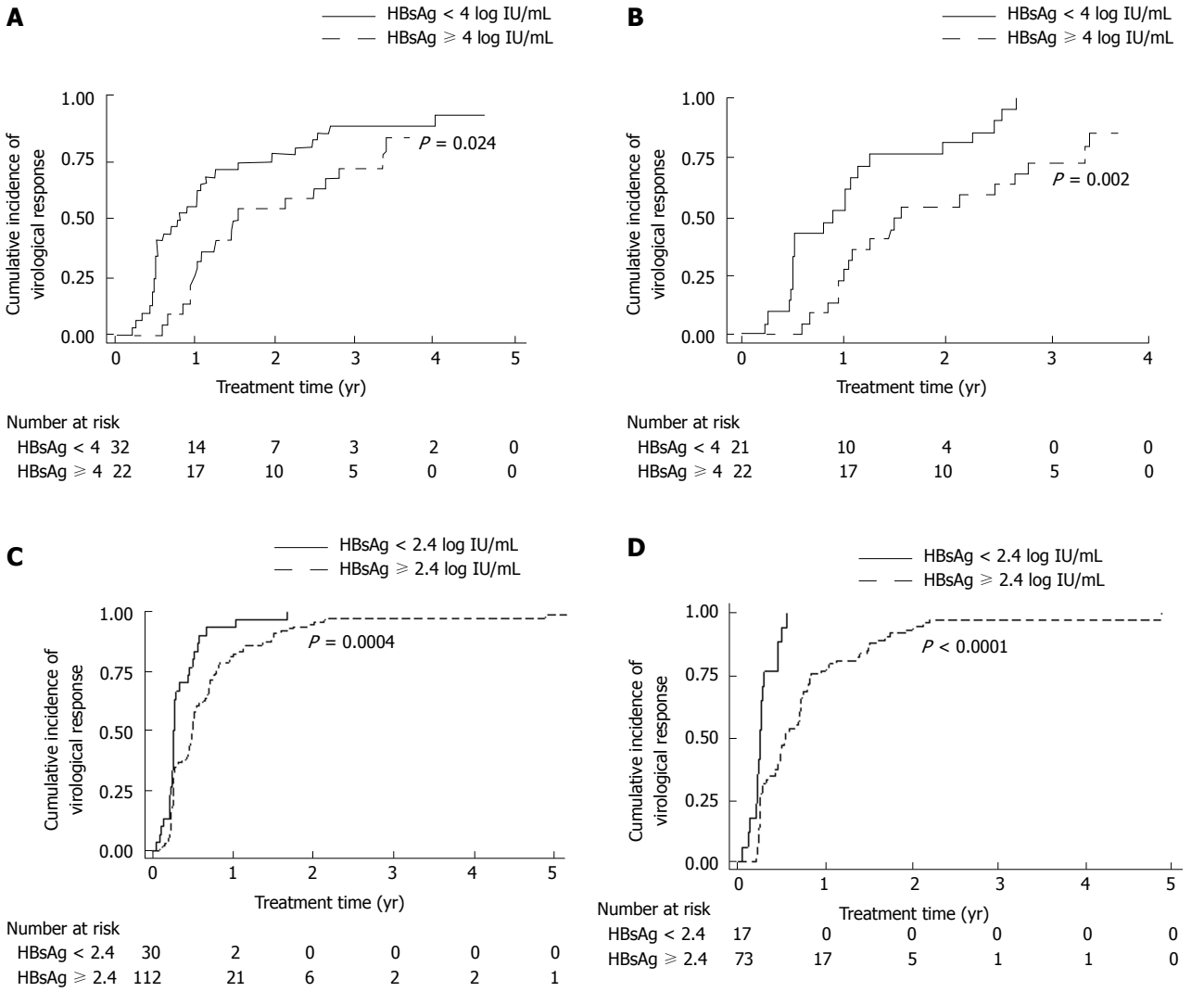


Figure 1 Cumulative incidence of virological response. A: Cumulative incidence of virological response in HBeAg-positive patients; B: Cumulative incidence of virological response in HBeAg-positive non-cirrhotic patients; C: Cumulative incidence of virological response in HBeAg-negative patients; D: Cumulative incidence of virological response in HBeAg-negative non-cirrhotic patients.

Table 2 Univariate and multivariate analyses of factors associated with virological response in HBeAg-positive patients

Factors	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Sex (female *vs* male)	1.31	0.73-2.36	0.37	2.95	1.33-6.57	0.008
Age (yr)	1.00	0.98-1.02	0.90	0.99	0.96-1.01	0.390
Cirrhosis (yes *vs* no)	0.91	0.42-1.96	0.81	0.94	0.32-2.79	0.910
HBV genotype (B *vs* C)[1]	1.14	0.62-2.09	0.67	2.04	0.99-4.21	0.053
ALT (× ULN)	1.02	0.98-1.06	0.25	1.04	1.00-1.08	0.060
HBV DNA (≤ 5 *vs* > 5 log IU/mL)	2.72	0.96-7.68	0.06	1.78	0.46-6.90	0.400
HBsAg (< 4 *vs* ≥ 4 log IU/mL)	1.96	1.08-3.55	0.03	4.92	2.10-11.51	< 0.001

[1]Only one HBeAg-positive patient had a mixed HBV genotype B and C infection; therefore, a genotype B + C subset was not included in this analysis. ALT: Alanine aminotransferase; CI: Confidence interval; HR: Hazard ratio; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; ULN: Upper limit of normal.

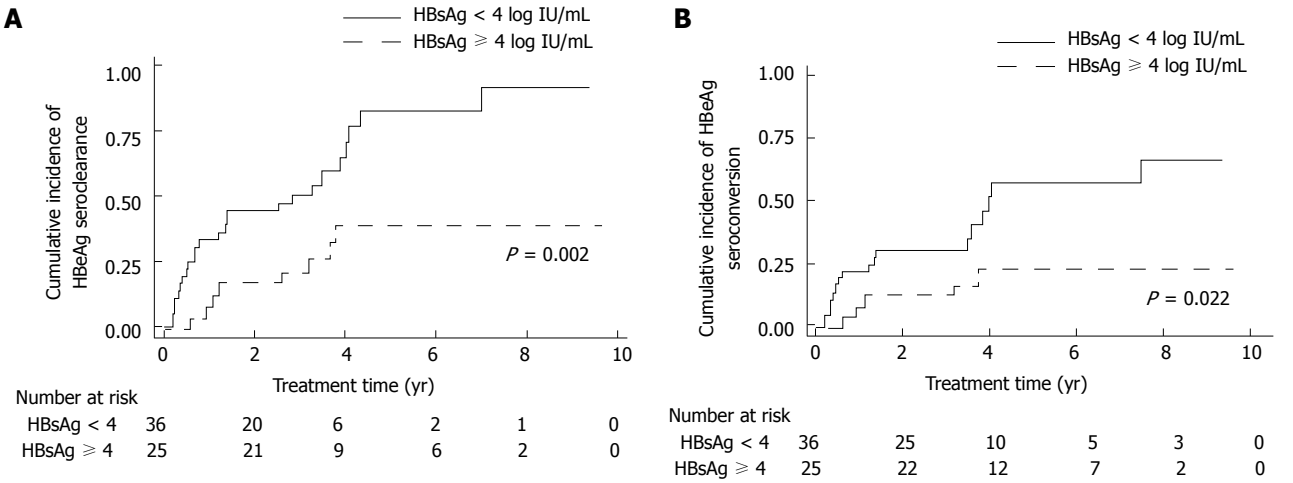


Figure 2 Cumulative incidence of HBeAg serological response. A: Cumulative incidence of HBeAg seroclearance; B: Cumulative incidence of HBeAg seroconversion.

considered statistically significant at $P < 0.05$.

RESULTS

Patient characteristics

A total of 211 treatment-naïve chronic hepatitis B patients receiving entecavir monotherapy were enrolled in this study. The median entecavir treatment time was 5.24 (2.00-9.62) years. The mean age was 50.4 ± 11.9 years. Most patients were men (69.7%), HBeAg-negative (70.6%), and non-cirrhotic (68.7%). Compared with HBeAg-negative patients, HBeAg-positive patients had a younger age, higher baseline HBV DNA and HBsAg levels, and lower proportions of liver cirrhosis, HCC diagnosed before or within half a year of entecavir therapy, and genotype B HBV infection (Table 1).

Virological response

One hundred and ninety-six patients were assessed for virological response to treatment. One hundred and eighty-eight patients (188/196, 95.9%) achieved virological response during treatment. The median time to virological response was 0.50 (0.04-4.88) years. Among HBeAg-positive patients (n = 54), those with a baseline HBsAg level of < 4 log IU/mL had an earlier virological response, compared with those with a baseline HBsAg level of ≥ 4 log IU/mL (P = .024, Figure 1A). Multivariate analysis revealed that the female sex and a baseline HBsAg level of < 4 log IU/mL were independently associated with an earlier virological response [female *vs* male: hazard ratio (HR): 2.95, 95% confidence interval (CI): 1.33-6.57, P = 0.008; HBsAg < 4 *vs* ≥ 4 log IU/mL: HR: 4.92, 95%CI: 2.10-11.51, P < 0.001, Table 2]. A subgroup analysis for HBeAg-positive non-cirrhotic patients showed that the female sex, a higher baseline ALT, and a baseline HBsAg level of < 4 log IU/mL were independently associated with an earlier virological response (Figure 1B and Supplemental Table 1A).

In HBeAg-negative patients (n = 142), a baseline HBsAg level of < 2.4 log IU/mL predicted virological response in the univariate analysis but not in the multivariate analysis (Figure 1C and Supplemental Table 1B). Therefore, a subgroup analysis for HBeAg-negative non-cirrhotic patients was performed. The results indicated that among HBeAg-negative non-cirrhotic patients (n = 90), those with a baseline HBsAg level of < 2.4 log IU/mL achieved virological response more easily (P < 0.0001, Figure 1D). Multivariate analysis

Table 3 Univariate and multivariate analyses of factors associated with virological response in HBeAg-negative non-cirrhotic patients

Factors	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Sex (female *vs* male)	1.14	0.72-1.81	0.57	1.39	0.78-2.47	0.27
Age (yr)	0.99	0.97-1.01	0.53	0.99	0.96-1.01	0.31
HBV genotype (B *vs* C)[1]	1.17	0.70-1.93	0.55	1.34	0.79-2.30	0.28
ALT (× ULN)	1.00	0.96-1.04	0.94	0.99	0.94-1.04	0.65
HBV DNA (≤ 4 *vs* > 4 log IU/mL)	1.33	0.73-2.40	0.35	0.63	0.19-2.07	0.45
HBsAg (< 2.4 *vs* ≥ 2.4 log IU/mL)	3.95	2.19-7.12	< 0.001	3.12	1.58-6.19	0.001

[1]None of the HBeAg-negative non-cirrhotic patients had a mixed HBV genotype B and C infection. ALT: Alanine aminotransferase; CI: Confidence interval; HR: Hazard ratio; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; ULN: Upper limit of normal.

Table 4 Univariate and multivariate analyses of factors associated with HBeAg seroclearance in HBeAg-positive patients

Factors	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Sex (female *vs* male)	0.74	0.36-1.54	0.43	1.14	0.49-2.67	0.76
Age (yr)	1.00	0.98-1.03	0.81	0.99	0.95-1.02	0.42
Cirrhosis (yes *vs* no)	1.42	0.64-3.15	0.39	0.71	0.24-2.07	0.53
HBV genotype (B *vs* C)[1]	1.17	0.57-2.39	0.67	2.04	0.90-4.62	0.09
ALT (× ULN)	1.02	0.96-1.09	0.51	1.04	0.98-1.09	0.17
HBV DNA (≤ 5 *vs* > 5 log IU/mL)	2.10	0.64-6.93	0.22	3.40	0.83-13.87	0.09
HBsAg (< 4 *vs* ≥ 4 log IU/mL)	3.32	1.49-7.43	0.003	5.74	2.19-15.00	< 0.001

[1]Only one HBeAg-positive patient had a mixed HBV genotype B and C infection; therefore, a genotype B + C subset was not included in this analysis. ALT: Alanine aminotransferase; CI: Confidence interval; HR: Hazard ratio; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; ULN: Upper limit of normal.

Table 5 Univariate and multivariate analyses of factors associated with HBeAg seroconversion in HBeAg-positive patients

Factors	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Sex (female *vs* male)	0.70	0.29-1.72	0.440	1.02	0.35-2.97	0.970
Age (yr)	0.99	0.95-1.02	0.440	0.98	0.94-1.02	0.310
Cirrhosis (yes *vs* no)	0.93	0.31-2.75	0.890	0.57	0.15-2.16	0.410
HBV genotype (B *vs* C)[1]	0.86	0.35-2.13	0.750	1.49	0.52-4.26	0.450
ALT (× ULN)	1.03	0.96-1.10	0.390	1.05	0.99-1.11	0.120
HBV DNA (≤ 5 *vs* > 5 log IU/mL)	3.16	0.93-10.76	0.070	4.15	1.05-16.44	0.043
HBsAg (< 4 *vs* ≥ 4 log IU/mL)	3.05	1.12-8.28	0.029	5.05	1.58-16.14	0.006

[1]Only one HBeAg-positive patient had a mixed HBV genotype B and C infection; therefore, a genotype B + C subset was not included in this analysis. ALT: Alanine aminotransferase; CI: Confidence interval; HR: Hazard ratio; HBeAg, hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; ULN: Upper limit of normal.

Table 6 Univariate and multivariate analyses of factors associated with new hepatocellular carcinoma development

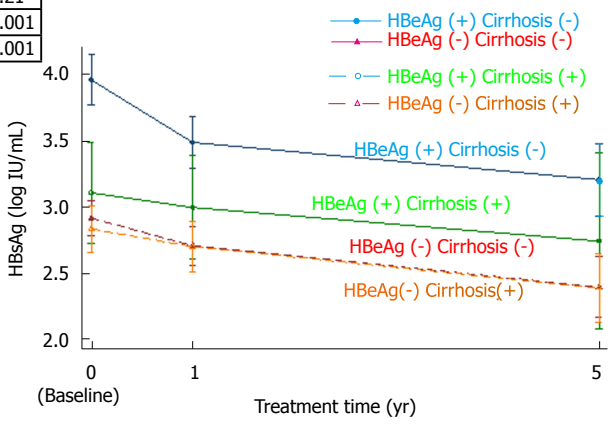
Factors	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Sex (female *vs* male)	0.42	0.09-1.90	0.26	0.31	0.05-1.94	0.21
Age (yr)	1.08	1.03-1.13	0.001	1.05	0.99-1.12	0.12
HBeAg (positive *vs* negative)	0.44	0.10-1.97	0.28	0.91	0.14-6.00	0.92
Cirrhosis (yes *vs* no)	11.32	3.11-41.24	< 0.001	13.02	2.00-84.99	0.007
HBV genotype (B *vs* C)[1]	0.58	0.18-1.90	0.37	0.63	0.12-3.28	0.58
ALT (× ULN)	1.02	0.95-1.09	0.55	1.06	0.97-1.16	0.20
HBV DNA (log IU/mL)	0.90	0.66-1.22	0.48	1.06	0.59-1.91	0.84
HBsAg (log IU/mL)	0.80	0.41-1.55	0.51	0.98	0.29-3.36	0.97

[1]Only two patients (one HBeAg-positive and one HBeAg-negative cirrhotic) had mixed HBV genotype B and C infection; therefore, a genotype B + C subset was not included in this analysis. ALT: Alanine aminotransferase; CI: Confidence interval; HR: Hazard ratio; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; ULN: Upper limit of normal.

A

Comparisons between different groups at one specific time point:			
Group	Baseline	1 yr	5 yr
HBeAg(+) Cirrhosis(-) *vs* HBeA (+) Cirrhosis(-)	$P < 0.001$	$P = 0.027$	$P = 0.21$
HBeAg(+) Cirrhosis(-) *vs* HBeAg (-) Cirrhosis (-)	$P < 0.001$	$P < 0.001$	$P < 0.001$
HBeAg(+) Cirrhosis(-) *vs* HBeAg (-) Cirrhosis (+)	$P < 0.001$	$P < 0.001$	$P < 0.001$

Comparisons between different time points for one specific group:			
Group	Baseline *vs* 1 yr	1 year *vs* 5 yr	Baseline *vs* 5 yr
HBeAg(+) Cirrhosis(-)	$P < 0.001$	$P = 0.050$	$P < 0.001$
HBeAg(+) Cirrhosis(+)	$P = 0.58$	$P = 0.46$	$P = 0.28$
HBeAg(-) Cirrhosis(-)	$P = 0.007$	$P = 0.010$	$P < 0.001$
HBeAg(-) Cirrhosis(+)	$P = 0.17$	$P = 0.021$	$P = 0.001$



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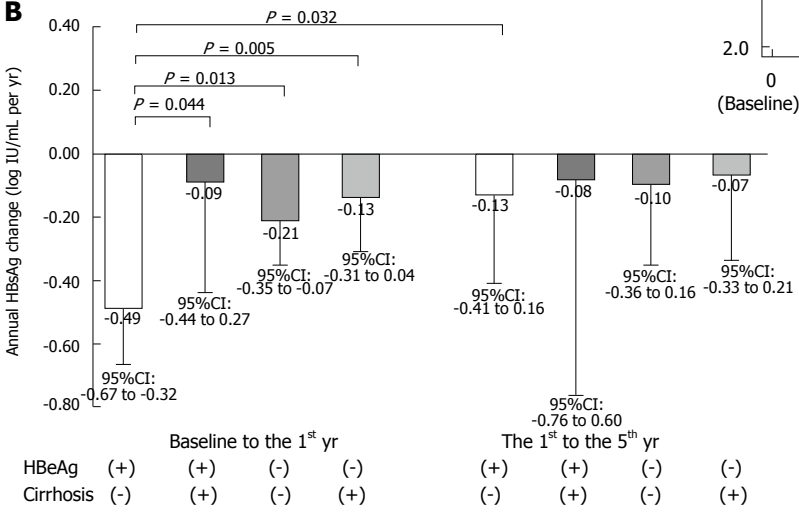

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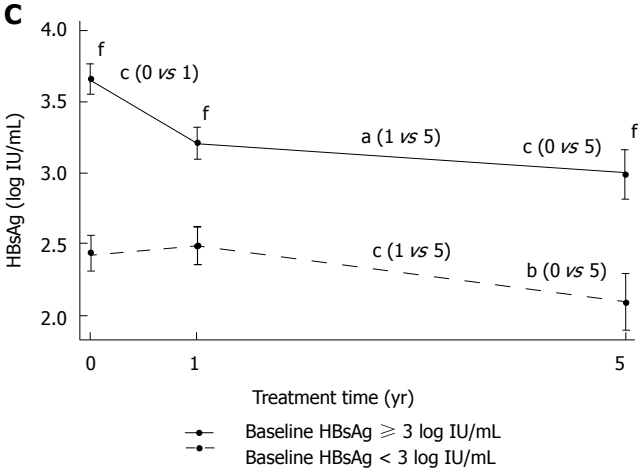

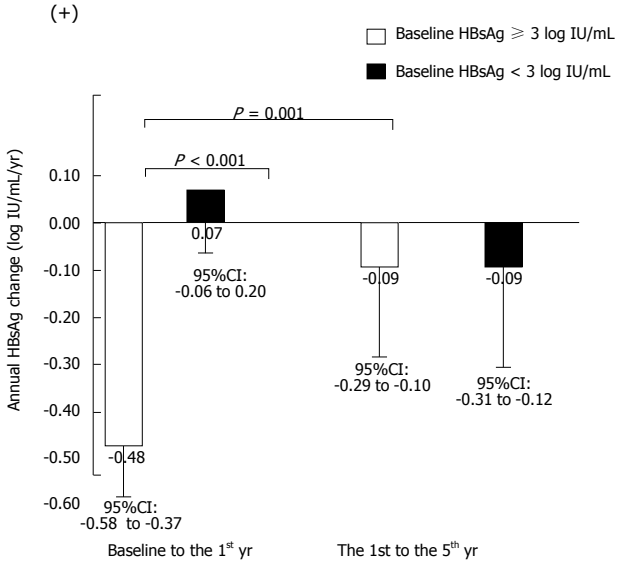

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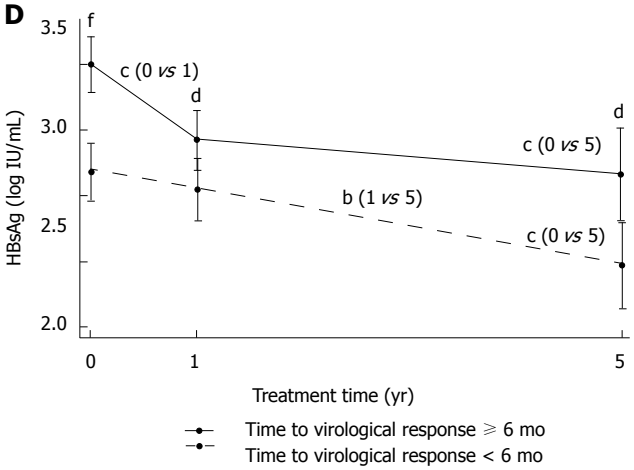

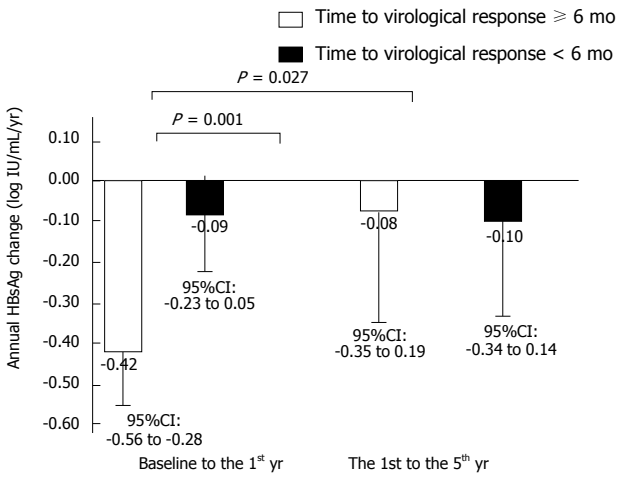

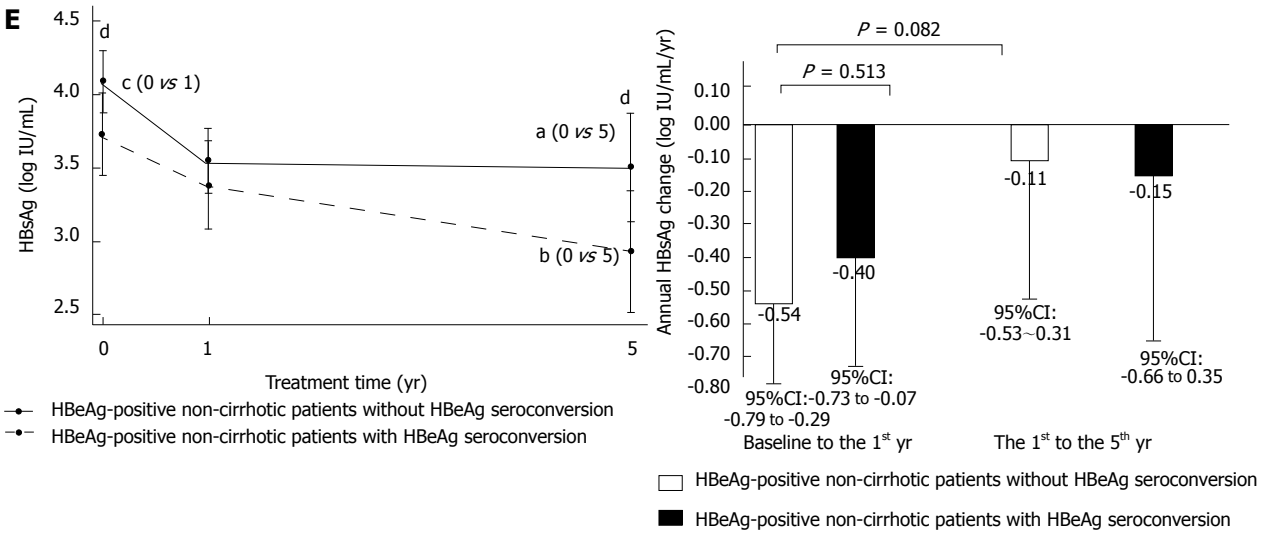

Figure 3 HBsAg kinetics during entecavir treatment. A: HBsAg levels at different time points, categorized by baseline HBeAg and cirrhosis status; B: Annual HBsAg changes in different periods, categorized by baseline HBeAg and cirrhosis status; C: HBsAg levels and annual HBsAg changes, categorized by baseline HBsAg < 3 log IU/mL and ⩾ 3 log IU/mL; D: HBsAg levels and annual HBsAg changes, categorized by time to virological response < 6 mo and ⩾ 6 mo; E: HBsAg levels and annual HBsAg changes, categorized by HBeAg seroconversion in HBeAg-positive non-cirrhotic patients. Error bars represent 95% confidence intervals. Comparisons between different time points or periods for one patient group: $^{a}P < 0.05$, $^{b}P < 0.005$, $^{c}P < 0.001$; Comparisons between different groups at one specific time point or period: $^{d}P < 0.05$, $^{e}P < 0.005$, $^{f}P < 0.001$.

showed that a baseline HBsAg level of < 2.4 log IU/mL was independently associated with an earlier virological response (HR: 3.12, 95%CI: 1.58-6.19, $P = 0.001$, Table 3). In HBeAg-negative cirrhotic patients ($n = 52$), baseline HBsAg levels failed to predict virological response.

Serological response

Sixty-one HBeAg-positive patients were assessed for serological response. Thirty-three patients (33/61, 54.1%) achieved HBeAg seroclearance during entecavir treatment. The median time to HBeAg seroclearance was 1.21 (0.19-6.99) years. HBeAg seroclearance occurred more rapidly in patients with a baseline HBsAg level of < 4 log IU/mL, compared with those with a baseline HBsAg level ⩾ 4 log IU/mL ($P = 0.002$, Figure 2A). Statistical significance remained after adjustment (multivariate: HR: 5.74, 95%CI: 2.19-15.00, $P < 0.001$, Table 4).

HBeAg seroconversion occurred in 22 patients (22/61, 36.1%). The median time to HBeAg seroconversion was 1.21 (0.21-7.49) years. Patients with a baseline HBsAg level of < 4 log IU/mL achieved HBeAg seroconversion more rapidly ($P = 0.022$, Figure 2B). Multivariate analysis showed that an HBV DNA level of ⩽ 5 log IU/mL and HBsAg level of < 4 log IU/mL were independently associated with earlier HBeAg seroconversion (HBV DNA ⩽ 5 log IU/mL *vs* > 5 log IU/mL: HR: 4.15, 95%CI: 1.05-16.44, $P = .043$; HBsAg < 4 log IU/mL *vs* ⩾ 4 log IU/mL: HR: 5.05, 95%CI: 1.58-16.14, $P = 0.006$, Table 5).

Biochemical response

One hundred and sixty-eight patients with elevated baseline ALT levels were assessed for biochemical response to treatment. One hundred and thirty-four patients (134/168, 79.8%) achieved biochemical response during entecavir treatment. The median time to biochemical response was 0.26 (0.04-3.09) years. In HBeAg-positive patients with elevated baseline ALT levels ($n = 52$), an HBsAg level of < 4 log IU/mL was not associated with an earlier biochemical response, as observed in Kaplan-Meier analysis and univariate analysis (Supplemental Figure 1 and Supplemental Table 2). However, multivariate analysis showed that HBV genotype B and an HBsAg level of < 4 log IU/mL were independently associated with more rapid biochemical response (genotype B *vs* C, HR: 4.59, 95%CI: 1.60-13.15, $P = 0.005$; HBsAg < 4 log IU/mL *vs* ⩾ 4 log IU/mL, HR: 4.00, 95%CI: 1.41-11.36, $P = 0.009$, Supplemental Table 2). In HBeAg-negative patients with elevated baseline ALT levels ($n = 116$), the baseline HBsAg level failed to predict biochemical response, irrespective of cirrhosis status.

Clinical event: new hepatocellular carcinoma development

New HCC development was defined as HCC diagnosed after half a year of entecavir treatment in patients without a history of HCC. One hundred and seventy-nine patients who had no HCC before and within a half a year of entecavir treatment were assessed for new HCC development. New HCC occurred in 13 patients (13/179, 7.3%). The median time to the development of new HCC was 5.16 (2.11-8.65) years. Liver cirrhosis was associated with increased risk of new HCC ($P < 0.001$, Supplemental Figure 2). Multivariate analysis revealed that liver cirrhosis was the only independent

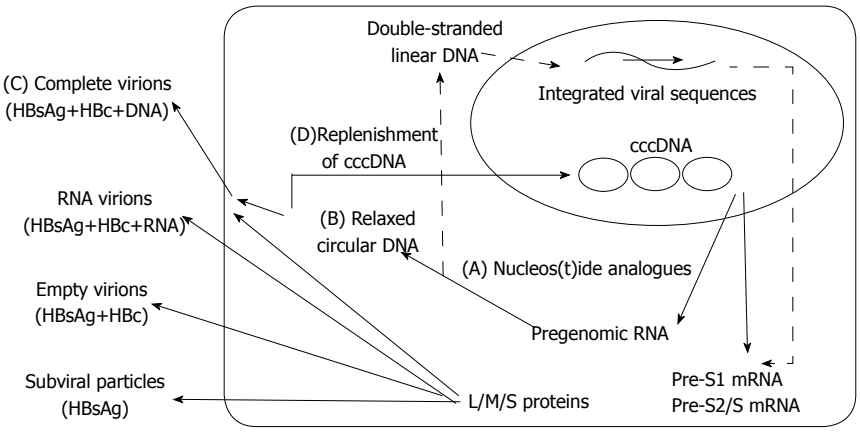

Figure 4 Possible mechanisms for HBsAg decline after nucleos(t)ide analogue treatment. A: Nucleos(t)ide analogues inhibit the activity of HBV reverse transcriptase; B: The production of relaxed circular DNA; C and D: The package andrelease of complete virions, and the replenishment of cccDNA. cccDNA: Covalently closed circular DNA; HBc: Hepatitis B core protein; HBsAg: Hepatitis B surface antigen; L/M/S proteins: Large, middle, and small envelope proteins; HBV: Hepatitis B virus.

risk factor for new HCC (HR: 13.02, 95%CI: 2.00-84.99, $P = 0.007$, Table 6).

Hepatitis B surface antigen kinetics

Serum HBsAg levels were determined at the baseline (211 patients; 211/211, 100%), 1-year (175 patients; 175/211, 82.9%), and 5-year time points (68 patients; 68/113, 60.2%) of entecavir treatment.

HBsAg levels at different time points, categorized by baseline HBeAg and cirrhosis status, are presented in Figure 3A. The HBeAg-positive non-cirrhotic group had significantly higher HBsAg levels at the baseline, 1-year, and 5-year time points, compared with the other groups, except for the HBeAg-positive cirrhotic group at the 5-year time point, which had a similar trend but did not reach statistical significance. Annual HBsAg changes in different periods, categorized by HBeAg and cirrhosis status, are presented in Figure 3B. The HBsAg decrease in the first year for the HBeAg-positive non-cirrhotic group was higher than that for the other three groups; moreover, the HBsAg decrease in the first year was higher than the decrease observed from the first to the fifth year for the HBeAg-positive non-cirrhotic group.

HBsAg levels at different time points and annual HBsAg changes in different periods, categorized by baseline HBsAg < 3 and ⩾ 3 log IU/mL, are presented in Figure 3C. Patients with baseline HBsAg levels ⩾ 3 log IU/mL had higher HBsAg at the baseline, 1-year and 5-year time points than those with baseline HBsAg levels < 3 log IU/mL. The annual HBsAg decrease in the first year for patients with baseline HBsAg levels ⩾ 3 log IU/mL was higher than that for patients with baseline HBsAg levels < 3 log IU/mL. Furthermore, the HBsAg decrease in the first year was higher than the decrease from the first to the fifth year for patients with baseline HBsAg levels ⩾ 3 log IU/mL.

HBsAg levels at different time points and annual HBsAg changes in different periods, categorized by time to virological response < 6 mo and ⩾ 6 mo, are presented in Figure 3D. Patients with a time to virological response of ⩾ 6 mo had higher HBsAg levels at the baseline, 1-year and 5-year time points, compared with those with a time to virological response of < 6 mo. The annual HBsAg decrease in the first year for patients with a time to virological response of ⩾ 6 mo was higher than for patients with a time to virological response of < 6 mo. Moreover, the HBsAg decrease in the first year was higher than that from the first to the fifth year for patients with a time to virological response of ⩾ 6 mo.

HBsAg levels at different time points and annual HBsAg changes in different periods, categorized by HBeAg-positive non-cirrhotic patients with and without HBeAg seroconversion, are presented in Figure 3E. HBeAg-positive non-cirrhotic patients without HBeAg seroconversion had higher baseline and five-year HBsAg levels than those with HBeAg seroconversion. HBeAg-positive non-cirrhotic patients without HBeAg seroconversion had a trend of greater annual HBsAg decrease in the first year than from the first to the fifth year, but these results did not reach statistical significance, which may be due to the limited case number at the 5-year time point.

DISCUSSION

Our data demonstrate that baseline HBsAg levels can be used to predict virological, serological, and biochemical responses in treatment-naïve chronic hepatitis B patients during entecavir treatment. Furthermore, our study provides a global view of HBsAg kinetics in chronic hepatitis B patients, categorized by baseline HBeAg and cirrhosis status during long-term entecavir treatment. The HBeAg-positive non-cirrhotic group had the highest HBsAg levels at the baseline and throughout entecavir treatment, compared with the other three patient groups. Although HBsAg levels decreased slowly during long-term entecavir treatment, a rapid rate of

HBsAg decrease was seen in the first year for HBeAg-positive non-cirrhotic patients.

Previous studies have shown that lower baseline HBsAg levels were also associated with higher chances of HBV DNA suppression[5,20,21,24], HBeAg seroclearance[21,24], and HBsAg seroclearance[25,26]. Because HBeAg-positive patients had higher HBsAg levels than HBeAg-negative patients (Table 1), the finding that the cut-off values of HBsAg for predicting treatment response were different between HBeAg-positive and HBeAg-negative patients is reasonable. In the current study, using an HBsAg cut-off value of 4 log IU/mL indicated that lower baseline HBsAg levels were associated with an earlier virological response, earlier HBeAg seroconversion, and earlier biochemical response in HBeAg-positive patients, and using a cut-off value of 2.4 log IU/mL, lower baseline HBsAg levels were found associated with an earlier virological response in HBeAg-negative non-cirrhotic patients.

HBsAg levels decreased slowly during entecavir treatment in most patients. In HBeAg-positive non-cirrhotic patients, a higher rate of HBsAg decrease was observed in the first year of treatment (Figure 3A and 3B). When patients were categorized according to baseline HBsAg levels, time to virological response, and time to HBeAg seroconversion, higher rates of HBsAg decrease were noted in the first year of treatment for patients with higher baseline HBsAg levels, patients with longer time to virological response, and patients without HBeAg seroconversion (Figure 3C, D, and E). These findings demonstrate that higher rates of HBsAg decrease occurred in the first year of treatment for patients who had higher baseline HBsAg levels. Therefore, rapid rates of HBsAg decline did not necessarily guarantee better clinical outcomes. When interpreting HBsAg changes, both HBsAg levels and decrease rates should be considered according to disease status. These findings are compatible to those of previous studies[27-29]. Because HBsAg levels decrease slowly during entecavir treatment, it could be checked at a 1-year interval if HBV DNA remains undetectable, as mentioned in a recent hepatitis B treatment guideline[1].

The difference in HBsAg kinetic patterns between HBeAg-positive and HBeAg-negative patients is appealing to consider. This could be related to the dissimilar activity of virus in patients with different HBeAg statuses. HBV cccDNA is the template for pre-S1 mRNA (2.4 kb), pre-S2/S mRNA (2.1 kb), preC mRNA (3.5 kb), pregenomic RNA (3.5 kb), and HBx mRNA (0.7 kb) transcription. Large (L) envelope proteins are translated from the pre-S1 mRNA, whereas middle (M) and small (S) envelope proteins are translated from the pre-S2/S mRNA[12,30]. Serum HBsAg consists of L, M, and S envelope proteins from complete virions (Dane particles), RNA virions, empty virions, and subviral particles (SVP, noninfectious HBsAg particles with spherical or filamentous forms). The amount of SVPs outnumbers complete virions by 1000-fold or greater[12, 30-32]. HBsAg is derived not only from cccDNA but also from integrated HBV DNA sequences[12, 30, 31]. NA inhibits the activity of HBV reverse transcriptase, posing an obstacle to the production of relaxed circular DNA, the packaging and release of complete virions, and the replenishment of cccDNA (Figure 4)[2,30,32]. Notably, these integrated sequences constitute a considerable part of the intrahepatic HBV DNA, and serum HBsAg circulates mainly as defective particles in HBeAg-negative patients[31,33]. This might account for the differences in HBsAg kinetics between HBeAg-positive and HBeAg-negative patients.

There are limitations to this study: it is a retrospective study of a single medical center, thus limiting the diversity of our patient population. In addition, serum samples were unavailable for 39.8% of enrolled patients at the five-year time point, which may have yielded some nonsignificant results for the HBeAg-positive cirrhotic patient group.

In conclusion, this study demonstrated that baseline HBsAg levels could be used to predict virological, serological, and biochemical responses during entecavir treatment. Although HBsAg levels decreased slowly during the treatment, a higher rate of HBsAg decrease was found in the first year of treatment for HBeAg-positive non-cirrhotic patients. Higher rates of HBsAg decrease were observed in the first year for patients with higher baseline HBsAg levels. A rapid HBsAg decline did not necessarily guarantee better outcomes. Clinicians interpreting HBsAg kinetics should consider HBsAg levels and decrease rates together according to a patient's disease status.

ARTICLE HIGHLIGHTS

Research Background

Hepatitis B surface antigen (HBsAg) levels have been studied in the natural course and pegylated interferon treatment course. During nucleos(t)ide analogue (NA) therapy, there are still controversies about using HBsAg to predict treatment responses, especially in HBeAg-negative patients. Besides, HBsAg kinetics and its relationships with outcomes during long-term entecavir therapy have not been fully elucidated.

Research motivation

We hoped to elucidate the utility of HBsAg in the prediction of treatment response in HBeAg-positive and HBeAg-negative patients. Furthermore, we would like to demonstrate the detailed HBsAg kinetics among different disease statuses and their relationships with the treatment outcomes.

Research objectives

We aimed to investigate the utility and kinetics of serum HBsAg in chronic hepatitis B patients during long-term entecavir treatment.

Research methods

We conducted this retrospective study to analyze the relationships between HBsAg levels and treatment responses in treatment-naïve chronic hepatitis B patients receiving at least two years of consecutive entecavir treatment. Patients were followed up at three to six month intervals with liver biochemistry, hepatitis B virus DNA, and abdominal sonography. Serum HBsAg levels were

determined at baseline, one year and five year time points. The cumulative incidence of treatment responses were obtained using the Kaplan-Meier analysis. Multivariate analysis was performed using Cox proportional hazards regression. A linear mixed model with a random intercept was used for analysis of longitudinal changes of HBsAg levels.

Research results

We demonstrated that baseline HBsAg levels could be used to predict treatment responses in HBeAg-positive patients with a cut-off value of 4 log IU/mL and in HBeAg-negative non-cirrhotic patients with a cut-off value of 2.4 log IU/mL. Furthermore, our study provides a global view of HBsAg kinetics in chronic hepatitis B patients during long-term entecavir therapy. The HBeAg-positive non-cirrhotic group had the highest HBsAg levels at the baseline and throughout entecavir treatment, as compared with the other three patient groups. Higher rates of HBsAg decrease were observed in the first year for patients with higher baseline HBsAg levels. A rapid HBsAg decline did not necessarily guarantee better outcomes

Research conclusions

Baseline HBsAg levels could be used to predict virological, serological, and biochemical responses. In the interpretation of HBsAg changes, HBeAg levels and decrease rates should be considered together according to a patient's disease status.

Research perspectives

HBsAg is a useful biomarker for chronic hepatitis B patients receiving NA therapy. It deserves to be studied in large prospective cohorts with different comorbidities for the future research.

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ORIGINAL ARTICLE

Retrospective Study

Performance of transient elastography in assessing liver fibrosis in patients with autoimmune hepatitis-primary biliary cholangitis overlap syndrome

Hui-Min Wu, Li Sheng, Qi Wang, Han Bao, Qi Miao, Xiao Xiao, Can-Jie Guo, Hai Li, Xiong Ma, De-Kai Qiu, Jing Hua

Hui-Min Wu, Li Sheng, Qi Wang, Han Bao, Qi Miao, Xiao Xiao, Can-Jie Guo, Hai Li, Xiong Ma, De-Kai Qiu, Jing Hua, Division of Gastroenterology and Hepatology, Renji Hospital, Shanghai 200127, China

Hui-Min Wu, Li Sheng, Qi Wang, Han Bao, Qi Miao, Xiao Xiao, Can-Jie Guo, Hai Li, Xiong Ma, De-Kai Qiu, Jing Hua, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China

Hui-Min Wu, Li Sheng, Qi Wang, Han Bao, Qi Miao, Xiao Xiao, Can-Jie Guo, Hai Li, Xiong Ma, De-Kai Qiu, Jing Hua, Shanghai Institute of Digestive Disease, Key Laboratory of Gastroenterology and Hepatology, Ministry of Health, Shanghai 200127, China

ORCID number: Hui-Min Wu (0000-0002-3991-3857); Li Sheng (0000-0002-3641-5211); Qi Wang (0000-0002-6937-8306); Han Bao (0000-0003-2934-3990); Qi Miao (0000-0003-3370-9692); Xiao Xiao (0000-0002-7936-336X); Can-Jie Guo (0000-0001-5951-299X); Hai Li (0000-0001-9937-8852); Xiong Ma (0000-0002-7047-1962); De-Kai Qiu (0000-0002-6312-1702); Jing Hua (0000-0002-6100-9477).

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Correspondence to: Jing Hua, MD, PhD, Professor, Division of Gastroenterology and Hepatology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai Institute of Digestive Disease, No. 160, Pujian Road, Shanghai 200127, China. hua-jing88@hotmail.com
Telephone: +86-21-68383113
Fax: +86-21-63200874

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Abstract

AIM

To investigate the performance of transient elastography (TE) for diagnosis of fibrosis in patients with

autoimmune hepatitis-primary biliary cholangitis (AIH-PBC) overlap syndrome.

METHODS

A total of 70 patients with biopsy-proven AIH-PBC overlap syndrome were included. Spearman correlation test was used to analyze the correlation of liver stiffness measurement (LSM) and fibrosis stage. Independent samples Student's *t*-test or one-way analysis of variance was used to compare quantitative variables. Receiver operating characteristics (ROC) curve was used to calculate the optimal cut-off values of LSM for predicting individual fibrosis stages. A comparison on the diagnostic accuracy for severe fibrosis was made between LSM and other serological scores.

RESULTS

Patients with AIH-PBC overlap syndrome had higher median LSM than healthy controls (11.3 ± 6.4 kPa *vs* 4.3 ± 1.4 kPa, $P < 0.01$). LSM was significantly correlated with fibrosis stage ($r = 0.756$, $P < 0.01$). LSM values increased gradually with an increased fibrosis stage. The areas under the ROC curves of LSM for stages F $\geqslant$ 2, F $\geqslant$ 3, and F4 were 0.837 (95%CI: 0.729-0.914), 0.910 (0.817-0.965), and 0.966 (0.893-0.995), respectively. The optimal cut-off values of LSM for fibrosis stages F $\geqslant$ 2, F $\geqslant$ 3, and F4 were 6.55, 10.50, and 14.45 kPa, respectively. LSM was significantly superior to fibrosis-4, glutaglumyl-transferase/platelet ratio, and aspartate aminotransferase-to-platelet ratio index scores in detecting severe fibrosis (F $\geqslant$ 3) (0.910 *vs* 0.715, $P < 0.01$; 0.910 *vs* 0.649, $P < 0.01$; 0.910 *vs* 0.616, $P < 0.01$, respectively).

CONCLUSION

TE can accurately detect hepatic fibrosis as a non-invasive method in patients with AIH-PBC overlap syndrome.

Key words: Liver stiffness measurement; Transient elastography; Liver fibrosis; Autoimmune hepatitis; Primary biliary cholangitis; Overlap syndrome

Core tip: Our research determined that transient elastography can accurately detect hepatic fibrosis as a non-invasive method in patients with autoimmune hepatitis-primary biliary cholangitis overlap syndrome. Liver stiffness measurements were significantly superior to fibrosis-4, glutaglumyl-transferase/platelet ratio, and aspartate aminotransferase-to-platelet ratio index scores for detecting severe fibrosis.

Wu HM, Sheng L, Wang Q, Bao H, Miao Q, Xiao X, Guo CJ, Li H, Ma X, Qiu DK, Hua J. Performance of transient elastography in assessing liver fibrosis in patients with autoimmune hepatitis-primary biliary cholangitis overlap syndrome. *World J Gastroenterol* 2018; 24(6): 737-743 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i6/737.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i6.737

INTRODUCTION

Liver fibrosis, characterized by the accumulation of the extracellular matrix, has been considered a common progressive stage and predictor of undesirable prognosis in various chronic liver diseases[1]. The evaluation of fibrosis is crucial in the assessment of chronic liver diseases. Liver biopsy remains the gold standard for evaluating fibrosis but has some drawbacks, which has led to the development of non-invasive methods.

Transient elastography (TE) by FibroScan based on ultrasound can noninvasively evaluate liver fibrosis *via* liver stiffness measurement (LSM)[2]. It has been introduced as a non-invasive surrogate to liver biopsy for detection of hepatic fibrosis in patients with various chronic liver diseases[3-6]. Numerous studies have shown that LSM values have a good correlation with histological fibrosis stages. Furthermore, compared to other serum biomarkers, such as aspartate aminotransferase (AST)-to-platelet ratio index (APRI), and fibrosis-4 (FIB-4) scores, LSM is probably the most validated non-invasive method to assess the stage of fibrosis with better diagnostic accuracy[3,7,8].

Autoimmune hepatitis (AIH) and primary biliary cholangitis (PBC) are two immune-mediated chronic liver diseases targeting hepatocytes and biliary epithelial cells, respectively[9]. The overlap syndrome of AIH-PBC shares clinical, biochemical, serological, and histological features of patients with AIH and PBC[10]. AIH is usually a chronic and long-term disease, and approximately 25% of patients with AIH progress to hepatic fibrosis or cirrhosis despite immunosuppression intervention[11]. Recently, studies reported that patients with AIH-PBC overlap syndrome exhibited a lower response rate to ursodeoxycholic acid and steroid combination therapy while having a more severe clinical prognosis compared with AIH patients[12]. Therefore, accurately assessing hepatic fibrosis and initiating anti-fibrotic intervention are essential for some AIH-PBC overlap syndrome patients with rapid disease progression. Our recent study revealed that TE can reliably stage liver fibrosis in AIH patients[13]. However, the evaluation of TE in assessing liver fibrosis in patients with AIH-PBC overlap syndrome remains limited.

In the current study, we assessed the diagnostic performance of TE in evaluating liver fibrosis in biopsy-proven AIH-PBC overlap syndrome patients and made a comparison with other non-invasive methods.

MATERIALS AND METHODS

Patients

Patients who were suspected to have AIH-PBC overlap syndrome and eventually underwent liver biopsy at

Renji Hospital (Shanghai, China) from September 2014 to July 2017 were recruited in this retrospective study. Ultimately, a total of 70 patients met the Paris AIH-PBC overlap syndrome criteria[10]. Briefly, the requirement of at least two out of three specific criteria in each condition was met. The PBC criteria included: (1) alkaline phosphatase (ALP) levels at least twice the upper limit of normal (ULN) or gamma glutamyltransferase (GGT) levels at least five times the ULN; (2) anti-mitochondrial antibody (AMA) positivity; and (3) florid bile duct lesion in liver biopsy. The AIH criteria comprised of: (1) alanine aminotransferase (ALT) levels at least five times the ULN; (2) IgG levels at least twice the ULN or anti-smooth muscle antibody (ASMA) positivity; and (3) moderate to severe periportal or periseptal lymphocytic piecemeal necrosis in histological examination. All patients received no immunosuppressive therapy before liver biopsy.

The exclusion criteria included chronic infection with hepatic virus B or C, primary sclerosing cholangitis (PSC), PBC, AIH, drug-induced liver disease, alcoholic or non-alcoholic fatty liver disease, hereditary metabolic liver disease, hepatobiliary parasitic infection, and severe systemic disease. Patients with acute severe attack with markedly elevated serum aminotransferase levels (ALT > 1000 U/L) and severe jaundice were excluded. Decompensated cirrhosis with ascites, hepatic encephalopathy, gastrointestinal bleeding, and hepatic carcinoma were excluded. Each participant of this study provided written informed consent.

Histological examination

Percutaneous liver biopsy guided by ultrasound was performed in all patients under local anesthesia using a 16G disposable needle. Liver specimens at least 1 cm in length with eight complete portal tracts were included. The specimens were fixed in 10% neutral formalin immediately and embedded in paraffin. Hematoxylin and eosin staining was used to observe the morphology of the liver, and Masson's trichrome and reticulin staining was performed to detect fibrosis. Liver fibrosis and necroinflammatory activity were evaluated by a single experienced pathologist who was blinded to the patients' clinical data. A METAVIR-derived scoring system was used for evaluating liver fibrosis and inflammation by a single senior pathologist[14]. In brief, the fibrosis stage description was F0, no fibrosis; F1, portal fibrosis without septa; F2, portal and periportal fibrosis with few septa; F3, portal and periportal fibrosis with numerous septa without cirrhosis; and F4, cirrhosis. Hepatic inflammatory activity was graded as follows: A0, none; A1, mild; A2, moderate; and A3, severe.

Clinical measurements

Medical records of all patients diagnosed with AIH-PBC overlap syndrome were reviewed, and clinical data and laboratory findings were collected and analyzed. Laboratory evaluations included liver biochemistry [*i.e.*, ALT, AST, ALP, GGT, total bilirubin, direct bilirubin, globulin, and albumin], serum immunoglobulins (IgG, IgM, and IgA), routine blood test (white blood cell count and platelet count), and prothrombin time. APRI score was calculated by using the formula of Wai *et al*[15] [(AST/ULN considered as 40 IU/L)/platelet count $\times 10^9$/L]. FIB-4 score was calculated by using the formula of Sterling *et al*[16] [(age × AST)/(platelet count × ALT$^{1/2}$)]. GGT/platelet ratio (GPR) was calculated by using the formula of Lemoine *et al* (GGT/platelet count × 10^9/L)[17]. The serum autoantibodies, including anti-nuclear antibody (ANA), AMA, and ASMA were detected by indirect immunofluorescence (Euroimmun AG, Hangzhou, China).

LSM by TE

TE measured with a FibroScan device and an M probe ultrasound transducer (both from Echosens, Paris, France) was performed in all patients who underwent liver biopsy on the same day. According to the ultrasonography images, we selected an appropriate area for detection that contained at least 6-cm-thick liver parenchyma with absence of main blood vessels and the gallbladder. We obtained 10 valid LSMs from each participant and considered LSM with an interquartile range $\leqslant$ 30% and a success rate $\geqslant$ 60% as reliable. The median value represented the value of LSM expressed in kilopascals (kPa). All the measurements were performed following the manufacturer's instructions. LSM assessment of 25 healthy people as normal controls was also performed.

Statistical analysis

Data were analyzed using SPSS software version 22.0 (SPSS Inc, Chicago, IL, United States). Normally distributed continuous variables are expressed as the mean ± SD. Quantitative variables were compared using independent samples Student's *t*-test or one-way analysis of variance when appropriate. The Spearman's rank correlation test was used to explore the correlation between the LSM and fibrosis grade. The diagnostic accuracy of LSM for the prediction of fibrosis stages was calculated using a receiver operator characteristic (ROC) curve. Optimal LSM cut-off values for F2-4 fibrosis were determined based on the highest combined sensitivity and specificity (Youden index). The performance characteristics of each cut-off value in terms of sensitivity and specificity were calculated. The areas under the ROC curves (AUROCs) were calculated to compare the diagnostic efficiency of each noninvasive predictor for severe fibrosis. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Characteristics of the patients

A total of 70 biopsy-proven AIH-PBC overlap syndrome patients were included, with a mean age of 46.6 ± 10.2, and 84.3% of patients were female. In all

Table 1 Baseline characteristics of the patients with autoimmune hepatitis- primary biliary cholangitis overlap syndrome *n* (%)

Characteristic	*n* = 70
Age	46.6 ± 10.2
Gender	
Male	11 (15.7)
Female	59 (84.3)
Autoantibody positive rate	94.3
Liver function test (mean ± SD)	
ALT (U/L)	185.6 ± 238.9
AST (U/L)	166.6 ± 190.7
GGT (U/L)	363.2 ± 393.3
ALP (U/L)	318.7 ± 245.9
Total bilirubin (μmol/L)	32.3 ± 33.9
Albumin (g/L)	40.1 ± 8.0
Serum IgG level (g/L)	17.0 ± 5.1
Serum IgM level (g/L)	4.2 ± 6.9
Biochemical score (mean ± SD)	
APRI	2.47 ± 3.85
FIB-4	3.22 ± 3.53
GPR	1.93 ± 2.28
Liver biopsy	
Fibrosis stage	
0	0 (0)
1	9 (12.9)
2	29 (41.4)
3	25 (35.7)
4	7 (10.0)
Hepatic inflammatory activity	
0	0 (0)
1	1 (1.4)
2	30 (42.3)
3	39 (55.7)
LSM value (kPa, maean ± SD)	11.3 ± 6.4

AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; APRI: Aspartate aminotransferase-to-platelet ratio index; FIB-4: Fibrosis-4; GGT: Gamma glutamyltransferase; GPR: Gamma glutamyltransferase/platelet ratio; LSM: Liver stiffness measurement.

patients, the prevalence of autoantibodies, including ANA, AMA, and ASMA, was 94.3%. AIH-PBC overlap syndrome was diagnosed based on the Paris criteria. The general characteristics of the patients are shown in Table 1.

Relationship between LSM and histological fibrosis stage

LSM was successfully performed in all patients. The mean LSM value of all AIH-PBC overlap syndrome patients was clearly higher than that of healthy normal controls (11.3 ± 6.4 kPa *vs* 4.3 ± 1.4 kPa, $P < 0.01$). LSM values for fibrosis stages F1, F2, F3, and F4 were 6.9 ± 3.4 kPa, 8.3 ± 2.0 kPa, 13.3 ± 5.5 kPa, and 22.8 ± 8.3 kPa, respectively. LSM was closely correlated with fibrosis stage ($r = 0.756$, $P < 0.01$). Patients with higher fibrosis stages usually had higher LSM values (Figure 1).

Diagnostic accuracy of LSM

The AUROC values of LSM in detecting significant fibrosis (F ⩾ 2), severe fibrosis (F ⩾ 3), and cirrhosis (F4) were 0.837, 0.910, and 0.966, respectively (Figure 2). The optimal cut-off values of LSM for fibrosis stages were 6.55 pKa for F ⩾ 2, 10.50 kPa for F ⩾ 3, and 14.45 pKa for F4 with the highest combined sensitivity and specificity (Table 2).

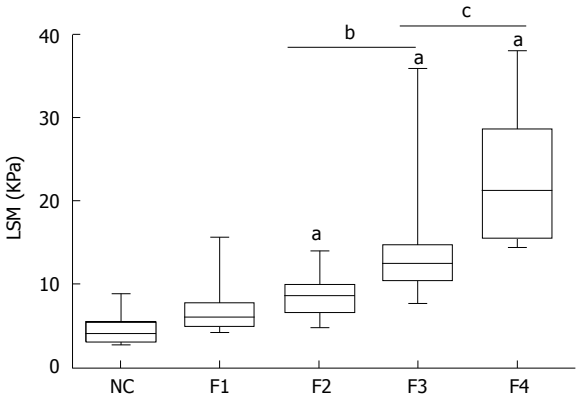

Figure 1 Correlation between liver stiffness measurement and histological fibrosis stage. LSM values were assessed by TE in healthy normal controls and AIH-PBC overlap syndrome patients with different liver fibrosis stages based on the results of liver biopsy (F0-4). [a]$P < 0.01$, F2, 3, and 4 *vs* NC; [b]$P < 0.01$, F3 *vs* F2; [c]$P < 0.01$, F4 *vs* F3. LSM: Liver stiffness measurement; NC: Normal controls; AIH-PBC: Autoimmune hepatitis-primary biliary cholangitis; TE: Transient elastography.

Comparison of the diagnostic accuracy of LSM for severe fibrosis with other serological scores

The biochemical scores FIB-4, GPR, and APRI were calculated for all patients based on their laboratory parameters. AUROCs of FIB-4, APRI, and GPR for detecting severe fibrosis (F ⩾ 3) were 0.715 (95%CI: 0.594-0.816), 0.649 (0.525-0.759), and 0.616 (0.0.492-0.730), respectively. LSM was superior to FIB-4, GPR, and APRI in detecting severe fibrosis (F ⩾ 3) by AUROC values (0.910 *vs* 0.715, $P < 0.01$; 0.910 *vs* 0.649, $P < 0.01$; 0.910 *vs* 0.616, $P < 0.01$, respectively) (Figure 3).

DISCUSSION

Timely and accurate assessment of the degree of liver fibrosis is crucial to the evaluation of disease progression and decision of therapeutic schedule in various chronic liver diseases[18]. LSM assessed by TE was introduced and widely used as an effective and promising non-invasive tool for assessment of liver fibrosis in patients with chronic hepatitis B and C, as well as non-alcoholic fatty liver diseases[19-21]. In the current study, we found that LSM had a strong correlation with histological fibrosis stage in patients with AIH-PBC overlap syndrome, while it was significantly superior to FIB-4, GPR, and APRI scores in detecting severe fibrosis.

Our study illustrated a favorable diagnostic performance of LSM for assessing different fibrosis stages in AIH-PBC overlap syndrome. The AUROCs of LSM in detecting significant fibrosis (F ⩾ 2), severe fibrosis (F ⩾ 3), and cirrhosis (F4) were 0.837, 0.910, and 0.966, respectively. The cut-off values for predicting

Table 2 Diagnostic accuracy of liver stiffness measurement in detecting liver fibrosis in patients with autoimmune hepatitis-primary biliary cholangitis overlap syndrome

stage	AUROC (95%CI)	Cut-off (kPa)	Sensitivity	Specificity	PPV	NPV	+LR	-LR
F ≥ 2	0.837 (0.729-0.914)	6.55	0.902	0.778	0.965	0.538	4.06	0.13
F ≥ 3	0.910 (0.817-0.965)	10.50	0.844	0.921	0.900	0.875	10.69	0.17
F = 4	0.966 (0.893-0.995)	14.45	1.000	0.889	0.500	1.000	9.00	0.00

AUROC: Areas under the receiver operator characteristic; PPV: Positive predictive value; NPV: Negative predictive value; +LR: Positive likelihood ratio; -LR: Negative likelihood ratio.

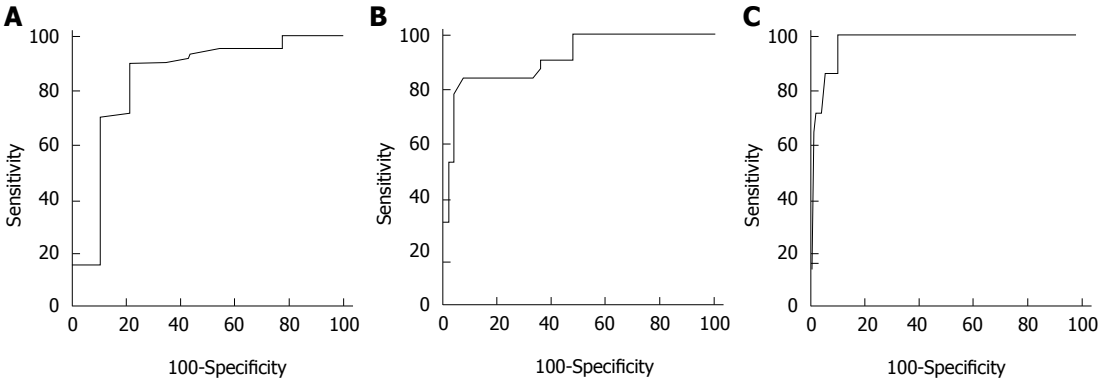

Figure 2 The receiver operator characteristic curve of liver stiffness measurement for the diagnosis of liver fibrosis stage. A: Significant fibrosis (F ⩾ 2); B: Severe fibrosis (F ⩾ 3); C: Cirrhosis (F4).

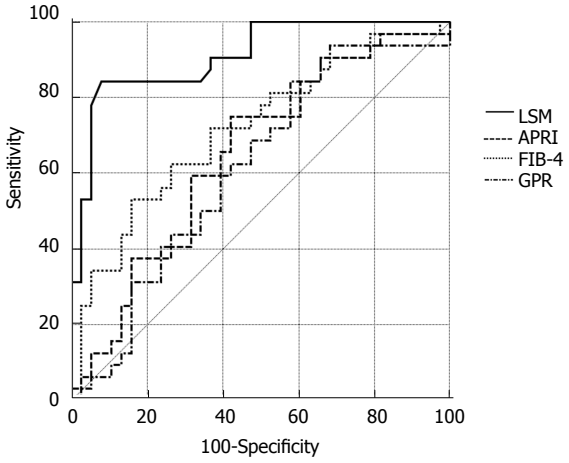

Figure 3 The receiver operator characteristic curves of liver stiffness measurement, fibrosis-4, aspartate aminotransferase-to-platelet ratio index, and GRP for the detection of severe fibrosis (F ⩾ 3). LSM: liver stiffness measurement; GPR: GGT/platelet ratio; FIB-4: Fibrosis-4; APRI: Aminotransferase -to-platelet ratio index.

F ⩾ 2, F ⩾ 3, and F4 were 6.55, 10.50, and 14.45 kPa, respectively. Compared with the cut-off values reported in our recent study in patients with AIH alone, in which the optimal LSM cut-off values for predicting significant fibrosis, severe fibrosis, and cirrhosis were 6.45, 8.75, and 12.5 kPa[13], respectively, the LSM cut-off values in AIH-PBC overlap syndrome were slightly higher, especially in severe fibrosis and cirrhosis. Another study reported that the optimal LSM cut-off values for significant fibrosis, severe fibrosis, and cirrhosis were 7.3 kPa, 9.8 kPa, and 17.3 kPa, respectively, in PBC and PSC patients[22]. It seemed that the patients with AIH-PBC overlap syndrome or PBC had relatively higher LSM values than AIH patients. The major reason is probably that AIH-PBC overlap syndrome patients have a poor treatment response and rapid disease progression. However, in a meta-analysis comprised of 22 studies and 4430 patients with different etiologies of liver disease, the estimated LSM cut-off values were 7.71 kPa for F ⩾ 2 and 15.08 kPa for F4[23]. Our findings are similar to these results, indicating that etiology of liver disease has no significant effect on LSM assessment.

LSM by TE had a favorable diagnostic performance in evaluating liver fibrosis as a non-invasive method. Compared with APRI and FIB-4, which are widely used as non-invasive serologic methods, LSM showed better accuracy in detecting liver fibrosis[6,24]. In the current study, we also found that LSM was significantly superior to FIB-4 and APRI in detecting severe fibrosis in AIH-PBC overlap syndrome patients, which is consistent with our previous result. In a recent study, GPR had exhibited a better accuracy than APRI and FIB-4 in detecting liver fibrosis in CHB patients[17]. Here, we found that GPR had a similar diagnostic performance for detecting severe fibrosis to APRI and FIB-4 scores but was inferior to LSM.

The major limitation of our study was the small patient sample size due to the low prevalence of AIH-PBC overlap syndrome. Larger studies are needed to confirm these results.

In conclusion, LSM by TE is an accurate and reliable

non-invasive method in evaluating liver fibrosis in patients with AIH-PBC overlap syndrome. LSM is significantly superior to APRI, GPR, and FIB-4 scores for detecting severe fibrosis.

ARTICLE HIGHLIGHTS

Research background

Transient elastography (TE) can reliably stage liver fibrosis *via* liver stiffness measurement (LSM) in chronic liver disease. However, the accuracy of TE for assessment of liver fibrosis in patients with autoimmune hepatitis-primary biliary cholangitis (AIH-PBC) overlap syndrome is still unclear.

Research motivation

It is important to identify non-invasive markers of liver fibrosis to predict disease progression.

Research objectives

We evaluated the performance and usefulness of TE for detection of fibrosis in these patients and compared TE with other non-invasive diagnostic tools.

Research methods

The diagnostic accuracy of LSM for the prediction of fibrosis stages was calculated using a receiver operator characteristic (ROC) curve. Optimal LSM cut-off values for F2-4 fibrosis were determined based on the highest combined sensitivity and specificity.

Research results

TE can accurately detect hepatic fibrosis as a non-invasive method in patients with AIH-PBC overlap syndrome.

Research conclusions

For the first time, the current study evaluated TE as a non-invasive assessment of liver fibrosis in patients with AIH-PBC overlap syndrome and demonstrated that it was a reliable tool that was superior to serum biomarker scores for predicting severe fibrosis.

Research perspectives

The impact of hepatic inflammation on LSM values was not analyzed due to the relatively small number of patients in each subgroup. Larger studies are needed to confirm these results.

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ORIGINAL ARTICLE

Clinical Practice Study

Value of contrast-enhanced ultrasound in the differential diagnosis of gallbladder lesion

Hui-Ping Zhang, Min Bai, Ji-Ying Gu, Ying-Qian He, Xiao-Hui Qiao, Lian-Fang Du

Hui-Ping Zhang, Min Bai, Ji-Ying Gu, Ying-Qian He, Xiao-Hui Qiao, Lian-Fang Du, Department of Ultrasound, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080, China

ORCID number: Hui-Ping Zhang (0000-0002-3890-6436); Min Bai (0000-0002-3513-3458); Ji-Ying Gu (0000-0002-0664-5753); Ying-Qian He (0000-0002-9758-0499); Xiao-Hui Qiao (0000-0003-2515-6477); Lian-Fang Du (0000-0001-5952-4368).

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Correspondence to: Lian-Fang Du, MD, Chief Doctor, Full Professor, Department of Ultrasound, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, 100 Haining Road, Shanghai 200080, China. du_lf@163.com
Telephone: +86-21-63240090

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Abstract

AIM

To describe contrast-enhanced ultrasound (CEUS) features and evaluate differential diagnosis value of CEUS and conventional ultrasound for patients with benign and malignant gallbladder lesions.

METHODS

This study included 105 gallbladder lesions. Before surgical resection and pathological examination, conventional ultrasound and CEUS were performed to examine for lesions. Then, all the lesions were diagnosed as (1) benign, (2) probably benign, (3) probably malignant or (4) malignant using both conventional ultrasound and CEUS. The CEUS features of these gallbladder lesions were analyzed and diagnostic efficiency between conventional ultrasound and CEUS was compared.

RESULTS

There were total 17 cases of gallbladder cancer and 88 cases of benign lesion. Some gallbladder lesions had typical characteristics on CEUS (*e.g.*, gallbladder adenomyomatosis had typical characteristics of small nonenhanced areas on CEUS). The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of CEUS were 94.1%, 95.5%, 80.0%, 98.8%

and 95.2%, respectively. These were significantly higher than conventional ultrasound (82.4%, 89.8%, 60.9%, 96.3% and 88.6%, respectively). CEUS had an accuracy of 100% for gallbladder sludge and CEUS helped in differential diagnosis among gallbladder polyps, gallbladder adenoma and gallbladder cancer.

CONCLUSION

CEUS may provide more useful information and improve the diagnosis efficiency for the diagnosis of gallbladder lesions than conventional ultrasound.

Key words: Contrast enhanced ultrasound; Conventional ultrasound; Gallbladder carcinoma; Gallbladder adenomyomatosis

Core tip: With the advent of ultrasound contrast agents, contrast-enhanced ultrasound (CEUS) is playing a more and more important role clinically. However, the value of CEUS in gallbladder lesions has not been widely accepted yet. In this study, we evaluated the differential diagnosis value of CEUS and conventional ultrasound for patients with benign and malignant gallbladder lesions. Our results showed that CEUS may provide more useful information and improve diagnosis efficiency for the diagnosis of gallbladder lesions than conventional ultrasound.

Zhang HP, Bai M, Gu JY, He YQ, Qiao XH, Du LF. Value of contrast-enhanced ultrasound in the differential diagnosis of gallbladder lesion. *World J Gastroenterol* 2018; 24(6): 744-751 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i6/744.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i6.744

INTRODUCTION

Conventional ultrasound is the primary and most important imaging modality for gallbladder diseases. The excellent image contrast between anechoic bile and gallbladder wall or gallbladder diseases, and the increasingly improved ultrasound spatial resolution ensure conventional ultrasound to have a high detection rate of gallbladder diseases[1]. With the advantages of real-time imaging, safety with no radiation, great cost effectiveness and great spatial resolution, conventional ultrasound makes itself more suitable than computed tomography (CT) and magnetic resonance imaging (MRI) for the detection of gallbladder diseases[2].

Despite the above-mentioned advantages of conventional ultrasound, the sensitivity and accuracy are not satisfactory, especially when stones or some other gallbladder lesions fill the gallbladder lumen[3,4]. With no information of microvascularity, it is very hard to differentiate some benign diseases, such as benign gallbladder wall thickening or motionless sludge, from malignant ones using conventional ultrasound. The application of microbubbles could help in the differential diagnosis by providing useful perfusion information in the lesions[5].

Contrast-enhanced ultrasound (CEUS) has been widely used in liver disease, with an excellent diagnostic efficiency comparable to contrast-enhanced CT[6-8]. The value of CEUS in other organs, such as kidney, breast, *etc.*, has also been well established and identified[9,10]. Although the value in gallbladder has not been recognized and accepted by the European Federation of Societies for Ultrasound in Medicine and Biology[11], there have been some studies which have shown the usefulness of CEUS in the differential diagnosis between benign and malignant gallbladder lesions[5,12].

In this study, we described CEUS features and evaluated differential diagnosis value of CEUS and conventional ultrasound for patients with benign and malignant gallbladder lesions.

MATERIALS AND METHODS

Study design

The Ethics Committee of our hospital approved this study. Before the sonographic examination, we obtained all patients' written informed consent. The features of gallbladder lesions in CEUS were analyzed and described retrospectively. The study and comparison of the diagnostic efficiency between CEUS and conventional ultrasound was designed prospectively.

Patients

Between December 2012 and October 2016, 136 gallbladder lesions in 133 patients were imaged using both conventional ultrasound and CEUS in our hospital. Of these, 31 lesions were excluded from this study because the patients did not undergo cholecystectomy and were without pathological diagnosis. Therefore, 105 gallbladder lesions in 103 patients (47 males and 56 females; mean age ± standard deviation, 42.5 ± 10.6 years) were included in this study.

Conventional ultrasound and CEUS

All the conventional ultrasound and CEUS examinations were performed by an ultrasound physician with thirteen years' experience in conventional ultrasound and five years' experience in CEUS. An Acuson S2000 diagnostic ultrasound system or an Acuson Sequoia 512 diagnostic ultrasound system (Siemens Medical Solutions, Mountain View, CA, United States) equipped with a transabdominal curvilinear transducer running on Cadence™ Contrast pulse sequence (CPS) software were used for all the ultrasound examinations. All the patients fasted at least for 8 h before the examinations.

Conventional ultrasound examinations were first performed to detect the gallbladder lesions.

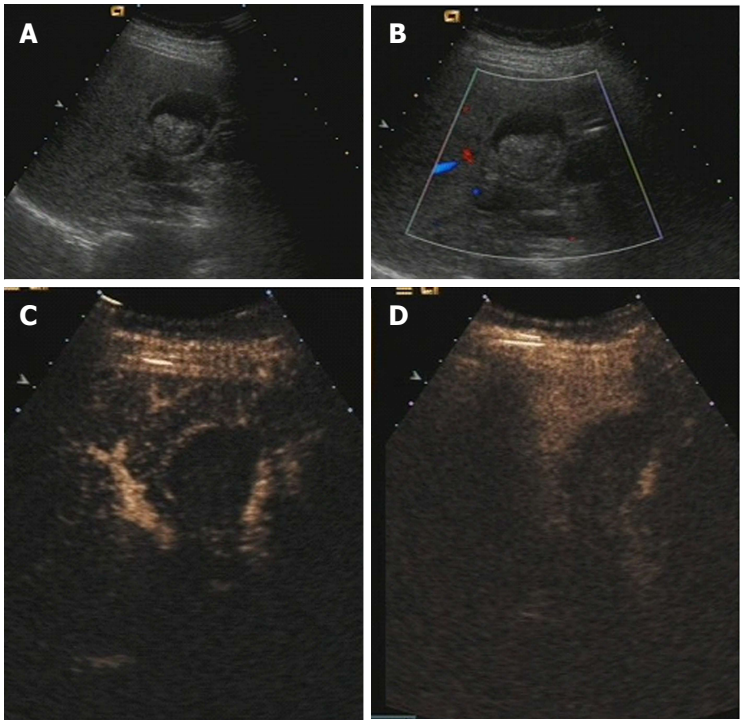

Figure 1 Gallbladder sludge in a 54-year-old female patient. A: B-mode sonography showed a hypoechoic, well-defined mass in the gallbladder, with an intact gallbladder wall; B: Color Doppler ultrasound showed no color Doppler signal in the lesion. According to A and B, a diagnosis of probably benign was made; C: CEUS showed complete nonenhancement on arterial phase; D: CEUS showed complete nonenhancement on venous phase. According to C and D, a diagnosis of benign gallbladder sludge was made. CEUS: Contrast-enhanced ultrasound.

Table 1 Diagnostic results of conventional ultrasound *n* (%)

	Benign		Malignant	
	definitely	probably	probably	definitely
Benign, *n* = 88	61 (69.3)	18 (20.5)	8 (9.1)	1 (1.1)
Malignant, *n* = 17	0 (0)	3 (17.6)	5 (29.4)	9 (52.9)

The lesion's size, location, shape, stalk, boundary, echogenicity and wall destruction were analyzed and recorded. Then, Doppler vascularity was observed using color Doppler ultrasound. A diagnosis of benign, probably benign, probably malignant or malignant was made according to conventional ultrasound features, by two radiologists with at least ten years' experience in both conventional ultrasound and CEUS. If they concluded different diagnosis, a third radiologist (with twenty-five years' experience in conventional ultrasound and twelve years' experience in CEUS) discussed together with them and decided on a final diagnosis.

For CEUS examinations, the same ultrasound machines were used. SonoVue (Bracco, Italy), the only microbubbles permitted for clinical use in China, was used in this study and was prepared following the appropriate guidelines before examinations. Every patient was instructed to take gentle and steady breaths to minimize the influence by respiratory movement. When the target lesion was shown clearly using conventional ultrasound, the CPS mode (MI: 0.21) was activated. A dose of 1.6 mL of SonoVue was administrated through the antecubital vein as a bolus immediately followed by 5 mL 0.9% saline solution. A stopwatch was started at the same time. The image was observed and recorded for 2 min and then the whole gallbladder and the liver were scanned to find other lesions and liver infiltration. After that, CEUS features of the lesion were analyzed and a diagnosis of benign, probably benign, probably malignant or malignant was made according to CEUS features by the above-mentioned radiologists.

After the resection of gallbladder lesions and the final pathological diagnosis was made, CEUS images were reviewed and the features of each kind of gallbladder lesions in CEUS were analyzed and summarized.

Statistical analysis

SPSS version 13.0 software (IBM Corporation, Chicago, IL, United States) was used for statistical analysis. $P < 0.05$ was considered a statistically significant difference. The diagnostic efficiency of conventional ultrasound and CEUS was assessed in terms of sensitivity, specificity, positive predictive value, negative predictive value and accuracy and was compared using chi-square test and Fisher's exact test.

RESULTS

Pathological results

There were 17 malignant and 88 benign gallbladder lesions in total in this study according to the histo-

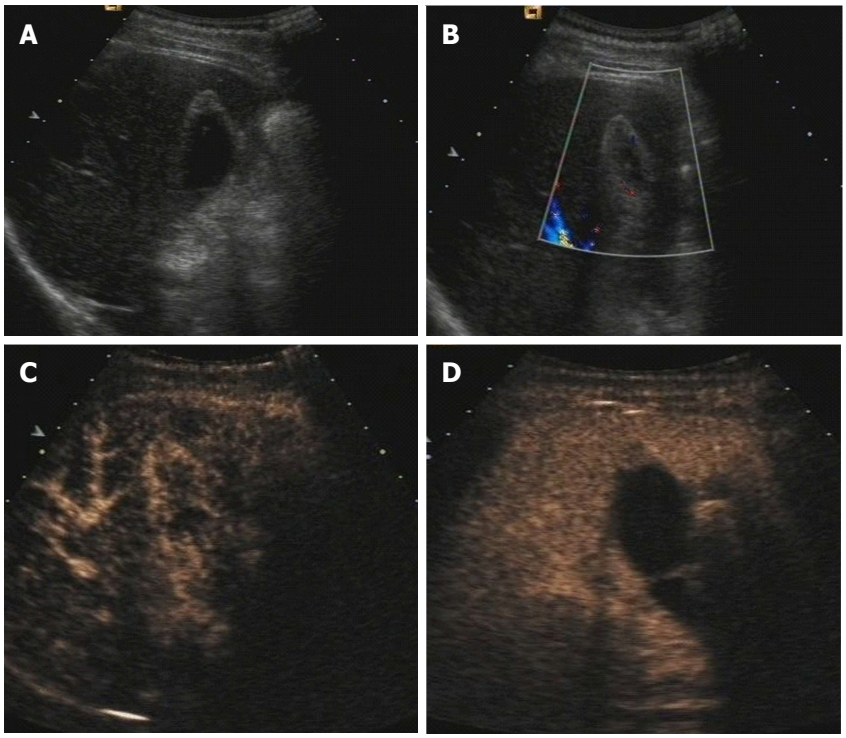

Figure 2 Gallbladder adenomyomatosis in a 62-year-old male patient. A: B-mode sonography showed a heterogeneously hypoechoic lesion on the gallbladder wall, with an intact gallbladder wall; B: Color Doppler ultrasound showed no color Doppler signal in the lesion. According to A and B, a diagnosis of probably benign was made. C: CEUS showed heterogeneously enhanced, with some small nonenhanced areas, on arterial phase; D: CEUS showed heterogeneously enhanced, with some small nonenhanced areas, on venous phase. According to C and D, a diagnosis of benign gallbladder adenomyomatosis was made. CEUS: Contrast-enhanced ultrasound.

Table 2 Diagnostic efficiency of conventional ultrasound and contrast-enhanced ultrasound between benign and malignant gallbladder lesions *n* (%)

Features of lesions	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
Conventional ultrasound	82.4	89.8	60.9	96.3	88.6
Contrast-enhanced ultrasound	94.1	95.5	80.0	98.8	95.2
P value	0.301	0.124	0.152	0.297	0.064

pathological diagnosis after cholecystectomy, including 17 cases of gallbladder cancer, 11 case of gallbladder sludge, 28 cases of gallbladder adenomyomatosis, 36 cases of gallbladder polyps and 13 cases of gallbladder adenoma.

Sonographic features on CEUS

All the cases of gallbladder sludge were shown as completely nonenhanced on CEUS, and the diagnostic accuracy was 100% (Figure 1).

Gallbladder adenomyomatosis was mostly shown as heterogeneously enhanced, with some small nonenhanced areas (represented as Rokitansky-Aschoff sinuses) on both arterial phase and venous phase (Figure 2). Some of them were together with echogenic foci and tail sign.

Gallbladder polyps and gallbladder adenoma were mostly shown as homogeneously hyperenhanced on arterial phase and isoenhanced on venous phase. The gallbladder wall was intact and the surrounding tissue was normal, with no invasion (Figure 3).

The appearances of gallbladder cancer on CEUS were various. It could be a mass in gallbladder which was heterogeneously hyperenhanced on arterial phase and washed out quickly (Figure 4). Or, the irregular thickness of gallbladder, which was also heterogeneously hyperenhanced on arterial phase and washed out quickly, could be a sign of malignancy. In some cases, the intact gallbladder wall was destroyed or the surrounding liver tissue was invaded.

Besides providing microvascular information, CEUS makes the contour of a lesion much clearer and the evaluation of a lesion's shape, size and boundary much more accurate.

Diagnostic efficiency of conventional ultrasound

The diagnostic results of conventional ultrasound are shown in Table 1. There were 3 malignant lesions

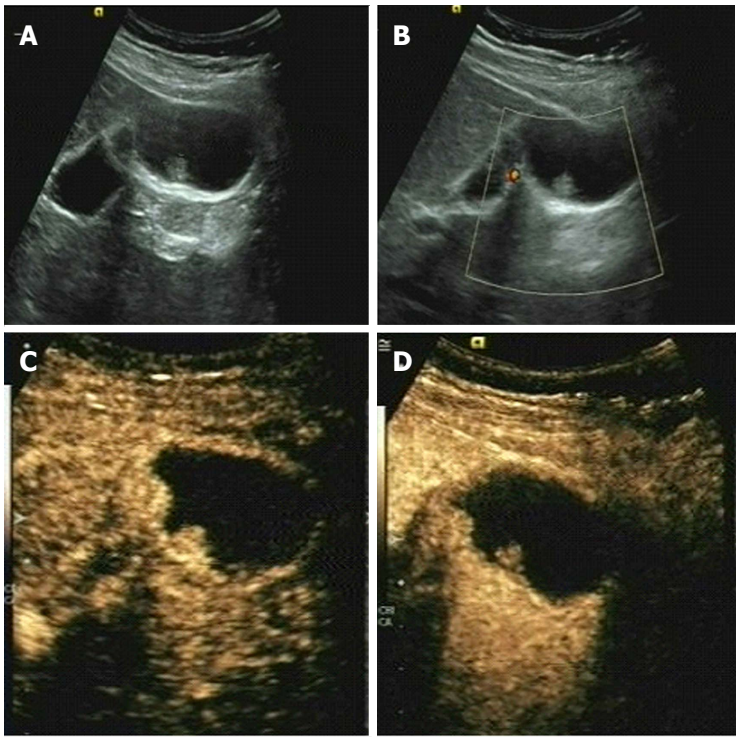

Figure 3 Gallbladder polyps in a 38-year-old male patient. A: B-mode sonography showed a homogeneously isoechoic lesion in the gallbladder, with an intact gallbladder wall; B: Color Doppler ultrasound showed no color Doppler signal in the lesion. According to A and B, a diagnosis of probably benign was made. C: CEUS showed a homogeneous and a little hyperenhanced lesion in the gallbladder on arterial phase; D: CEUS showed the enhancement of the lesion is similar to the surrounding gallbladder wall on venous phase. According to C and D, a diagnosis of benign lesion was made. CEUS: Contrast-enhanced ultrasound.

Table 3 The Diagnostic results of contrast- enhanced ultrasound *n* (%)

	Benign		Malignant	
	Definitely	Probably	Probably	Definitely
Benign, *n* = 88	78 (88.6)	6 (6.8)	4 (4.5)	0 (0)
Malignant, *n* = 17	0 (0)	1 (5.9)	6 (35.3)	10 (58.8)

misdiagnosed as probably benign and 5 diagnosed as probably malignant. There were 8 benign lesions (2 cases of sludge, 3 cases of adenomyomatosis, 2 cases of polyps and 1 case of gallbladder adenoma) misdiagnosed as probably malignant, and one benign lesion misdiagnosed as definitely malignant (1 case of adenoma). A total of 18 benign lesions (3 cases of sludge, 5 cases of adenomyomatosis, 5 cases of polyps and 5 cases of gallbladder adenoma) were diagnosed as probably benign.

The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of conventional ultrasound were shown in Table 2.

Diagnostic efficiency of CEUS

The diagnostic results of CEUS are shown in Table 3. Two malignant lesions which were misdiagnosed as probably benign by conventional ultrasound were correctly diagnosed as probably malignant by CEUS, and one malignant lesion which was diagnosed as probably malignant by conventional ultrasound was confirmed as malignant by CEUS. For benign lesions, all the cases of sludge were confirmed as benign. All cases of adenomyomatosis but 3 (1 diagnosed as probably malignant and 2 as probably benign) and all cases of polyps but 3 (1 diagnosed as probably malignant and 2 as probably benign) were confirmed as benign. Two cases of adenoma were misdiagnosed as probably malignant and another two cases of adenoma were diagnosed as probably benign. The rest 9 of the cases of adenoma were confirmed as benign.

The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of CEUS are shown in Table 2. The diagnostic efficiencies of CEUS were all significantly higher than those of conventional ultrasound, though the differences were not statistically significant.

DISCUSSION

In this study, we compared the value of CEUS in the differential diagnosis of benign and malignant gallbladder lesions with conventional ultrasound. Our results showed that the diagnostic efficiencies of CEUS were much higher than those of conventional ultrasound, though the differences were not statistically significant. With all the advantages and information of conventional ultrasound, CEUS provides more information about the important microvascularity in lesions. Also, with the application of microbubbles, the contour, the boundary and the shape of a lesion, the

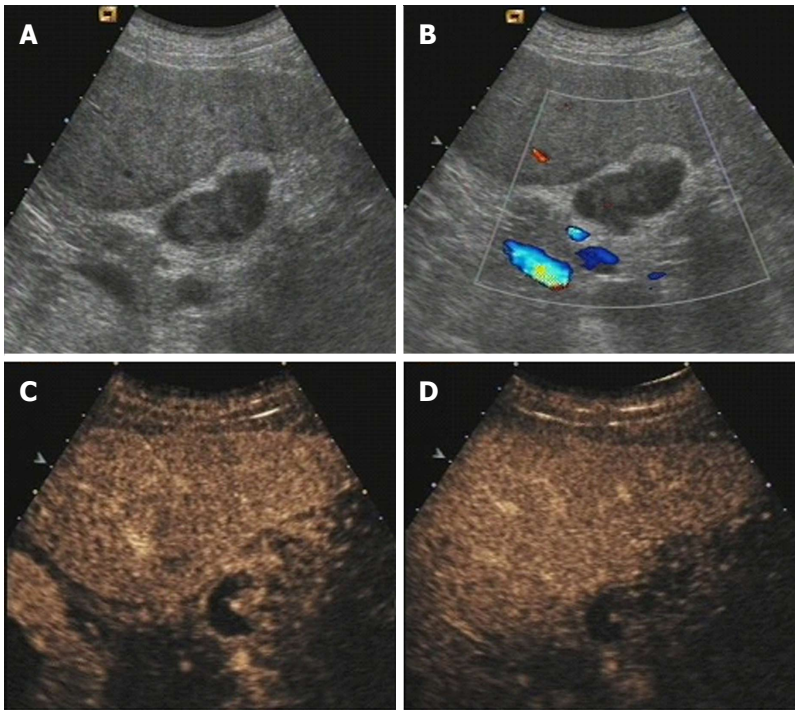

Figure 4 Gallbladder cancer in a 46-year-old male patient. A: B-mode sonography showed a heterogeneously hypoechoic mass in the gallbladder, and the posterior wall of the gallbladder was not very clear; B: Color Doppler ultrasound showed no color Doppler signal in the lesion. According to A and B, a diagnosis of probably malignant was made. C: CEUS showed a heterogeneously hyperenhanced mass with tortuous-type tumor vessel on arterial phase and the boundary of the mass was not clear; D: CEUS showed the enhancement of the lesion is much lower than that of the surrounding gallbladder wall on venous phase. According to C and D, a diagnosis of malignant mass was made. CEUS: Contrast-enhanced ultrasound.

intactness of gallbladder wall and the invasion of the surrounding tissue could be revealed more clearly. So, the diagnostic efficiencies were highly improved, though the differences between the diagnostic efficiencies were not statistically significant.

Although the clinical significance of gallbladder sludge has not been confirmed yet, the accurate diagnosis is still of importance to avoid unnecessary examination and treatment[13]. Gallbladder sludge is usually shown on ultrasound as movable, echogenic matter, which could be easily diagnosed. However, sometimes gallbladder sludge could be shown as an intraluminal mass and imitates tumors such as gallbladder cancer or adenoma[14]. Then, the differential diagnosis is very difficult using conventional ultrasound. CEUS is very useful at such a time. As sludge has no blood supply inside it, it shows a complete nonenhancement on both arterial phase and venous phase. The diagnostic accuracy was 100% in our study, and the result was similar with some previous studies[11,15].

Gallbladder adenomyomatosis is a noninfectious and nontumorous disease of gallbladder which is usually found accidentally, with no malignant potential and which needs no specific treatment[16]. It has some typical characteristics on CEUS, too. With the small nonenhanced areas on arterial phase and venous phase (represented as Rokitansky-Aschoff sinuses), together with echogenic foci and tail sign or not, the correct diagnosis would be easily made[17,18]. The study by Tang *et al*[17] showed that small anechoic spaces or intramural echogenic foci were 100% detected using CEUS, which made the diagnostic accuracy much higher than conventional ultrasound. In this study, besides one case with no small anechoic spaces that was misdiagnosed as probably malignant, the rest of the cases were all diagnosed correctly as gallbladder adenomyomatosis.

The differential diagnosis among gallbladder polyps, gallbladder adenoma and gallbladder cancer was not easy on CEUS. However, some studies showed that some CEUS features were useful and significant for differentiating malignancy from benignity. The study of Xu *et al*[19] showed that focal gallbladder wall thickening, inner layer discontinuity and outer layer discontinuity were associated with gallbladder malignancy. Branched or linear intralesional vessels, tortuous-type tumor vessel, enhanced heterogeneously in the artery phase and washed out quickly in the late phase were usually considered as signs for malignancy[20-22]. On the contrary, gallbladder polyps or gallbladder adenoma was usually enhanced homogenously and the microbubbles inside the lesions washed out together with normal gallbladder wall. Recently, the study of the differential diagnosis of localized gallbladder lesions using contrast-enhanced harmonic endoscopic ultrasonography also confirmed the value of CEUS for the evaluation and differentiation of localized gallbladder lesions[23]. Although CEUS provides the microvascular information, conventional ultrasound is still very important and is the foundation of CEUS. The size, shape and boundary

of a lesion, the intactness of gallbladder wall and the invasion of surrounding tissue are very important for the differential diagnosis. Besides providing microvascular information, CEUS makes the contour of a lesion much clearer and the evaluation of a lesion's shape, size and boundary much more accurate. That is an important reason for the improvement of CEUS diagnostic efficiency, compared with conventional ultrasound.

Our study has some limitations. First, the sample was not large enough, especially for the malignant lesions. The pathological types of the lesions were not enough in number, either. For example, all the gallbladder adenomyomatosis in our study were of localized type, and no segmental or diffuse types were included. And, there were only a few early-stage cancers in this study, making it hard to compare the difference between benign lesions and early-stage cancers on CEUS. Second, the CEUS features were not analyzed using quantitative analysis software, but by naked eyes. No quantitative parameters were acquired and analyzed. Furthermore, the interobserver agreement in CEUS and conventional ultrasound was not compared in this study.

In conclusion, gallbladder sludge and gallbladder adenomyomatosis had special features on CEUS and the diagnostic accuracy was very high. CEUS helped the differential diagnosis among gallbladder polyps, gallbladder adenoma and gallbladder cancer. The diagnostic efficiency of CEUS was highly improved compared to conventional ultrasound.

ARTICLE HIGHLIGHTS

Research background

With the advent of ultrasound contrast agents, contrast-enhanced ultrasound (CEUS) is playing a more and more important role clinically. CEUS is a safe, convenient and repeatable imaging method, with no risk of serious allergy and radiation. CEUS has an excellent diagnostic efficiency for hepatic focal lesions, which is comparable with contrast-enhanced computed tomography. However, the value of CEUS in gallbladder lesions was not widely accepted yet.

Research motivation

The European Federation of Societies for Ultrasound in Medicine and Biology guidelines 2011 did not recognize the value of CEUS for the differential diagnosis of gallbladder lesions. However, there were still some studies published which showed the usefulness of CEUS in the differential diagnosis between benign and malignant gallbladder diseases. So, the value of CEUS for gallbladder is still unclear.

Research objectives

We aim to describe CEUS features and evaluate differential diagnosis value of CEUS and conventional ultrasound for patients with benign and malignant gallbladder lesions.

Research methods

This study included 105 gallbladder lesions, which were examined using conventional ultrasound and CEUS before surgical resection and pathological examination in our hospital between December 2012 and October 2016. Each lesion was diagnosed as (1) benign, (2) probably benign, (3) probably malignant or (4) malignant using both conventional ultrasound and CEUS by two radiologists with at least ten years' experience in both conventional ultrasound and CEUS. CEUS features of these gallbladder lesions were analyzed. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of conventional ultrasound and CEUS was calculated and compared.

Research results

Gallbladder sludge was completely nonenhanced on CEUS. Gallbladder adenomyomatosis had typical characteristics of small nonenhanced areas on CEUS, together with echogenic foci and tail sign sometimes. Gallbladder cancer on CEUS was usually heterogeneously hyperenhanced on arterial phase and washed out quickly. Besides providing microvascular information, CEUS makes the contour of a lesion much clearer and the evaluation of a lesion's shape, size and boundary much more accurate.

The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of CEUS were 94.1%, 95.5%, 80.0%, 98.8% and 95.2%, respectively; these values were significantly higher than conventional ultrasound (82.4%, 89.8%, 60.9%, 96.3% and 88.6%, respectively).

Research conclusions

CEUS helped in the differential diagnosis between among different kinds of gallbladder lesions. The diagnostic efficiency of CEUS was highly improved compared with conventional ultrasound. According to our results, for a gallbladder lesion, when a definite diagnosis could not be made using conventional ultrasound, CEUS examination could be used as a further diagnostic method.

Research perspectives

In this study, we demonstrated the value of CEUS for gallbladder lesions. Prospective study with large numbers of patients and different kinds of gallbladder lesions will be needed to confirm the results. The application of endoscopic CEUS may provide more useful information for differentiating between benign and malignant gallbladder lesions.

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ORIGINAL ARTICLE

Observational Study

High prevalence of hepatitis B-antibody loss and a case report of *de novo* hepatitis B virus infection in a child after living-donor liver transplantation

Palittiya Sintusek, Nawarat Posuwan, Piyaporn Wanawongsawad, Suttiruk Jitraruch, Yong Poovorawan, Voranush Chongsrisawat

Palittiya Sintusek, Suttiruk Jitraruch, Yong Poovorawan, Voranush Chongsrisawat, Division of Gastroenterology and Hepatology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Nawarat Posuwan, Yong Poovorawan, Center of Excellence in Clinical Virology, Chulalongkorn University, Bangkok10330, Thailand

Piyaporn Wanawongsawad, Excellence Center of Organ Transplantation, King Chulalongkorn Memorial Hospital, Bangkok 10330, Thailand

ORCID number: Palittiya Sintusek (0000-0003-4441-0151); Nawarat Posuwan (0000-0002-7022-1541); Piyaporn Wanawongsawad (0000-0001-7259-8441); Suttiruk Jitraruch (0000-0003-2766-2453); Yong Poovorawan (0000-0002-2337-6807); Voranush Chongsrisawat (0000-0002-6106-0504).

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at voranush.c@chula.ac.th. Consent was not obtained but the presented data are anonymized and the risk of identification is low.

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Correspondence to: Voranush Chongsrisawat, MD, Associated Professor, Chief, Division of Gastroenterology and Hepatology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, 1873 Rama 4 Road, Pathumwan, Bangkok 10330, Thailand. voranush.c@chula.ac.th
Telephone: +66-2-2564951
Fax: +66-2-2564911

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Abstract

AIM

To assess the seroprevalence of hepatitis B virus (HBV) immunity among previously vaccinated pediatric liver transplant recipients and present a case report of *de novo* hepatitis B infection after liver transplantation.

METHODS

This study focused on children with chronic liver diseases who received primary hepatitis B immunization and had a complete dataset of anti-HBs before and after liver transplantation between May 2001 and June 2017. Medical records were retrospectively reviewed for potential factors relating to HBV immunity loss.

RESULTS

In total, 50 children were recruited. The mean time from liver transplantation to anti-HBs testing was 2.53 ± 2.11 years. The mean anti-HBs levels before and after liver transplantation were 584.41 ± 415.45 and 58.56 ± 6.40 IU/L, respectively. The rate of non-immunity (anti-HBs < 10 IU/L) in the participants was 46% (n = 26) at one year, 57% (n = 7) at two years and 82% (n = 17) at > three years following liver transplantation. The potential factors relating to HBV immunity loss after liver transplantation were identified as anti-HBs (P = 0.002), serum albumin (P = 0.04), total bilirubin (P = 0.001) and direct bilirubin (P = 0.003) before liver transplantation. A five-year-old boy with biliary cirrhosis received 4 doses of HBV vaccine with an anti-HBs titer of > 1000 IU/L and underwent liver transplantation; his anti-HBc-negative father was the donor. After liver transplantation, the boy had stenosis of the hepatic artery up to the inferior vena cava anastomosis and underwent venoplasty three times. He also received subcutaneous injections of enoxaparin for 5 mo and 20 transfusions of blood components. Three years and ten months after the liver transplantation, transaminitis was detected with positive tests for HBsAg, HBeAg, and anti-HBc (2169.61, 1706 and 8.45, respectively; cutoff value: < 1.00) and an HBV viral load of 33212320 IU/mL.

CONCLUSION

The present study showed that loss of hepatitis B immunity after liver transplantation is unexpectedly common. In our case report, despite high levels of anti-HBs prior to transplantation, infection occurred at a time when, unfortunately, the child had lost immunity to hepatitis B after liver transplantation.

Key words: Hepatitis B vaccine; Liver transplantation; *De novo* hepatitis B infection; Anti-HBs antibody; Immunity

Core tip: Despite the completion of hepatitis B vaccination, loss of hepatitis B immunity in children after liver transplantation is common and we encountered a case of *de novo* hepatitis B virus (HBV) infection following liver transplantation. Serum anti-HBs, albumin, total bilirubin, and direct bilirubin prior to liver transplantation were identified as potential factors related to HBV immunity loss after liver transplantation. A booster dose of hepatitis B vaccine and raising serum albumin to normal levels could delay the rapid loss of HBV immunity after liver transplantation but may not prevent *de novo* hepatitis B. Consequently, strategies are required to maintain anti-HBs antibody above the protective level after liver transplantation. Regular assessment of anti-HBs after liver transplantation should also be considered along with revaccination to guarantee long-term protection from HBV infection.

Sintusek P, Posuwan N, Wanawongsawad P, Jitraruch S, Poovorawan Y, Chongsrisawat V. High prevalence of hepatitis B-antibody loss and a case report of *de novo* hepatitis B virus infection in a child after living-donor liver transplantation. *World J Gastroenterol* 2018; 24(6): 752-762 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i6/752.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i6.752

INTRODUCTION

Hepatitis B virus (HBV) infection is considered a great burden worldwide owing to its chronicity and the increased risk of hepatocellular carcinoma. Moreover, antiviral therapy might not completely eradicate HBV from the human liver[1]. Since the 1980s, primary HBV immunization has been implemented to reduce HBV transmission and has shown high efficacy and good serological correlates for protective immunity[2,3]. The rapid and robust response, which usually develops 5-8 d after re-exposure to the HBsAg and peaks after approximately 14 d, indicates the long-lasting protective property of the vaccine despite the undetectable anti-HBs titer[4-6]. In contrast, a more rapid decline of anti-HBs antibody level has been observed in children post-liver transplantation compared to healthy children[7,8], which corresponds to a loss of protection as *de novo* HBV infection was evident in some cases[9-11]. This evidence implied that these immunocompromised patients might need a higher protective level of anti-HBs antibody with which to prevent HBV infection following liver transplantation[11-14].

The assessment of anti-HBs titers is recommended in patients who have undergone liver transplantation[12-14], but there is a lack of data regarding an appropriate schedule for revaccination. Moreover, there is insufficient data supporting the disease burden of *de novo* hepatitis B infection after liver transplantation, especially if the liver is from an antiHBc-negative donor[9,10].

The present study aimed to assess anti-HBs

immunity loss in children who received primary vaccination and also possessed anti-HBs immunity above the protective level prior to liver transplantation. In addition, we also present a case of *de novo* hepatitis B infection after liver transplantation despite the fact that the patient had high titers of pre-transplantation anti-HBs and received an anti-HBc-negative liver from his father.

MATERIALS AND METHODS

Recruitment of participants

All children who underwent liver transplantation and received ⩾ 3 doses of hepatitis B vaccine prior to transplantation between May 2001 and June 2017 were invited to participate in this study. Participants over 18 years of age at the time of the study and with no history of anti-HBs or anti-HBs < 10 IU/L before liver transplantation were excluded. Medical records were retrospectively reviewed to collate the following information: (1) demographic data [gender, age, body weight (BW), height, body mass index (BMI)] and (2) history of hepatitis B vaccination and booster prior to liver transplantation. Furthermore, the pediatric end-stage liver disease (PELD) score, or the model for end-stage liver disease (MELD) score, was calculated in children aged ⩽ 13 years old and > 13 years old at the time of liver transplantation, respectively, every 3 mo after children were placed on the transplant waiting list. The current PELD/MELD score at the time of albumin infusion has not been initiated was used for data analysis. Laboratory data that might reflect immune status and disease severity was collated, including complete blood count, albumin, total bilirubin (TB), direct bilirubin (DB), immunosuppressant use and hepatitis B profiles before/after transplantation (HBsAg, anti-HBs, and anti-HBc). Follow-up time and donor characteristics were also collated, including gender; hepatitis B profiles (HBsAg, anti-HBs, and anti-HBc), and also whether the donor was alive or cadaveric. Samples were obtained from all participants who did not undergo hepatitis B profile testing after transplantation. Nutritional status (BW, height, BMI) and immunosuppressant use at the same time of hepatitis B profile testing were also recorded. Participants were classified into two subgroups: immune and non-immune. Hepatitis B immunity was defined as when anti-HBs level > 10 IU/L. *De novo* hepatitis B infection was defined as positive HBsAg and HBV DNA serological tests after liver transplantation despite a negative test prior to transplantation. A high anti-HBs titer was defined as when anti-HBs > 1000 IU/L.

Ethical considerations

Routine history taking and physical examination, including weight and height measurement, were carried out by physicians. Anti-HBs, anti-HBc, and HBsAg were tested concurrent with routine laboratory testing during the follow-up visit to the transplant clinic. Verbal consent was obtained from the caregivers. Ethical approval was granted by the Ethics Committee, Faculty of Medicine, Chulalongkorn University (IRB number: 614/60).

Statistical analysis

Continuous and categorical data were presented as mean ± SD/median (range) and proportion or percentage, respectively. The Mann-Whitney *U* test and unpaired *t*-test were used to compare continuous data, as appropriate. The Chi-square test was used to compare categorical data. Analysis of variance (ANOVA) was used to compare more than 2 continuous variables. Multiple linear regression was performed to investigate the influence of significant parameters on the loss of anti-HBs after liver transplantation. A *P*-value > 0.05 was regarded as being statistically significant. Data analyses were performed using SPSS version 24.0.0 (SPSS, Inc., Chicago, IL, United States).

RESULTS

Recipient and donor characteristics

Seventy-two children underwent liver transplantation between May 2001 and June 2017. All children were negative for HBsAg. Twenty-two cases were excluded for the following reasons: incomplete anti-HBs data (n = 12), anti-HBs < 10 IU/L (n = 8) before transplantation, and age > 18 years (n = 2). Finally, 50 children were recruited into the present study with a mean age of 6.67 ± 4.63 years; 54% were female (Figure 1). The indications for liver transplantation were biliary atresia (n = 39), Alagille syndrome (n = 2), progressive familial intrahepatic cholestasis (PFIC) (n = 2), primary bile acid deficiency (n = 1), fulminant Wilson's disease (n = 1), hepatoblastoma (n = 1), glycogen storage disease (n = 1) and cryptogenic cirrhosis (n = 3). All children received primary hepatitis B vaccination at birth and at 1 and 6 mo of age. Twenty-three subjects received one booster dose 1-2 mo before transplantation. Twenty-three subjects had high anti-HBs (> 1000 IU/L) before liver transplantation. Most subjects received a living donor liver transplantation from their parents (n = 44, 88%), while 6 (12%) received a liver from a cadaveric donor. Children who received an anti-HBc-positive liver from the 6 cadaveric donors were required to take lifelong lamivudine. The severity of chronic liver disease, or PELD score and MELD score, were 18.38 ± 8.47 (n = 47) and 15 (range: 11-19; n = 3), respectively. All children received at least two immunosuppressants early after transplantation and only one immunosuppressant subsequently, with the exception of immunosuppressive agent-withdrawal in one female patient. This particular patient developed post-transplantation lymphoproliferative disease

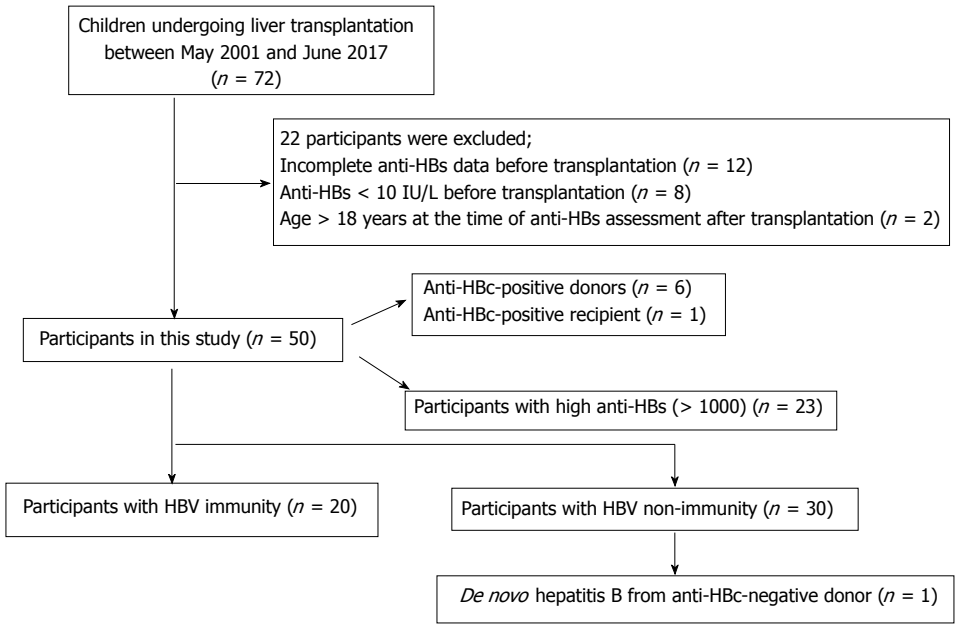

Figure 1 Algorithm showing our study participants. HBV: Hepatitis B virus.

(PTLD) and ongoing cholestasis, and is thus awaiting re-transplantation. The clinical and demographic characteristics of all participants and donors are shown in Table 1.

Recipient anti-HBs before and after liver transplantation

Anti-HBs titers before and after liver transplantation were 584.41 ± 415.45 and 58.56 ± 6.40 IU/L, respectively. Thirty participants (60%) experienced the loss of HBV immunity after transplantation. The rates of non-immunity (anti-HBs < 10 IU/L) in the participants were 46% (n = 26) at one year, 57% (n = 7) at two years and 82% (n = 17) at > three years following liver transplantation. The age at liver transplantation and the time from transplant to anti-HBs testing were 3.06 ± 3.97 years and 2.53 ± 2.11 years, respectively. One patient was diagnosed with *de novo* hepatitis B infection three years after liver transplantation.

Nutritional status, disease severity and the immune status of subjects before and after liver transplantation

After transplantation, the body weight and height, not including BMI, were increased compared to pre-transplant status (P < 0.001). TB, DB, and albumin levels, which were reflective of disease severity, improved after liver transplantation in both the short-term (at 3 mo) and long-term (2.53 ± 2.11 years; P < 0.001). With respect to immune status, white blood cell count (P = 0.002), neutrophils (P < 0.001) and lymphocytes (P = 0.01) were reduced, while platelet count (P = 0.005) increased after liver transplantation, in both the short-term (at 3 mo) and long-term (2.53 ± 2.11 years). The number of prescribed immunosuppressants decreased in the long-term post-liver transplantation (P < 0.001; Table 2).

Comparing participants with HBV immunity and loss of immunity

The age at transplantation and the time of anti-HBs testing after transplantation were lower in participants with HBV immunity (5.27 ± 3.74 years and 2.09 ± 2.03 years, respectively) than in those with loss of HBV immunity (7.60 ± 4.98 years and 3.71 ± 4.78 years, respectively; P = 0.082). No significant differences were observed in terms of BW, height, and BMI that might reflect the nutritional status of either of the groups. In terms of disease severity, participants with HBV immunity had a lower PELD score (P = 0.086), TB (P = 0.003), DB (P < 0.001), and higher albumin (P = 0.04) levels. There were no differences in terms of white blood cell, neutrophil or lymphocyte count before and in the short- or long-term since liver transplantation. Even the number of prescribed immunosuppressants decreased in the long-term after liver transplantation, and there was no difference between the two groups in this respect. We further studied the effect of a booster vaccine before liver transplantation, as twenty-three patients received the booster vaccine. Of these, 14 (61%) still had HBV immunity while 9 (39%) showed a loss in HBV immunity. Twenty-seven patients did not receive the booster vaccine before liver transplantation; of these, six (22%) still had HBV immunity while 21 (78%) did not. We compared all potential factors associated with the loss of HBV immunity between patients who received a booster dose with (n = 14) and without HBV immunity (n = 9), and there were no significant differences with respect to any of the parameters tested (Table 2). One patient who received a booster dose, and whose anti-HBs was more than 1000 IU/L before liver transplantation, was diagnosed with *de novo* hepatitis three years and ten months

Table 1 Patients' demographic data and characteristics mean ± SD or *n* (%)

Characteristics	All participants (*n* = 50)
Age (yr)	6.67 ± 4.63
Age at liver transplantation (yr)	3.06 ± 3.97
Gender - female	27 (54)
Before liver transplantation	
Anti-HBs level (IU/L)	584.41 ± 415.45
Anti-HBc positive	1 (2)
After liver transplantation	
Anti-HBs level (IU/L)	58.56 ± 6.40
Length of stay in hospital after transplant (d)	44.10 ± 29.30
ABO incompatibility	3 (6)
PELD/MELD score	18.38 ± 8.47/15 (11-19)
Time since transplantation (yr)	2.53 ± 2.11
Medical complications	
Acute rejection	22 (44)
Cytomegalovirus infection	15 (30)
Posttransplant lymphoproliferative disorder	14 (28)
De novo hepatitis B infection	1 (2)
De novo food allergy	9 (18)
Surgical complications	
Vascular stricture	20 (40)
Biliary stricture	12 (24)
Chylous ascites/chylothorax	7 (14)
Donor characteristics	
Gender - female	22 (44)
Cadaveric	6 (12)
Living	44 (88)
Anti-HBs	
Negative	13 (26)
Positive	
1-9 IU/L	30 (60)
> 10 IU/L	7 (14)
Anti-HBc positive	6 (12)

PELD: Pediatric end-stage liver disease; MELD: Model for end-stage liver disease.

after liver transplantation (Table 3).

A patient with de novo hepatitis B

A five-year-old boy with biliary cirrhosis and an unsuccessful Kasai's operation underwent liver transplantation at 14 mo of age; his anti-HBc-negative father was the donor. He received 4 doses of HBV vaccine prior to liver transplantation, and his pre-existing anti-HBs antibody titer was > 1000 IU/L. His parent's viral profiles were negative for HBV infection (HBsAg, anti-HBc, and anti-HBs were all negative). After liver transplantation, he developed stenosis at the hepatic artery leading to the inferior vena cava anastomosis and underwent venoplasty with balloon dilatation three times. Furthermore, he was given subcutaneous enoxaparin injections every 12 h for 5 mo and transfused 20 times with blood components. Three years and ten months after the liver transplantation, transaminitis was detected, with positive tests for HBsAg, HBeAg, and anti-HBc (2169.61, 1706, and 8.45, respectively; cutoff value: < 1.00), and an HBV viral load of 33212320 IU/mL. The timeline of this patient is shown in Figure 2.

Several possible routes of HBV infection after liver transplantation were investigated. His father and mother were both tested for HBsAg, anti-HBc, and anti-HBs titers and serum HBV viral load; all results were negative. There were no apparent HBsAg carriers in the patient's family. Another strong possibility might be from blood transfusion, in which the blood component was partly derived from a recently infected donor. However, the handling of blood and blood products in Thailand is extremely safe, as we employ the universally-accepted nucleic acid amplification test (NAT) to screen for HBV, hepatitis C virus, and HIV from all blood donors. All 15 donors who donated blood to this patient had undergone repeat NAT-based HBV testing and none had any evidence of HBV occult infection. The *de novo* activation of HBV with escape mutations from hepatitis B surface antibody after living donor liver transplantation has been documented previously, however, no vaccine escape mutants were found when the HBs gene was screened in our patient (Figure 3).

DISCUSSION

Transmission of the HBV core from hepatitis B core antibody-positive donors was first reported in 1998 by Uemoto *et al*[15]. In Uemoto *et al*'s, HBV existing in the liver of healthy donors who were hepatitis B core antibody-positive, but not in the blood, was shown to be transmitted to recipients by liver grafts following liver transplantation. Moreover, livers from hepatitis B core antibody-positive donors exerted influence on graft survival as this was lower in the recipients of hepatitis B core antibody-positive tissue compared to those receiving tissue from hepatitis B core antibody-negative donors, especially among HBsAg-negative recipients[16]. As a result, robust strategies have been developed to prevent viral activation and *de novo* hepatitis B infection in recipients receiving liver grafts from hepatitis B core antibody-positive donors. These strategies involve passive immunization with hyper-immune hepatitis B immunoglobulin (HBIG), with or without antiviral agent, or the administration of hepatitis B vaccine[17-20]. In contrast, the prevention of *de novo* hepatitis B infection in recipients of tissue from hepatitis B core antibody-negative donors has generally been disregarded because evidence to support *de novo* hepatitis B infection from the loss of HBV immunity after liver transplantation is scarce[9,10].

In this study, we report a high prevalence of the loss of HBV immunity following liver transplantation, and its association with disease severity and anti-HBs titer levels before liver transplantation. Our study demonstrated that 60% of pediatric liver transplant patients who were previously immunized were non-immune after their transplants. This was comparable to the 67% of patients reported in a previous study who also showed a loss of immunity[8]. While patients with HBV immunity had a significantly higher titer of anti-HBs before liver transplantation than participants showing

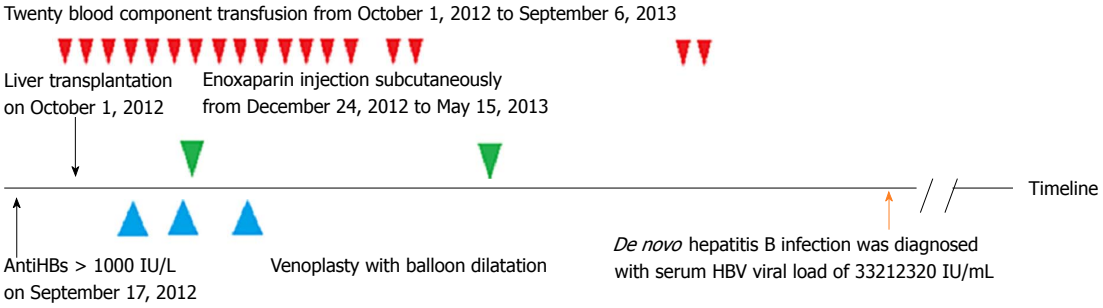

Figure 2 Timeline of the case report from pre-liver transplantation to diagnosis of HBV infection. HBV: Hepatitis B virus.

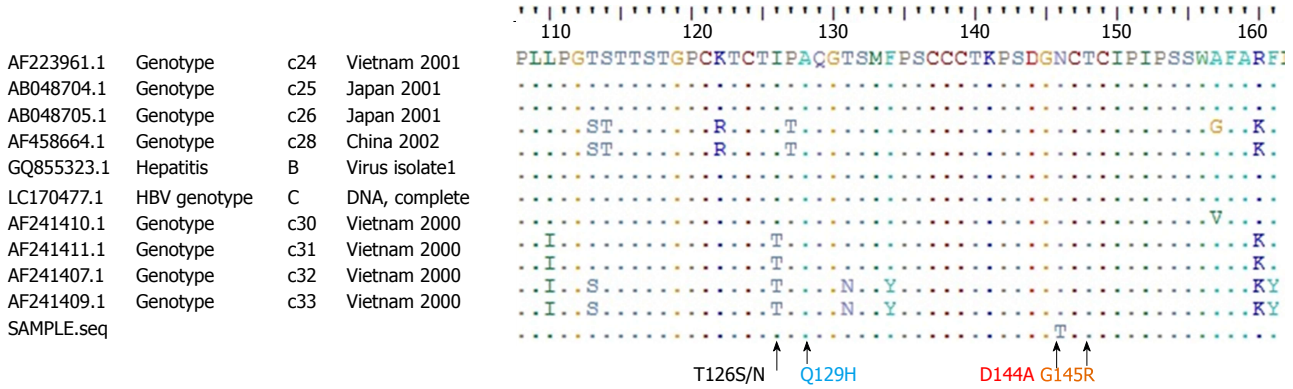

Figure 3 Amino acid sequence of "a" determinant of the HBs gene (position 110-160) showing no significant escape mutants in common regions (T126S/N, Q129H, D144A, and G145R).

Table 2 Nutritional and laboratory results of pre and post liver transplantation mean ± SD or *n* (%)

	Pre-transplantation	Post-transplantation		*P* value
		Short-term (3 mo)	Long-term	
Body weight (kg)	11.66± 8.01	-	21.32 ± 12.07	< 0.001
Height (meter)	0.81 ± 0.23	-	1.07 ± 0.28	< 0.001
BMI (kg/m^2)	16.59 ± 1.67	-	16.63 ± 2.71	0.76
Complete blood count				
White blood cell (/μL)	10492 ± 4627	8559 ± 2495	7729 ± 3843	0.002
Neutrophil (/μL)	4938 ± 2565	3385 ± 1679	3418 ± 1912	< 0.001
Lymphocyte (/μL)	4263 ± 2222	3982 ± 1722	3109 ± 1762	0.01
Platelet (/μL)	172880 ± 94557	199720 ± 82777	233957 ± 94136	0.005
Liver function test				
Total bilirubin(mg/dL)	18.50 ± 12.68	2.41 ± 0.91	0.84 ± 0.91	< 0.001
Direct bilirubin (mg/dL)	13.41 ± 10.28	0.83 ± 0.71	0.38 ± 0.51	< 0.001
Albumin (g/dL)	3.29 ± 0.97	3.94 ± 0.48	4.08 ± 0.35	< 0.001
Number of immunosuppressant				
0	0		1 (2)	
1	5 (10)		27 (54)	< 0.001
2	23 (46)		17 (34)	
3	22 (44)		5 (10)	

BMI: Body mass index.

a loss of HBV immunity after liver transplantation, a higher titer of anti-HBs before liver transplantation cannot guarantee protective HBV immunity following liver transplantation, as evidenced by the case report described in the present study. This finding contrasts with a previous study by Su *et al*[9] who reported that an anti-HBs titer of > 200 IU/L before liver transplantation might be sufficient to prevent *de novo* HBV infection in an HBsAg-negative recipient. One possible cause of HBV infection in our patient is that the loss of HBV immunity could have occurred after liver transplantation at a time when the patient was most likely exposed

Table 3 Potential factors associated with immunity in participants with HBV immunity and immunity loss mean ± SD or *n* (%)

	Nonimmunity (*n* = 30)	Immunity (*n* = 20)	*P* value
Age (yr)	7.60 ± 4.98	5.27 ± 3.74	0.08
Age at liver transplantation (yr)	3.71 ± 4.78	2.09 ± 2.03	0.11
Gender:Female	15 (50)	12 (60)	0.58
Body weight (kg)	13.07 ± 9.91	9.56 ± 2.74	0.07
Height (m)	0.85 ± 0.28	0.75 ± 0.12	0.09
Body mass index (kg/m^2)	16.45 ± 1.62	16.45 ± 1.62	0.47
Anti-HBs level before transplantation (IU/L)	441.39 ± 408.88	798.94 ± 330.47	0.24
Length of hospital stay after transplantation (d)	47.77 ± 34.89	38.80 ± 17.45	0.24
PELD/MELD score	20.19 ± 8.50 (*n* = 28)	15.90 ± 7.98 (*n* = 19)	0.09
Time since transplant to anti-HBs testing (yr)	2.83 ± 2.00	2.06 ± 2.22	0.22
Donor characteristics			
Gender: Female	14 (47)	8 (40)	0.43
Living	26 (87)	18 (90)	0.54
Medical complication			
Acute rejection	11 (37)	11 (55)	0.16
CMV infection	6 (20)	9 (45)	0.11
PTLD	7 (23)	7 (35)	0.28
De novo hepatitis B	1 (3)	0 (0)	0.65
De novo food allergy	5 (16)	4 (20)	0.52
Surgical complication			
Vascular stricture	13 (43)	7 (35)	0.39
Biliary stricture	8 (27)	4 (20)	0.46
Chylous ascites/chylothorax	5 (17)	2 (10)	0.41
Laboratory data before transplantation			
White blood cell (/μL)	10237 ± 4167	10875 ± 5334	0.63
Neutrophil (/μL)	4780 ± 2137	5174 ± 3145	0.60
Lymphocyte (/μL)	4372 ± 2515	4100 ± 1742	0.65
Platelet (/μL)	159366 ± 99431	193156 ± 85132	0.22
Total bilirubin (mg/dL)	22.80 ± 13.72	12.04 ± 7.37	0.003
Direct bilirubin (mg/dL)	16.51 ± 11.37	8.73 ± 6.08	0.001
Albumin (g/dL)	3.07 ± 0.65	3.63 ± 1.25	0.04
Laboratory data 3 mo after transplantation			
White blood cell (/μL)	8312 ± 2596	8931 ± 3440	0.64
Neutrophil (/μL)	3221 ± 1307	3630 ± 2136	0.45
Lymphocyte (/μL)	3879 ± 1851	4136 ± 1542	0.61
Platelet (/μL)	193300 ± 86221	208600 ± 78591	0.53
Albumin (g/dL)	3.96 ± 0.51	3.91 ± 0.44	0.72
Total bilirubin (mg/dL)	0.99 ± 1.12	0.59 ± 0.35	0.07
Direct bilirubin (mg/dL)	0.37 ± 0.15	0.32 ± 0.17	0.12
Laboratory data after long-term transplantation			
White blood cell (/μL)	7114 ± 2845	8815 ± 5083	0.22
Neutrophil (/μL)	3196 ± 1412	3809 ± 2576	0.38
Lymphocyte (/μL)	3039 ± 1553	3232 ± 2128	0.72
Platelet (/μL)	217033 ± 87395	263823 ± 100739	0.12
Albumin (g/dL)	4.12 ± 0.35	4.00 ± 0.35	0.26
Total bilirubin (mg/dL)	0.98 ± 1.10	1.10 ± 0.31	0.08
Direct bilirubin (mg/dL)	0.45 ± 0.62	0.25 ± 0.13	0.20
Immunosuppressant 3 mo after transplantation			
None	0	0	
1	3 (10)	2 (10)	0.41
2	16 ((53)	7 (35)	
3	11 (37)	11 (50)	
Immunosuppressant after long-term liver transplantation			
None	1 (3)	0	
1	17 (56)	10 (50)	0.77
2	9 (30)	8 (40)	
3	3 (10)	2 (10)	

PELD: Pediatric end-stage liver disease; MELD: Model for end-stage liver disease; PTLD: Post-transplantation lymphoproliferative disease.

to HBV. Our data also showed a rapid reduction in the level of anti-HBs after liver transplantation with titers below the protective threshold. Regular assessment of anti-HBs, and revaccination after liver transplantation, should therefore be considered to maintain the anti-HBs titer level above the protective threshold and therefore prevent *de novo* hepatitis B. Lin *et al*[10] recommended maintaining a high level of anti-HBs (> 1000 IU/L), which may prevent *de novo* HBV infection in pediatric patients undergoing liver transplantation who had

efficient primary vaccination. A booster vaccine appears to be the most simple and effective regimen with which to maintain high titers of anti-HBs. However, this previous study reported that patients had to receive 1-19 injections, within a period of 4-42 mo after liver transplantation to maintain adequate titers[10]. Potential factors for the rapid loss of anti-HBs loss should therefore be considered in order to avoid patients undergoing multiple injections.

In the present study, anti-HBs level, cholestasis, and low albumin levels before liver transplantation were identified to be significant factors contributing to the loss of immunity after liver transplantation. Contrast to a previous study that found statistical significance in terms of age or time since liver transplantation instead[8]. Anti-HBs level before liver transplantation may represent one of the main factors with which to predict the loss of anti-HBs after liver transplantation; age and time since liver transplantation should not be relied upon. Although the hepatitis B vaccine is highly immunogenic and very effective, there is a gradual reduction of anti-HBs titer to below the protective threshold after one or two decades of life, even in healthy individuals[21,22]. In children with chronic liver diseases, anti-HBs immunity decreases more rapidly than in healthy children, as demonstrated in the present study; indeed, at least 8 of the 72 children on the waiting list for liver transplantation in our institution had anti-HBs < 10 IU/L and were excluded from the present study. In a previous study, Leung *et al*[8] stated that a major limitation of their study was that they could not definitively state whether the loss of HBV immunity in their study was due to a loss of immunity before or after transplantation. We believe that the present study has addressed this shortfall by focusing on anti-HBs level before liver transplantation and then divided patients into high- and low-titer groups. A booster vaccine before liver transplantation is necessary in order to maintain a higher titer of anti-HBs in children following liver transplantation. However, the cutoff for a protective anti-HBs level that is more than 10 IU/L after vaccination might not be enough to protect these vulnerable patients from HBV[23], owing to the rapid decline of anti-HBs over time. Further functional cellular studies are now required to address the most appropriate cutoff level for the protective threshold of anti-HBs in children following liver transplantation.

However, it is not just strategies to delay HBV immunity loss that are needed; we also need to develop methods to re-establish active immunity against HBV after liver transplantation. Lu *et al*[20] reported the improved feasibility of vaccination combined with nucleoside analogues in the prevention of HBV reinfection after orthotopic liver transplantation compared to regular HBIG administration. In this previous study, long-term and repetitive vaccine stimulation was shown to be an important method with which to create and cultivate an enhanced immune response in these immunocompromised patients. Similar to this study, a few pediatric studies have reported the use of intermittent vaccination reinforcement, or booster vaccination, to maintain spontaneous anti-HBs production in children after liver transplantation[10,24,25]. For example, Ni[25] studied both the humoral and cellular immunity of booster hepatitis B vaccines in children after liver transplantation and demonstrated that the immunological response following a booster dose appeared to be adequate, at least over the short term (2 mo assessment period). However, Bauer *et al*[26] conducted a pilot study of cellular immune response investigating HBsAg-specific T and B cells in adults after liver transplantation compared with controls and highlighted the role of the strong inhibitory effect of regulatory T cells upon immunological response after hepatitis B revaccination over a period of long term assessment (> one year). The best rationale for HBV revaccination in liver transplantation patients has yet to be elucidated. Future studies are required to identify an appropriate HBV immunization protocol for children after liver transplantation which will effectively re-establish both cellular and humoral immunities to HBV.

Other predictive parameters responsible for rapid anti-HBs loss are cholestasis and low albumin levels. Low albumin levels might reflect the poor synthetic function of the liver, or severe malnutrition, or both. A previous study confirmed that albumin infusion could restore the immunological function of patients with decompensated cirrhosis by increasing circulating PGE_2, a potent immunomodulator, both *in vitro* and *in vivo*[27-29]. However, a study by Leung *et al*[8], and this present study, could not demonstrate a significant difference in terms of disease severity when comparing PELD score between patients in immune and non-immune groups. While the mean PELD score in Leung *et al*'s[8] study was higher than the present study, the mean serum albumin level, which is one of the parameters used to calculate the PELD score, was in the upper normal level in Leung *et al*'s[8] study compared to the low albumin level in the present study. This upper normal level of serum albumin might imply that the subjects involved in this previous study had already received albumin infusion at the time of data collection. In the present study, we evaluated the PELD score every three months from when the patients joined the waiting list for transplantation and chose the most recent PELD score, at which point, albumin infusion had not been initiated. One limitation of our method is that our PELD score might be lower than the actual PELD score prior to liver transplantation; however, we obtained actual serum albumin data for analysis and that might be why our data showed lower PELD scores, with significantly low albumin levels in patients with anti-HBs loss, than the data reported by Leung *et al*[8]. As a result, while waiting for liver transplantation, albumin infusion is an effective treatment option to treat not only hepatorenal syndrome and spontaneous bacterial peritonitis, but also gain better immunity; this practice represents common practice for most chronic

liver diseases in children.

In the present study, we were unable to demonstrate a significant effect of nutritional status upon anti-HBs immunity after liver transplantation. In our transplant unit, children were not routinely tested for lipid soluble vitamins A, D, and E. Furthermore, body weight and BMI are not the best parameters to perform nutritional assessment, as some children suffered from edema and huge abdominal distension as a result of ascites. Moreover, the immunosuppressants and complications after liver transplantation were not shown to be significantly associated with the loss of anti-HBs; in this respect, our study was consistent with Leung *et al*[8].

Our study has some limitations which need to be considered, particularly the low number of participants, inadequate data reflect the nutritional status, and the heterogeneity of immunosuppressant-use among patients. A larger, well-designed, multicenter study, using the same protocol of care after liver transplantation, is now needed to validate our present results.

In summary, significant loss of anti-HBs after liver transplantation is unexpectedly common. An anti-HBs level above 1000 IU/L before liver transplantation cannot prevent *de novo* hepatitis B. Boosters or a full-series vaccination is required for children after liver transplantation, concurrent with close monitoring of anti-HBs level. Further studies should aim to identify the best rationale for a HBV re-immunization program based upon strong clinical evidence. The potential factors that can affect anti-HBs levels, and which can be modulated prior to liver transplantation and therefore delay the rapid loss of anti-HBs include booster hepatitis B vaccines and the early administration of albumin.

ARTICLE HIGHLIGHTS

Research background

A more rapid decline of anti-HBs antibody was observed in children after liver transplantation compared to healthy children who had been previously immunized. The loss of anti-HBs might not indicate a loss of hepatitis B virus (HBV) immunity in healthy subjects. However, viral reactivation and *de novo* hepatitis B infection were clearly demonstrated in an HBsAg-negative recipient who received a liver from hepatitis B core antibody-positive or negative donors, suggesting the loss of HBV protection in such immunocompromised subjects. The present study provided strong evidence of HBV immunity loss in 60% of children after liver transplantation and one case of *de novo* hepatitis B infection. This was despite a high titer of anti-HBs prior to transplantation and the receipt of a hepatitis B core-negative liver. The present study highlighted the importance of developing strategies to re-establish active immunity to HBV following liver transplantation.

Research motivation

HBV infection in patients after liver transplantation can lead to chronic hepatitis, shorter graft survival and graft loss. However, a routine strategy for HBV reimmunization after liver transplantation, and an appropriate cutoff for the prevention of *de novo* hepatitis B infection, have yet to be elucidated. This study demonstrated a decline of anti-HBs level after liver transplantation and provided valuable data relating to the factors which can affect the rapid loss of anti-HBs and which can be modulated before liver transplantation in order to delay rapid anti-HBs loss. Such factors include booster hepatitis B vaccines and the early administration of albumin. A revaccination program is recommended for children after liver transplantation in order to re-establish active immunity to HBV.

Research objectives

Regular assessment of anti-HBs and revaccination after liver transplantation to maintain an anti-HBs titer level above the protective threshold should be considered to prevent *de novo* hepatitis B. Further studies should aim to identify the best rationale for a reimmunization program to effectively re-establish active immunity to HBV.

Research methods

The authors enrolled a total of 50 children who had undergone liver transplantation between May 2001 and June 2017. Demographic data, types of donor and liver transplant, anti-HBs level, time since liver transplantation, complications and immunosuppressant-use were collated and analyzed using SPSS version 24.0.0 software. To this end, the authors reported an observational study of a five-year-old boy who had received full HBV vaccination previously, underwent liver transplantation with his father's anti-HBc-negative liver, but was then diagnosed with *de novo* hepatitis B three years after transplantation.

Research results

The authors found that the loss of hepatitis B immunity after liver transplantation was unexpectedly common and that 60% of subjects had an anti-HBs level < 10 IU/L after a mean period of 2.53 years after transplantation. The potential factors relating to the loss of HBV immunity were anti-HBs (P = 0.002), serum albumin (P = 0.04), total bilirubin (P = 0.001) and direct bilirubin (P = 0.003) prior to liver transplantation. We also report a case of *de novo* hepatitis B, who received a hepatitis B core antibody-negative liver from his father, and had a high titer of anti-HBs (> 1000 IU/L) prior to transplantation. Future studies should aim to develop strategies to re-establish active immunity to HBV after liver transplantation.

Research conclusions

The new findings of this study are the high prevalence of hepatitis B immunity loss, and a case report of *de novo* hepatitis B, in a previously immunized recipient who received a liver from a hepatitis B core antibody-negative donor. *De novo* hepatitis B in a previously immunized recipient who received a liver from a hepatitis B core antibody-negative donor, could have been initiated when exposed to HBV during the period following transplantation coincident with the loss of HBV immunity. High anti-HBs loss after liver transplantation is unexpectedly common. High anti-HBs (> 1000 IU/L) prior to liver transplantation cannot prevent *de novo* hepatitis. Serum anti-HBs, albumin, total bilirubin and direct bilirubin prior to liver transplantation were the potential factors associated with the loss of HBV immunity after liver transplantation. Anti-HBs levels below the protective level in children after liver transplantation might reflect the loss of immunity or the loss of protection against HBV. A re-immunization program for all liver-transplanted children in order to prevent *de novo* hepatitis B. Disease severity, nutritional status, immune status and HBV immunity as a result of HBV immunization might represent potential factors to consider for re-establishing active HBV immunity following liver transplantation. *De novo* hepatitis B could have occurred after liver transplantation at a point when the recipient was likely to have been exposed to HBV.

Research perspectives

Re-assessment of anti-HBs levels and vaccination to maintain levels of anti-HBs above the protective level might prevent de novo hepatitis B after liver transplantation. Studying the immunological response of HBsAg-specific T and B cells following HBV exposure in order to establish an appropriate cutoff for the protective level of anti-HBs to prevent HBV infection in children after liver transplantation will be merit. Moreover, setting up an appropriate HBV immunization protocol to re-establish active HBV immunity by assessing cellular and humoral immunity response after HBV immunization protocols over short- and long-term follow-up periods also should be considered.

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CASE REPORT

Gastroenteritis in an adult female revealing hemolytic uremic syndrome: Case report

Paulina Chinchilla-López, Vania Cruz-Ramón, Oscar Ramírez-Pérez, Nahum Méndez-Sánchez

Paulina Chinchilla-López, Vania Cruz-Ramón, Oscar Ramírez-Pérez, Nahum Méndez-Sánchez, Liver Research Unit, Medica Sur Clinic and Foundation, Mexico City 14050, Mexico

ORCID number: Paulina Chinchilla-López (0000-0001-8367-4657); Vania Cruz-Ramón (0000-0002-5149-9636); Oscar Ramírez-Pérez (0000-0002-5808-3705); Nahum Méndez-Sánchez (0000-0001-5257-8048).

Author contributions: Chinchilla-Lopez P, Cruz-Ramón V and Ramirez-Pérez O analyzed the data and wrote the paper; Méndez-Sánchez N designed the report, diagnostic approach to patient with suspected gastroenteritis associated with hemolytic uremic Syndrome.

Informed consent statement: The patient involved in this study gave her written informed consent authorizing use and disclosure of her protected health information.

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Correspondence to: Nahum Méndez-Sánchez, MD, MSc, PhD, Professor, Liver Research Unit, Medica Sur Clinic and Foundation, Puente de Piedra 150, Col. Toriello Guerra, México City 14050, México. nmendez@medicasur.org.mx
Telephone: +525-55-4247200-4215
Fax: +525-55-6664031

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Abstract

Nowadays acute gastroenteritis infection caused by *Escherichia coli* (*E. coli*) O157:H7 is frequently associated with hemolytic uremic syndrome (HUS), which usually developed after prodromal diarrhea that is often bloody. The abdominal pain accompanied by failure kidney is a suspicious symptom to develop this disorder. Their pathological characteristic is vascular damage which manifested as arteriolar and capillary thrombosis with abnormalities in the endothelium and vessel walls. The major etiological agent of HUS is enterohemorragic (*E coli*) strain belonging to serotype O157:H7. The lack of papers about HUS associated to gastroenteritis lead us to report this case for explain the symptoms that are uncommon. Furthermore, this report provides some strategies to suspect and make an early diagnosis, besides treatment approach to improving outcomes and prognosis for patients with this disorder.

Key words: Gastroenteritis; Gastrointestinal hemorrhage; Hemolytic-uremic syndrome; *Escherichia coli* O157; Shiga-toxigenic *Escherichia coli*

Core tip: Bloody diarrhea and Hemolytic uremic syndrome are frequent caused by *E. coli* serotype O157:H7. The most causes of gastroenteritis are diagnosed as non-infectious illness and this could be the reason that clinicians did not usually associated with the hemolytic uremic syndrome development. This case report not only represents the importance of the diagnosis and the

treatment approach, but also is one of the few studies where it is emphasized the gastrointestinal role and the critical symptoms that the clinical has to recognize it.

Chinchilla-López P, Cruz-Ramón V, Ramírez-Pérez O, Méndez-Sánchez N. Gastroenteritis in an adult female revealing hemolytic uremic syndrome: Case report. *World J Gastroenterol* 2018; 24(6): 763-766 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i6/763.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i6.763

INTRODUCTION

Thrombotic thrombocytic purpura (TTP) and hemolytic-uremic syndrome (HUS) are acute fulminant disorders characterized by thrombocytopenia and microangiopathic hemolytic anemia. Gastroenteritis is recognized as a precursor of the HUS, which is often accompanied by pain abdominal, vomiting and bloody stools. Other specific symptoms to establish the diagnosis are kidney failure and cognitive impairment[1].

CASE REPORT

A 61-year-old woman with a history of hypertension, osteoarthritis and irritable bowel syndrome presented to the emergency department (ED) after 5 d of bloody diarrhea without mucus, after eating high-fat food. Her vital signs upon arrival in ED were within normal limits. Physical examination revealed diffuse abdominal tenderness on deep palpation without signs of peritonitis, spasms in the upper extremities, and muscle weakness.

The results of laboratory testing were: hemoglobin, 12.3 g/dL; platelet count, 250 × 10^3/μL; serum creatinine, 0.76 mg/dL; serum sodium, 133 mmol/L; serum potassium, 3.04 mmol/L; serum calcium, 6.2 mmol/L; and serum magnesium, 0.54 mmol/L. She was diagnosed with hydroelectric disequilibrium *as* a consequence *of* diarrheic syndrome and admitted for immediate treatment of hypocalcemia with fluids and electrolytes *via* central venous access; in addition intravenous antibiotic therapy with ertapenem was begun because culture showed the presence of extended-spectrum beta-lactamase (ESBL)-producing *E. coli*. Despite aggressive antibiotic therapy, she continued to clinically deteriorate with petechiae and ecchymoses appearing on her lower legs and her biochemical profile did not improve (Table 1). At that point, the results of blood tests were: hemoglobin, 9.8 g/dL; platelet count, 11 × 10^3; serum creatinine, 3.5 mg/dL; and lactate dehydrogenase, 3182 units/mL. She was diagnosed with microangiopathic hemolytic anemia. Her uremia required immediate hemodialysis after the insertion of a Mahurkar catheter. Overall, she required seven hemodialysis sessions and seven plasmaphereses during the 13 d after admission. Hematological tests showed a slight increase in her erythrocyte count, so she did not need erythrocyte transfusion until the sixth day of her hospital stay. Altogether, she required three transfusions of packed cells.

Finally, the patient showed slow improvement in her renal function, recovering diuresis after 3 wk. She was discharged with a platelet count of 213 × 10^3/μL.

DISCUSSION

TTP and HUS have been considered rare diseases with high mortality. They continue to be rare in adults, although we have seen them more frequently than in the past. The prevalence of TTP is around 30%-40%, whereas HUS rate is approximately 4%-10%; their mortality ranges around 90% and 15%, respectively[2]. The most important reason that our patient had a positive outcome was that we never excluded HUS as a possible diagnosis. The unusual features that led us to the diagnosis were the bloody diarrhea, abdominal and diffuse pain evolving over 5 d after eating high-fat food, and the abnormal blood test results, specifically the presence of hemolytic anemia and acute kidney failure[3].

Karpac *et al*[4] reported that the most common symptom associated with typical HUS is diarrhea; however, the majority of gastrointestinal diseases can cause this. In addition, the bloody diarrhea is often caused by *Campylobacter, E. coli* O157:H7, and other Shiga toxin-producing *E. coli*, *Salmonella, Shigella,* and *Yersinia*; however, *E. coli* O157:H7 is the most important pathogen, which usually causes abdominal tenderness, more than 5 watery stools in the last 24 h, and especially bloody diarrhea that frequently persists during first 8 h[5]. Incidentally, Kuehne *et al*[6] demonstrated that the true STEC gastroenteritis incidence in a computed estimate was 32.3-fold higher than the incidence based on notified HUS cases.

In addition, Pedersen *et al*[7] showed that the incidence rate per 100000 persons of STEC infections was highest in children < 5 years of age (32.17) and in adults ⩾ 65 years of age (11.64) compared to 15-64 years old population (7.89).

Regarding to the first symptoms of our patient, the appearance of ecchymoses and petechiae, besides the sudden decrease in platelets and the increase in serum creatinine confirmed our diagnosis of microangiopathy.

As indicated in the guidelines for management of microangiopathies, our first-line therapy was plasma exchange[2] combined with hemodialysis and aggressive antibiotic therapy because ESBL-producing *E. coli* was detected in blood cultures. However, at that time we could not differentiate between HUS and TTP. Later, the normal levels of ADAMTS 13 protease led us to the definitive diagnosis of HUS[8].

Differentiation of HUS and TTP remains complex.

Table 1 Biochemistry Test evolution of patient with gastroenteritis linked to hemolytic uremic syndrome

Test	Initial evaluation	Middle evaluation	Final evaluation
Hemoglobin	12.3 g/dL (13-17 g/dL)	6.8 g/dL	9.7 g/dL
Platelets	250 × 103/μL (150-450 × 10^3/μL)	37 × 10^3/μL	213 × 10^3/μL
Leukocytes	7.9 × 103/μL (4.5-11 × 10^3/μL)	6.6 × 10^3/μL	5.3 × 10^3/μL
Neutrophils	84.3 % (40%-75%)	75%	88%
Lymphocytes	10.7% (12%-46%)	14%	8.00%
Blood urea nitrogen	4.9 mg/dL (8.0-20 mg/dL)	70.4 mg/dL	59.5 mg/dL
Urea	10.5 mg/dL (17.1-42.8 mg/dL)	150.7 mg/dL	127.3 mg/dL
Creatinine	0.76 mg/dL (0.44-1.03 md/dL)	6.24 mg/dL	1.97 mg/dL
Sodium	133 mmol/L (136-144 mmol/L)	129 mmol/L	139 mmol/L
Potassium	3.04 mmol/L (3.60-5.10 mmol/L)	2.95 mmol/L	4.27 mmol/L
Calcium	6.7 mg/dL (8.9-10.3 mmol/dL)	6.9 mg/dL	8.5 mg/dL
Magnesium	0.52 mg/dL (1.80-2.50 mg/dL)	2.03 mg/dL	1.41 mg/dL
Partial thromboplastin time	31.9 s (24.8-31.8 s)	29.4 s	25.3 s
Fibrinogen	249 mg/dL (177-410 mg/dL)	223 mg/dL	221 mg/dL
D-dimer	4620 ng/mL (0-199 ng/mL)	2520 ng/mL	321 ng/ mL
Alanine aminotransferase	48 U/L (14-54 U/L)	37 U/L	20 U/L
Aspartate aminotransferase	251 U/L (15-41 U/L)	121 U/L	35 U/L
Dehydrogenase lactic	3182 U/L (98-192 U/L)	1781 U/L	314 U/L
Albumin	4.0 g/dL (3.5-4.8 g/dL)	2.5 g/dL	3.5 g/dL

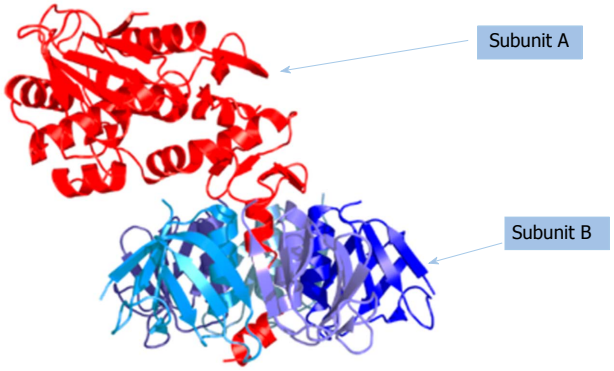

Figure 1 Tridimensional structure of Shiga toxin. Shiga toxins are a family of related toxins with two major groups, Stx1 and Stx2, expressed by genes considered to be part of the genome of lambdoidprophages. The most common sources for Shiga toxin are the bacteria S. dysenteriae and the shigatoxigenic serotypes of Escherichia coli, which includes serotypes O157:H7, O104:H4, and other enterohemorrhagic *E. coli*.

Acute renal failure is usually more severe in HUS[9], while TTP more frequently results in damage to the central nervous system. Moake[5,8] reported that systemic aggregation of platelets, especially in the central nervous system, usually indicates TTP because platelet aggregation in HUS is predominantly confined to the renal circulation[8]. In relation to ADMTS 13, a protein responsible for cleaving Von Willebrand factor, our patient had normal levels and this was another characteristic that assisted our diagnosis.

The key features of HUS treatment are firstly, fluid therapy with isotonic solutions to avoid the occurrence of oliguria, anuria, and the requirement for dialysis[10]. In our patient, diuresis was less than 0.5 mL/kg/h for more than 12 h, meaning that hemodialysis was necessary[11]. Secondly, the guidelines recommend blood transfusion when hemoglobin drops to 6 mg/dL, therefore, our patient required three transfusions of packed cells. Thirdly, patient needed antihypertensive therapy because patients with HUS develop arterial hypertension caused by an overexpansion of the intravascular volume and/or ischemia-induced activation of the renin–angiotensin system[12]. Finally, management with low-molecular-weight heparin was necessary to prevent a thrombotic event.

In summary, our patient presented typical clinical features of HUS. This is an uncommon disease in adults, which has a relatively good prognosis with low mortality. The gastroenterologist may encounter the HUS as it presents with primarily intestinal symptoms or may assist in the management of the abdominal complications. Anticipation of the broad clinical scope of the HUS is essential for the optimal management of this serious entity.

After contaminated food is ingested, the *Shiga* toxin (Figure 1) is released into the blood circulation, which leads to damage of the intestinal mucosa and the renal endothelium. Damaged renal endothelial cells promote a prothrombin state with an increase in platelet adhesion and formation of microthrombi[13]. Clinicians should consider *E. coli* O157:H7 when evaluating patients with diarrhea, especially those with a history of bloody diarrhea, and should be aware that patients with *E. coli* O157:H7 infection can get a wrong diagnosis with a non-infectious disease. Afterwards, that is the reason why it is necessary to perform culture stools for *E coli* 0157:H7 since may lead to early recognition of outbreaks and to implement public health control measures. Definitely, surveillance on the organism is needed to define more clearly the clinical illness, populations at risk of infection and risks and benefits of treatment methods.

ARTICLE HIGHLIGHTS

Case characteristics

A 61-year-old woman, with hypertension, osteoarthritis and irritable bowel syndrome, presented to emergency department after 5 d of bloody diarrhea without mucus.

Clinical diagnosis

The diagnosis of hemolytic uremic syndrome (HUS) was based on the presence of diarrheal prodrome, thrombocytopenia and the development of acute renal failure.

Differential diagnosis

Thrombotic thrombocytic purpura and other causes of thrombocytopenia.

Laboratory diagnosis

Moderate hydroelectrolytic disequilibrium, thrombocytopenia and microangiopathic hemolytic anemia.

Pathological diagnosis

The culture showed the presence of *E. coli* producing extended-spectrum β-lactamases.

Treatment

Fluid and electrolyte replacement, plasma exchange, hemodialysis and intravenous antibiotic therapy with ertapenem.

Related reports

HUS associated to gastroenteritis is a rare event in adults due to very few cases had been detected. However, this disorder has high mortality but good prognosis when it is diagnosed in early stages. The treatment should be based on syndromic approach according to guidelines.

Term explanation

ADAMTS13 - a disintegrin-like metalloproteinase with thrombospondin motif type 1 member 13 - cleaves Von Willebrand factor anchored on the endothelial surface, in circulation, and at the sites of vascular injury.

Experiences and lessons

The gastroenterologist may encounter the HUS as it presents with primarily intestinal symptoms or may assist in the management of the abdominal complications. Anticipation of the broad clinical scope of the HUS is essential for the optimal management of this serious entity.

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