Effect of intestinal microbiota imbalance associated with chronic hepatitis B virus infection on the expression of microRNA-192 and GLP-1

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Abstract. It has been reported that hepatitis B virus (HBV) infection has an impact on intestinal microbiota imbalance to induce diabetes mellitus (DM), but the underlying mechanisms still remain to be explored. The present study aimed to investigate the regulatory role of microRNA-192 (miR-192-5p) and glucagon-like peptide-1 (GLP-1) in intestinal microbiota imbalance by recruiting patients with DM infected with HBV. In the present study, patients with HBV infection and different levels of alanine transaminase (ALT) were recruited and divided into three groups. Intestinal microbiota analysis was performed to evaluate the fecal bacterial composition of patients in various groups. Quantitative PCR was performed to explore the differential expression of miR-192-5p and GLP-1 in the feces, peripheral blood and intestinal mucosal tissue samples of each patient. Immunohistochemistry was used to assess the expression of GLP-1 protein in the intestinal mucosal tissue samples. Luciferase assays were performed by cell transfection of miR-192-5p mimics/precursors/inhibitors to study the inhibitory effect of miR-192-5p on GLP-1 expression. Intestinal microbiota imbalance was observed in hepatitis B surface antigen (HBsAg)-positive patients with high ALT. The expression of miR-192-5p was significantly elevated in the feces, peripheral blood and intestinal mucosal tissue samples of HBsAg-positive patients with high ALT along with decreased GLP-1 mRNA and protein expression. Luciferase activity of GLP-1 vector was inhibited by miR-192-5p mimics and promoted by miR-192-5p inhibitors. Transfection of miR-192-5p precursors resulted in upregulation of miR-192-5p and downregulation of GLP-1, while miR-192-5p inhibitors remarkably suppressed the expression of miR-192-5p and notably induced the expression of GLP-1. These results showed a regulatory network involving HBV infection, intestinal microbiota imbalance, and miR-192-5p and GLP-1 expression.

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Introduction

Chronic infection by hepatitis B virus (HBV), or chronic HBV infection (CHBVI), is a major global health problem despite the introduction of effective anti-viral therapies and vaccines (1). According to statistics released by the United Nations, there were >250 million CHBVI patients in 2015 who were positive for hepatitis B surface antigen (HBsAg). Moreover, >50% of patients with CHBVI are located in the Pacific region countries, including Vietnam, Philippines, Japan, Korea and China. For example, the incidence of CHBVI was ~10% in Korea during 2009-2017, although it has been gradually decreasing to a rate of <3% (2,3). DM is associated with the progression of severe liver outcomes in adults with HBV (3).

There is a complex community of intestinal microbiotas, including *Bacteroides*, *Escherichia coli*, *Enterococcus faecalis* and *Bifidobacteria*, living in the human digestive system, including the gut and intestines (4). These intestinal microbiotas are crucial for human health, although the change in the composition of intestinal microbiotas may occur in certain pathological situations to cause bacterial translocation and pathological symptoms (4). In particular, since the liver and intestinal microbiotas are connected by the blood circulation system, the abnormality in intestinal microbiotas can easily affect the liver (5). A previous study showed reduced levels of *Lactobacillus* and *Bifidobacteria* infection in patients with liver cirrhosis and CHBVI, thus weakening the protection provided by the intestinal barrier and resulting in bacterial translocation, microbiota imbalance and endotoxemia (6). As a result, promoting the growth of intestinal bacteria, including *Bifidobacterium* and *Lactobacillus*, while suppressing the reproduction of potential intestinal pathogens, including Enterobacteriaceae, are of great significance in improving the intestinal microbiota imbalance as well as delaying and preventing liver cirrhosis (6).

MicroRNAs (miRNAs/miRs) are a type of non-coding RNA implicated in a number of diseases and the serum levels of certain miRNAs are utilized as markers to aid the prognosis and classification of tumors (7). miR-192-5p, a miRNA related to inflammatory bowel disease, can suppress the expression of nucleotide-binding oligomerization domain-containing

protein 2 while regulating inflammatory reactions in epithelial cells in the colon (8). In addition, miR-215-5p can be utilized as a prognostic biomarker to predict the transformation of non-penetrating Crohn's disease (CD) into penetrating CD (9). Moreover, the expression levels of miR-215-5p and miR-192-5p have been found to be reduced during the progression of colon cancer (10-12). It has also been demonstrated that the miRNAs released from the epithelial cells in the intestine can regulate the composition of local microbiota. Another study also investigated the effect of stomach microbiota on the expression of miRNAs in epithelial cells in the intestine (13). It has previously been demonstrated that the microbiota regulates gene expression in the colon of mice (14). Therefore, it has been further suggested that the miRNAs synthesized by epithelial cells in the intestine are involved in the growth of stomach microbiota (15). Detection of miRNAs isolated from the feces of rodents may help to monitor the relationship between stomach microbiota and miRNA expression in live animals in a time-dependent manner (13). Moreover, miR-192-5p expression is increased in diabetes mellitus (DM) to inhibit insulin production by promoting the apoptosis of β-cells in the pancreas. Moreover, miR-192-5p can promote type 1 DM by reducing the expression of glucagon-like peptide-1 (GLP-1) (16).

It has been shown that HBV infection may cause intestinal microbiota imbalance and deregulation of miR-192-5p expression (17-19). Furthermore, intestinal microbiota imbalance and deregulation of miR-192-5p may elevate the risk of DM by altering the expression of GLP-1 (16,20). In the present study, samples from HBV-infected subjects with or without high alanine transaminase (ALT) levels were collected and the association among HBV infection, liver function and the expression of miR-192-5p and GLP-1 were investigated.

Materials and methods

Human sample collection. This study included subjects undergoing HBsAg testing and stool sample testing at The Third Hospital of Hebei Medical University (Shijiazhuang, China). A total of 60 DM patients with HBV infections were recruited in the research between December 2015 and August 2016. A total of 5 ml peripheral blood was collected from each subject after they were informed that those samples would be used for academic study. These patients were further divided into the following three groups according to their levels of HBsAg and ALT: i) Group A, HBsAg-negative control (NC) group (n=20); ii) group B, HBsAg-positive group with a normal level of ALT (n=20); and iii) group C, HBsAg-positive group with a high level of ALT (n=20). The demographic and clinicopathological characteristics of all participants, such as their age, gender, body mass index (BMI), fat content, systolic blood pressure (BP), diastolic BP, glucose, total cholesterol (C), high-density lipoprotein (HDL)-C, low-density lipoprotein (LDL)-C, triglycerides, total bilirubin, aspartate transaminase (AST), ALT, white blood cell (WBC), platelet, homeostatic model assessment (HOMA)-insulin resistance (IR) and fatty liver symptoms, were collected, summarized and compared, as presented in Table I. Mucosal biopsy samples were obtained from the jejunum or descending colon by dissection under a microscope, removing the overlying smooth muscle and associated myenteric innervation, and then they were directly snap frozen in liquid nitrogen and stored at -18˚C until further analysis. All procedures were approved by the ethics committee of The Third Hospital of Hebei Medical University (approval no. CTHHMU2015037) and written informed consent was obtained from participants before the initiation of this study.

Criteria for enrollment. This research used a case vs. control design as described in a previous article (13). Among all candidates who showed positive reactions in the HBsAg testing, the subjects with the following exclusion criteria were excluded: i) A history of antibiotics usage; ii) a history of probiotics use; iii) a history of treatment using C-lowering drugs; iv) a history of diabetes mellitus; and v) patients with liver cirrhosis. All patients included in the present study who showed positive reactions in the HBsAg testing were matched by control subjects of the same gender and a similar age. All procedures were approved by the institutional ethics committee.

Bacterial enumeration using quantitative (q)PCR. A sample of ~1.0 g feces collected from each participant was directly put in a test tube holding 2 ml RNA later buffer (Ambion; Thermo Fisher Scientific, Inc.) to achieve RNA stabilization. Then, the composition of stomach microbiota in all samples was evaluated with a YIF-SCAN instrument (Yakult Honsha Co., Ltd.) in conjunction with a SYBR Green kit (Thermo Fisher Scientific, Inc.) to measure the expression of bacterial 23S and 16S ribosomal RNA (rRNA) (21-25). It is known that the bacterial species in the digestive system includes six anaerobic species of bacteria, including *Prevotella*, the *Bacteroides fragilis* group, the *Atopobium* cluster, *Bifidobacterium*, the *Clostridium leptum* subgroup and the *Clostridium coccoides* group. In addition, there were five potential pathogens, including *Pseudomonas*, *Staphylococcus*, *Enterococcus*, Enterobacteriaceae and *Clostridium perfringens*, along with eight types of *Lactobacilli*, including the *L. sakei* group, the *L. reuteri* group, the *L. ruminis* group, the *L. plantarum* group, the *L. casei* group, *L. fermentum*, *L. brevis* and the *L. gasseri* group (26,27). The bacteria strains investigated in this research were chosen according to their high rate of incidence in the human digestive system. To test the presence of various bacterial strains in the patient samples, qPCR was performed to confirm the number of 23S and 16S rRNA gene copies related to the quantity of cultured bacteria following a protocol described in previous studies (26,27). Since the YIF-SCAN instrument can be used to quantify the accurate numbers of cells in each of the bacterial species described above, the broad spectrum of bacteria in the gut and intestine, including rare bacteria, can be accurately detected.

Reverse transcription (RT)-qPCR. Total RNA in the feces, peripheral blood (serum samples) and intestinal mucosal tissue samples collected from the patients in various groups was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). Then, RT was carried out using a RT assay kit (Thermo Fisher Scientific, Inc.) according to the manufacture's protocol and then amplified using qPCR carried out on a CFX Real-Time PCR machine (Bio-Rad Laboratories, Inc.) with a GoTaq® Master Mix assay kit (Promega Corporation) following the manufacturer's instructions. The thermocycling

Table I. Demographic and clinical data of the participants included in the present study.

Characteristics	Group A (n=20)	Group B (n=20)	Group C (n=20)	P-value
Age, years	42.5±5.3	43.1±6.2	43.0±5.8	0.842
Male, n (%)	12 (0.6)	10 (0.5)	12 (0.6)	0.846
BMI, kg/m^2	25.6±3.4	25.4±3.8	25.9±3.4	0.326
Fat percent, %	24.1±5.2	23.4±3.5	23.9±3.1	0.645
SBP, mmHg	104.5±5.1	105.8±6.5	105.4±5.2	0.624
DBP, mmHg	68.1±5.3	66.9±5.2	69.3±5.6	0.415
Glucose, mg/dl	93.5±11.2	94.3±7.3	114.6±9.3	<0.01
Total-C, mg/dl	194.2±25.1	199.8±31.6	189.5±25.5	0.342
HDL-C, mg/dl	52.3±3.8	52.8±6.4	53.8±5.8	0.727
LDL, mg/dl	124.5±23.8	124.5±21.8	113±25.2	0.854
Triglycerides, mg/dl	125.6±17.7	128.6±12.4	133.7±6.8	0.251
Total bilirubin, mg/dl	0.91±0.17	0.92±0.13	0.88±0.16	0.532
AST, IU/l	20.3±4.2	21.2±6.8	54.8±7.4	<0.01
ALT, IU/l	20.8±7.2	21.5±6.7	65.8±9.1	<0.01
WBC, x10^3/mm^3	6.3±1.2	6.6±0.5	5.9±0.9	0.335
Platelet, x10^3/mm^3	225.6±58.4	242.1±37.9	237±6.5	0.242
HOMA-IR	1.2±0.4	1.5±0.4	1.2±0.2	0.224
Fatty liver, n (%)	8 (0.4)	8 (0.4)	10 (0.5)	0.425

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; C-, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate transaminase; ALT, alanine transaminase; WBC, white blood count; HOMA-IR, homeostatic model assessment-insulin resistance.

conditions were 10 min at 95˚C, 30 sec at 95˚C (40 cycles), 30 sec at 60˚C and 30 sec at 72˚C. The relative expression of miR-192-5p (Forward: 5'-CTGACCTATGAATTGACA G-3'; Reverse: 5'-GAACATGTCTGCGTATCTC-3') and GLP-1 (Forward: 5'-CGTTCCCTTCAAGACACAGAGG-4'; Reverse: 5'-ACGCCTGGAGTCCAGATACTTG-3') was quantified using the $2^{-\Delta\Delta Cq}$ method (28). U6 (Forward: 5'-GTG CTCGCTTCGGCAGCA-3' Reverse: 5'-CAAAATATGGAA CGCTTC-3') and GAPDH mRNA (Forward: 5'-GTCTCC TCTGACTTCAACAGCG-3'; Reverse: 5'-ACCACCCTGTTG CTGTAGCCAA-3') were utilized as the internal standard for miR-192-5p and GLP-1, respectively.

Cell culture and treatment. CAPAN-1 and HPAC cells were acquired from American Type Culture Collection, and then cultured in high-glucose DMEM (HyClone; Cytiva) with 100 g/ml streptomycin, 2 mM/ml L-glutamine, 100 U/ml penicillin and 10% FBS (HyClone; Cytiva). Then, the cells were divided into the following three groups: i) Negative control (NC) group; ii) a group of miR-192-5p precursors; and iii) a group of miR-192-5p inhibitors. Cells were cultured at 37˚C in a humidified tissue culture incubator containing 5% CO_2. On the day of the experiment, 1x10^4 cells/well of CAPAN-1 and HPAC cells were transfected with 30 nM NC miRNA (5'-CAGUACUUUUGUGUAGUACAA-3'), miR-192-5p precursors (sense, 5'-CUGACCUAUGAAUUGACAGCC-3' and anti-sense, 5'-CUGUCAAUUCAUAGGUCAGUU-3') or miR-192-5p inhibitors (sense, 5'-GGCUGUCAAUUCAUA GGUCAG-3' and anti-sense, 5'-GGCUGUCAAUUCAUA GGUCAG-3') manufactured by Guangzhou RiboBio Co., Ltd., using FuGENE® 6 transfection reagent (Promega Corporation) according to the manufacturer's instructions. Scrambled miRNA was used as the NC. The transfected cells were harvested after 48 h of transfection for subsequent assays.

Vector construction, mutagenesis and luciferase assay. The potential targets of miR-192-5p were identified using TargetScan (http://www.targetscan.org/vert_72/) (29). Then, GLP-1 was identified as a potential miR-192-5p target, and the 3' UTR fragment of GLP-1 containing the miR-192-5p binding site was inserted into a pcDNA3.1 luciferase vector (Promega Corporation) to generate the wild-type 3' UTR plasmid of the GLP-1 gene. In the next step, site-directed mutagenesis was carried out using a QuikChange mutagenesis kit (Stratagene) following the manufacturer's instructions, and the mutant 3' UTR fragment of GLP-1 containing the mutated miR-192-5p binding site was inserted into another pcDNA3.1 vector to generate the mutant plasmid of GLP-1 3' UTR. Then, CAPAN-1 and HPAC cells were transfected with wild-type or mutant plasmid of GLP-1 3' UTR in conjunction with miR-192-5p precursors using FuGENE 6 transfection reagent. At 48 h after the start of the transfection, the activity of firefly luciferase was normalized to *Renilla* luciferase activity and measured using a Luciferase assay kit (Cytiva) in conjunction with a Synergy plate reader (BioTek Instruments, Inc.).

Western blotting. Cell lysate containing 1% NP-40, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl, 150 mM NaCl and protease inhibitors (Roche Diagnostics) was prepared in an ice-cold lysis buffer (pH 7.4). The protein concentration was measured with a BCA assay kit (Bio-Rad Laboratories, Inc.)

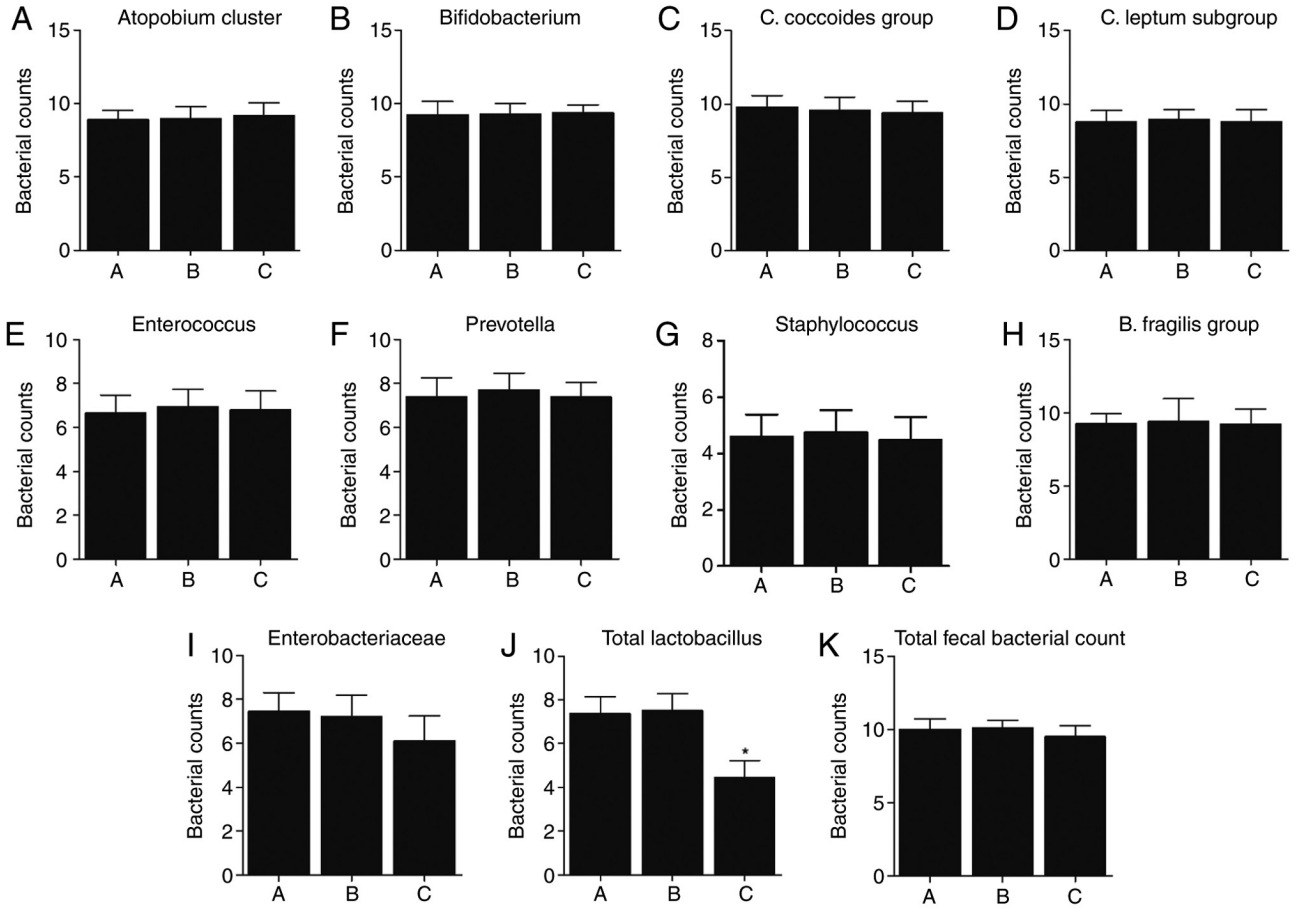

Figure 1. Microbiota imbalance was observed in HBsAg-positive patients with high ALT levels. (A-K) Differences in bacterial counts among the following groups of patients: i) Group A, HBsAg-negative control; ii) group B, HBsAg-positive patients with normal ALT levels; and iii) group C, HBsAg-positive patients with high ALT levels. *P<0.05 vs. Group A. HBsAg, hepatitis B surface antigen; ALT, alanine transaminase.

and total protein (50 μg/lane) was separated via SDS-PAGE on 6% gel, which was then electrotransferred onto a nitrocellulose membrane. After being blocked in phosphate buffered saline (PBS) and 5% non-fat dry milk at room temperature for 1 h, the membrane was incubated at 4˚C for 12 h in PBS containing primary anti-GLP-1 antibody (cat. no. ab240494; 1:10,000; Abcam) and β-actin (cat. no. ab8226; Abcam) as the internal control (1:10,000; Abcam). After being washed twice in PBS and incubated at room temperature for 2 h with secondary antibodies conjugated to horseradish peroxidase (cat. no. ab6721; 1:15,000; Abcam), the protein level of GLP-1 was visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) and subsequently semi-quantified using Quantity One software (v4.6.6, Bio-Rad Laboratories, Inc.).

Immunohistochemistry. The immunohistochemistry staining of feces, peripheral blood and intestinal mucosal tissue samples (5 μm sections blocked in 3% hydrogen peroxide for 15 min and incubated at room temperature for 120 min) collected from various groups of patients was performed using an anti-GLP-1 staining kit including the corresponding primary and secondary antibodies (cat. no. AB3244, EMD Millipore), following the manufacturer's instructions, to determine the GLP-1 protein expression in various samples. The samples were counterstained with hematoxylin at 37˚C for 2 h and observed under an Olympus light microscope (magnification, x200; Olympus Corporation).

Statistical analysis. All data was tested for heterogeneity using the I^2 statistical method. Inter-group comparisons were performed using one-way analysis of variance (ANOVA), followed by the Scheffe post hoc test. All statistical calculations were carried out using Stata software version 12.0 (StataCorp LP). P<0.05 was considered to indicate a statistically significant difference. All data are presented as the mean ± standard deviation and each experiment was repeated in triplicate.

Results

Demographic and clinicopathological characteristics of the participants recruited in this study. A total of 60 patients with HBV infections were recruited in this research. These patients were further divided into three groups according to their levels of HBsAg and ALT: Group A, B and C. The demographic and clinicopathological characteristics of all participants, including their age, gender, BMI, fat content, systolic BP, diastolic BP, glucose, total-C, HDL-C, LDL-C, triglycerides, total bilirubin, AST, ALT, WBC, platelet, HOMA-IR and fatty liver symptoms, were collected, summarized and compared in Table I. One-way ANOVA was utilized to compare the

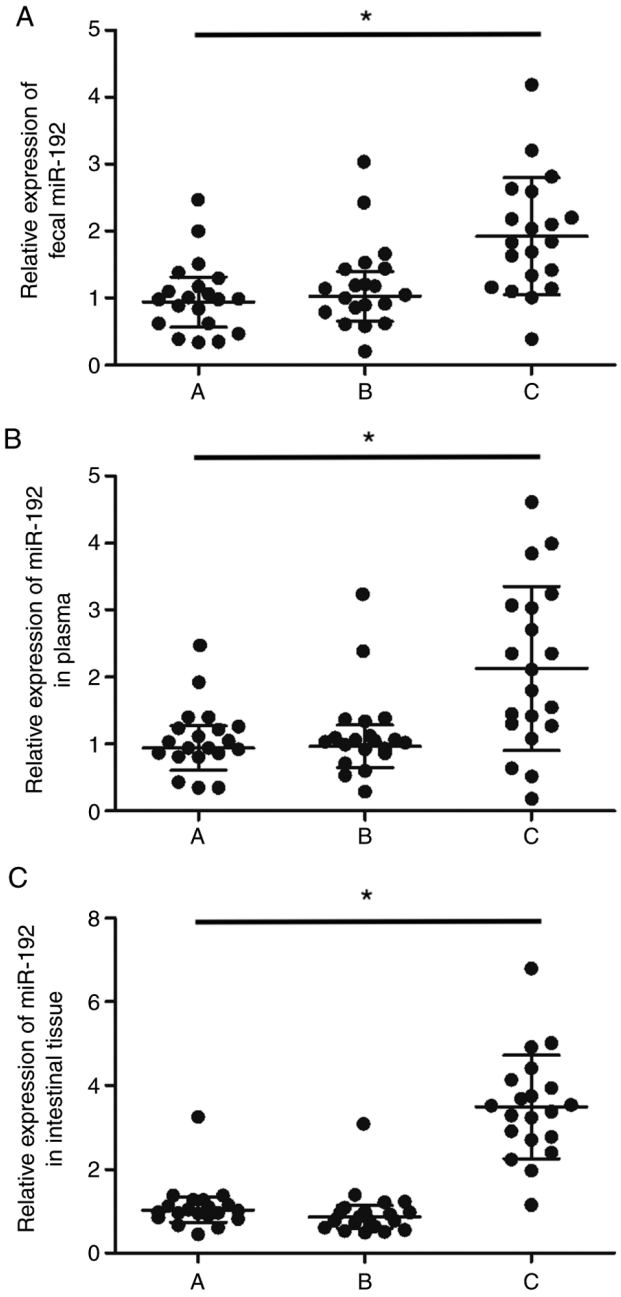

Figure 2. miR-192-5p expression is upregulated in the feces, peripheral blood and intestinal mucosal tissue samples of HBsAg-positive patients with high ALT levels. miR-192-5p expression in the (A) feces, (B) peripheral blood and (C) intestinal mucosal tissue was elevated in patients in Group C who were HBsAg-positive with high ALT levels. *P<0.05 vs. Group A. miR, microRNA; HBsAg, hepatitis B surface antigen; ALT, alanine transaminase.

differences among the three groups. The results showed that only the glucose level and liver function (indicated by AST and ALT level) in the HBsAg-positive group with high ALT was much higher than that in the other two groups, whereas the other characteristics showed no obvious differences among the three groups.

Reduced total Lactobacillus in HBsAg-positive patients with high ALT. Fecal sampling was carried out to analyze intestinal microbiotas in the patients. No obvious difference was found in terms of the number of majority of bacteria among

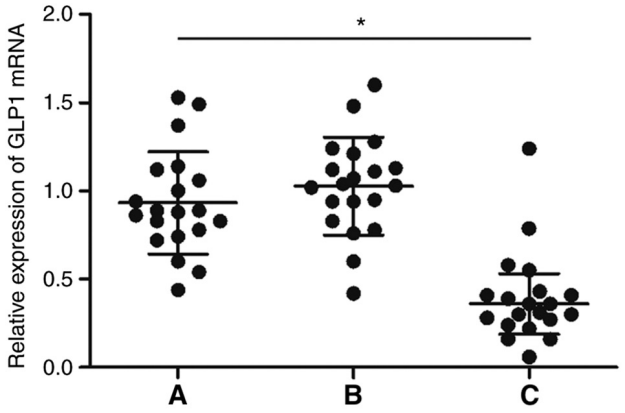

Figure 3. Expression of GLP-1 mRNA in the intestinal mucosal tissue is inhibited in hepatitis B surface antigen-positive patients with high alanine transaminase levels. *P<0.05 vs. Group A. GLP-1, glucagon-like peptide-1; HBsAg, hepatitis B surface antigen; ALT, alanine transaminase; NC, negative control; Group A, HBsAg-NC group; Group B, HBsAg-positive group with a normal level of ALT; Group C, HBsAg-positive group with a high level of ALT.

the three groups, except that the counts of total *Lactobacillus* were significantly lower in Group C compared with that in Groups A and B (Fig. 1A-K).

Upregulated miR-192-5p expression in the feces, peripheral blood and intestinal mucosal tissue samples of HBsAg-positive patients with high ALT. Then, the peripheral blood, intestinal mucosal tissue and fecal samples were collected from every patient to measure their miR-192-5p expression. It was found that the expression of miR-192-5p was significantly upregulated in the feces (Fig. 2A), peripheral blood (Fig. 2B) and intestinal mucosal tissue (Fig. 2C) samples from the HBsAg-positive patients with a high level of ALT compared with other patients.

GLP-1 expression is suppressed in the intestinal mucosal tissue samples of HBsAg-positive patients with high ALT. RT-qPCR was performed to analyze the differential expression of GLP-1 mRNA in the intestinal mucosal tissue samples collected from patients of the three groups. The results clearly showed that the expression of GLP-1 mRNA was significantly lower in patients in Group C compared with that in Groups A and B (Fig. 3). Furthermore, immunohistochemistry was carried out to explore the differential expression of GLP-1 proteins in a case representative of each of the three groups of patients. Consistently, GLP-1 protein was evidently downregulated in patients in Group C (Fig. 4).

miR-192-5p inhibits GLP-1 expression by binding to the 3' UTR of GLP-1. In order to explore the regulatory role of miR-192-5p in GLP-1 expression, luciferase plasmids of wild-type GLP-1 3' UTR were constructed to co-transfect CAPAN-1 and HPAC cells with miR-192-5p precursors, miR-192-5p inhibitors, anti-miR-control and miR-control, respectively. miR-192-5p effectively suppressed the luciferase activity of wild-type GLP-1 3' UTR in CAPAN-1 (Fig. 5A) cells. Then, miR-192-5p precursors and miR-192-5p inhibitors were transfected into CAPAN-1 cells, and miR-192-5p

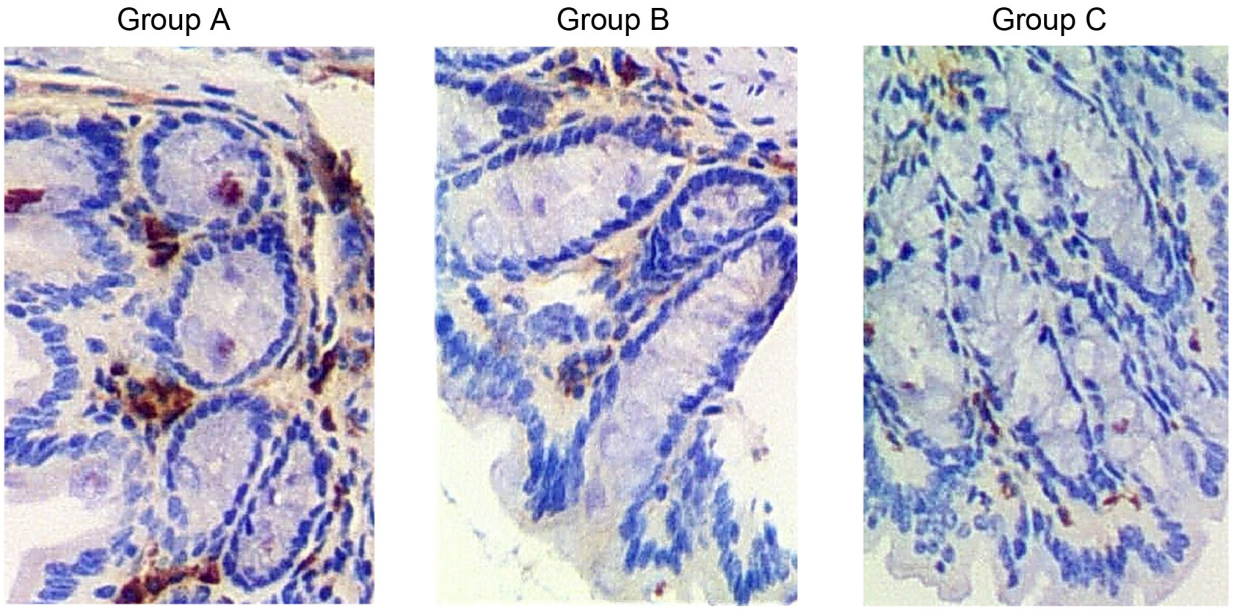

Figure 4. Expression of GLP-1 protein in the intestinal mucosal tissue was inhibited in hepatitis B surface antigen-positive patients with high alanine transaminase levels. GLP-1, glucagon-like peptide-1; HBsAg, hepatitis B surface antigen; ALT, alanine transaminase; NC, negative control; Group A, HBsAg-NC group; Group B, HBsAg-positive group with a normal level of ALT; Group C, HBsAg-positive group with a high level of ALT.

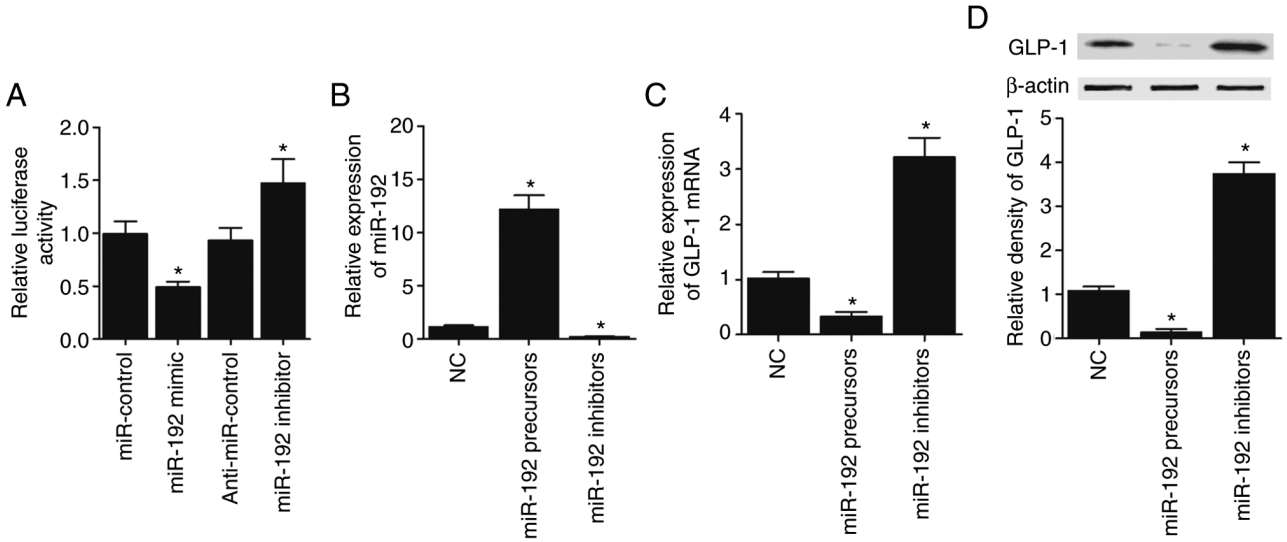

Figure 5. Inhibition of GLP-1 by miR-192-5p in CAPAN-1 cells. (A) Luciferase activity was decreased in CAPAN-1 cells transfected with miR-192-5p precursors and increased in CAPAN-1 cells transfected with miR-192-5p inhibitors. *P<0.05 vs. miR-control group. (B) The expression of miR-192-5p was upregulated in CAPAN-1 cells transfected with miR-192-5p precursors and reduced in CAPAN-1 cells transfected with miR-192-5p inhibitors. *P<0.05 vs. NC group. (C) The expression of GLP-1 mRNA was downregulated in CAPAN-1 cells transfected with miR-192-5p precursors and upregulated in CAPAN-1 cells transfected with miR-192-5p inhibitors. *P<0.05 vs. NC group. (D) The expression of GLP-1 protein was downregulated in CAPAN-1 cells transfected with miR-192-5p precursors and upregulated in CAPAN-1 cell transfected with miR-192-5p inhibitors. *P<0.05 vs. NC group. GLP-1, glucagon-like peptide-1; miR, microRNA; NC, negative control.

expression was significantly elevated in CAPAN-1 cells transfected with miR-192-5p precursors but decreased in CAPAN-1 cells transfected with miR-192-5p inhibitors (Fig. 5B). On the contrary, GLP-1 mRNA (Fig. 5C) and protein (Fig. 5D) expression was significantly decreased in CAPAN-1 cells transfected with miR-192-5p precursors and significantly increased by miR-192-5p inhibitors. Furthermore, all the results described above were confirmed in HPAC cells (Fig. 6A-D).

Discussion

HBV can induce severe liver disorders, such as hepatocellular carcinoma, cirrhosis, and chronic and acute hepatitis (30). In particular, a number of patients with CHBVI are infected during early childhood, whereas infection with HBV in adults can be caused by sharing syringes and sexual intercourse (21,22,31). In a previous controlled case study, it was found that CHBVI apparently elevated the risks of pancreatic cancer in patients

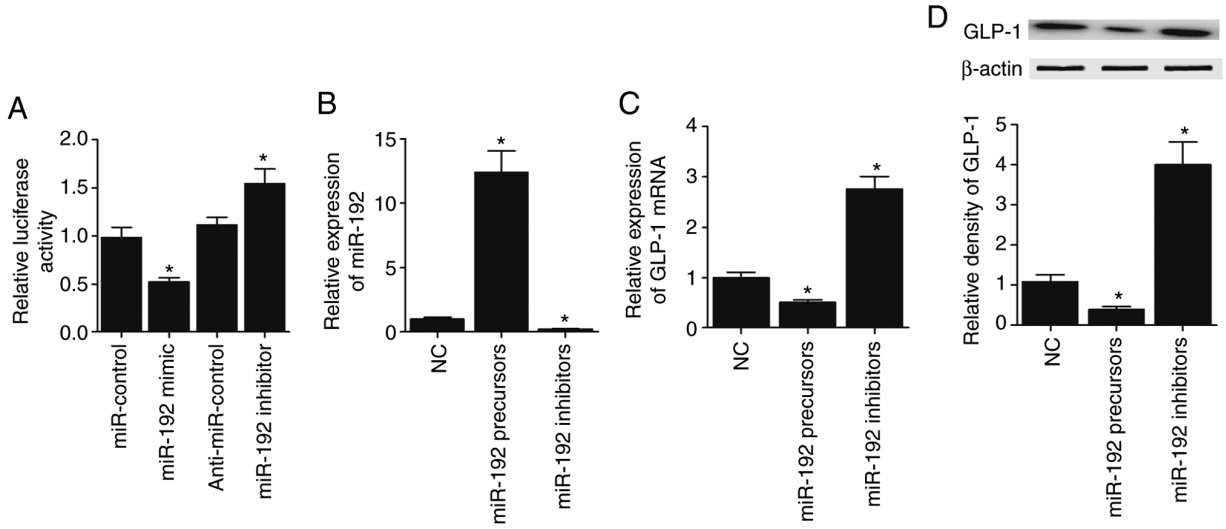

Figure 6. Inhibition of GLP-1 by miR-192-5p in HPAC cells. (A) Luciferase activity was decreased in CAPAN-1 cells transfected with miR-192-5p mimics and increased in CAPAN-1 cells transfected with miR-192-5p inhibitors. *P<0.05 vs. miR control group. (B) The expression of miR-192-5p was upregulated in CAPAN-1 cells transfected with miR-192-5p precursors and reduced in CAPAN-1 cells transfected with miR-192-5p inhibitors. *P<0.05 vs. NC group. (C) The expression of GLP-1 mRNA was downregulated in CAPAN-1 cells transfected with miR-192-5p precursors and upregulated in CAPAN-1 cells transfected with miR-192-5p inhibitors. *P<0.05 vs. NC group. (D) The expression of GLP-1 protein was downregulated in CAPAN-1 cells transfected with miR-192-5p precursors and upregulated in CAPAN-1 cell transfected with miR-192-5p inhibitors. *P<0.05 vs. NC group. GLP-1, glucagon-like peptide-1; miR, microRNA; NC, negative control.

in Korea and Taiwan (23). Moreover, DM patients showed an apparently higher rate of CHBVI than patients with DM who were not positive for PC. By contrast, it was also shown that the patients with CHBVI had a lower level of *B. catenulatum*. Therefore, it was suggested that *B. catenulatum* could help to protect the liver against acute injuries by restoring the normal status of intestinal microbiotas to decrease the levels of endotoxin and cytokines in the stomach (24,25). In the present study, three groups of patients were recruited, Group A (HBsAg-negative control), group B (HBsAg-positive with normal ALT levels) and group C (HBsAg-positive with high ALT levels), to analyze the profiles of intestinal microbiota in different groups. Total lactobacillus count was obviously elevated in Group C patients. ALT levels are a signal of liver lesions, and in patients with high or low ALT, the microbiota was different, which interfered with miR-192/GLP-1 signaling. In addition, RT-qPCR was performed to evaluate the expression of miR-192-5p in the feces, peripheral blood and intestinal mucosal tissue samples of patients in different groups, and the miR-192-5p expression was notably increased in the patients in Group C. miR-192-5p-5p expression is reduced in various types of cancer, such as colorectal, renal and lung cancer. In addition, the activation of miR-192-5p-5p has been shown to promote renal cell apoptosis (10,32,33). Moreover, Boni *et al* (26) demonstrated that increased miR-192-5p expression also elevated the efficacy of 5-fluoruracil in killing tumor cells of colon cancer by inducing cell cycle arrest. Moreover, Hu *et al* (27) reported that miR-192-5p expression was reduced in breast cancer. The present study compared the expression of GLP-1 as a target of miR-192-5p-5p in the intestinal mucosal tissue samples of patients in various groups, and GLP-1 expression was found to be remarkably decreased in patients in Group C.

It has been demonstrated that fecal miRNAs are essential components in the lumen of the stomach (34). Moreover, fecal miRNAs are mainly produced by epithelial cells in the intestine (35). In particular, fecal miRNAs help to regulate stomach microbiota by targeting the genes of various bacteria (34). The aforementioned results indicate the important role of stomach microbiota in maintaining the homeostasis of its host (36). Moreover, miR-192-5p expression is increased in DM to suppress the secretion of insulin and the proliferation of β-cells (37). Moreover, miR-192-5p can suppress the proliferation of NIT-1 cells, while promoting their apoptosis (16). In the present study, luciferase assays were performed to explore the regulatory relationship between miR-192-5p and GLP-1, and the results showed that miR-192-5p suppressed the luciferase activity of GLP-1 vector. In addition, CAPAN-1 and HPAC cells were transfected with miR-192-5p precursors and inhibitors. The transfection of miR-192-5p precursors effectively upregulated the expression of miR-192-5p, but suppressed the expression of GLP-1, while the transfection of miR-192-5p inhibitors inhibited the expression of miR-192-5p but promoted the expression of GLP-1. In line with this, a previous study showed suppressed expression of GLP-1 in the presence of miR-192-5p (37), suggesting that miR-192-5p can promote DM development by negatively regulating GLP-1 expression.

GLP-1 is released by L-cells living in small intestines and can increase the secretion of insulin from β-cells while reducing the production of glucose in the liver (38,39). GLP-1 can also participate in the physiological process of weight loss (40,41). The dosing of GLP-1 in mice was found to reduce sucrose responses, while the suppression of GLP-1 expression increased food intake (42,43).

Increased GLP-1 expression can promote insulin secretion to accelerate the metabolism of glucose, while the release of GLP-1 is elevated by adding fatty acids, glucose, dietary fibers and amino acids into the diet (41). In addition, using a diet containing fatty acids, proteins and glucose rather than glucose by itself, the prognosis of patients with DM can be improved (41).

In conclusion, the present results demonstrated the association between HBV infection and the composition of gut microbiota in patients with DM. HBV infection can affect the composition of the gut microbiota, leading to an imbalanced proportion of *Lactobacillus*. Therefore, the gut microbiota composition was altered in patients with different HBV-infection statuses and serum ALT levels, indicating the presence of a potential link between the severity of DM and the expression of miR-192-5p and GLP-1.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author's contributions

YL designed the study, collected the experimental data, analyzed the data, confirmed the authenticity of all the raw data and wrote the manuscript.

Ethics approval and consent to participate

All procedures were approved by the ethics committee of The Third Hospital of Hebei Medical University (approval no. CTHHMU2015037) and written informed consent was obtained from participants before the initiation of this study.

Patient consent for publication

Not applicable.

Competing interests

The author declares that he has no competing interests.

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