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Original Article

Serum sphingosine-1 phosphate level is increased in patients with hepatitis B and displays a positive association with liver fibrosis

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Abstract: Objective: To investigate the difference in serum sphingosine-1 phosphate (S1P) concentration between the HBV hepatitis patients and healthy controls, and its relevant association with serum liver fibrosis indicators. Methods: A total of 28 HBsAg (+) HBeAg (+) Anti-HBc (+) hepatitis B patients, 42 HBsAg (+) Anti-HBe (+) Anti-HBc (+) hepatitis B patients, and 21 healthy subjects with normal liver function were included. Liquid chromatography-tandem mass method was used to detect the level of serum S1P. Results: SerumS1P concentration of Anti-HBe (+) hepatitis B patients was higher than that of the control group (P=0.017) and HBeAg (+) patients (P=0.007). At the same time, there was no significant difference in the serum S1P concentration between HBeAg (+) hepatitis B patients and the control group (P>0.05). Moreover, serum S1P concentration was positively correlated with liver fibrosis indices, Collage Type IV Protein (r=0.264; P=0.011), and Chitosanase 3-like protein 1 (r=0.295; P=0.004). Conclusions: Serum S1P level is increased in patients with hepatitis B and displays a positive association with liver fibrosis.

Keywords: Hepatitis B, hepatic fibrosis, sphingosine 1-phosphate, collagen type IV protein, chitosanase 3-like protein 1

Introduction

Chronic hepatitis B (CHB) affects about 350 million people worldwide. Furthermore, China suffers from hepatitis B greatly, and there are mainly two serotypes occurring in China: HBsAg (+) HBeAg (+) Anti-HBc (+) and HBsAg (+) Anti-HBe (+) Anti-HBc (+). CHB is one of the most common causes of chronic liver injury, which can advance to liver fibrosis. It was reported that 600,000 cases die of complication due to chronic HBV infection every year [1-3]. Liver fibrosis has been documented as a reversible process, and restraining this pathologic process may reduce the incidence of post-hepatitis B cirrhosis and liver cancer.

Sphingosine-1 phosphate (S1P), a bioactive sphingolipid produced by ceramide through ceramidase action and then phosphorylated by sphingosine kinase 1 or 2 (SphK1 or SphK2) [4-6], was found in 1960s and is mainly produced by erythrocytes. S1P primarily binds apolipoprotein M (ApoM) and albumin *in vivo* and performs biological functions through sphingosine-1 phosphate receptors (S1PRs) residing on the cell membrane [7].

S1P is connected with many diseases. In particular, it has been reported to be related to hepatitis: It was indicated that there was a positive correlation between S1P and Nod-like receptor family protein 3 (NLRP3) inflammasome in mouse hepatitis models [8]; Knockout of SphK1 can alleviate the liver injury after ischemia-reperfusion by reducing inflammatory response [9]; S1P receptor activator KRP203 could target CXCR4 (+) CD4 (+) T lymphocytes in peripheral blood and inhibit concanavalin A (ConA) - induced immune hepatitis [10]. These findings indicate that S1P is correlated to hepatitis.

Moreover, other studies revealed that SphK was up-regulated in human liver fibrosis, which promoted the expression of S1P level and fur-

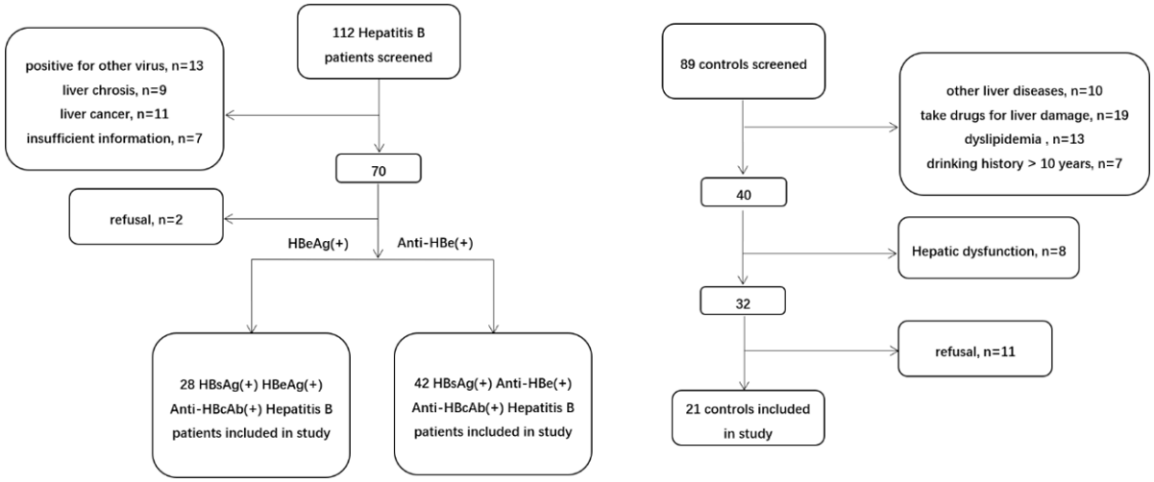

Figure 1. Flow chart of the inclusion and exclusion criteria in this study. 70 patients with hepatitis B were selected according to the principle of the method, and 42 hepatitis B patients with HBsAg (+) HBeAg (+) Anti-HBc (+) and 28 patients with HBsAg (+) Anti-HBe (+) Anti-HBc (+) were selected. In addition, 21 subjects were included in the control group of this study.

ther improved the directional migration ability of myofibroblasts [11]. Reducing S1P can mitigate the development of liver fibrosis in mice [12]. It is suggested that S1P is also related to the development of liver fibrosis. However, no references described the changes in S1P in hepatitis B patients or analyzed the relationship of S1P to liver fibrosis in hepatitis B patients.

Therefore, this study detected the change of serum S1P concentration between hepatitis B patients and normal controls and explored the relationship between serum S1P and liver fibrosis markers, illustrating whether S1P can be employed as a new non-invasive diagnostic or therapeutic index of liver fibrosis.

Materials and methods

Research subjects

112 hepatitis B patients were recruited from The Second Xiangya Hospital of Central South University (Changsha, Hunan, People's Republic of China) from January to October 2021. 28 HBsAg (+) HBeAg (+) Anti-HBc (+) hepatitis B patients and 42 HBsAg (+) Anti-HBe (+) Anti-HBc (+) hepatitis B patients were finally included in this study (**Figure 1**). The median age of the HBsAg (+) HBeAg (+) Anti-HBc (+) group was 32 (25-45) years old, which consists of 14 males and 14 females. The median age of HBsAg (+) Anti-HBe (+) Anti-HBc (+) group was 35 (27, 47) years old, including 25 males and 17 females. Screening criteria for hepatitis B patients were as follows: (1) Other viral hepatitis; (2) Alcoholic/nonalcoholic fatty liver disease, autoimmune liver disease, parasitic hepatitis, and other liver diseases; (3) Liver cirrhosis and liver cancer; (4) Antiviral therapy. The control group was screened during the period between January to October 2021 at the health management center of the Second Xiangya Hospital of Central South University. We recruited 89 people with the virological assessment test all negative, and finally, 21 subjects were included (**Figure 1**). All subjects were provided with signed informed consent, and the study protocol was approved by the investigation and review committee of the Xiangya Second hospital.

Virological assessment

Levels of serum HBsAg, Anti-HBs, HBeAg, Anti-HBe, Anti-HBc were determined by chemiluminescence immunoassay using an ARCHITECT i2000sr (Abbott Laboratories, USA).

Liver biochemical assays

The concentration of serum alanine aminotransferase (ALT) was determined by IFCC method; aspartate transaminase (AST), total protein (TP) and albumin (ALB) were determined by colorimetric method; globulin (GLO) is the subtraction of TP and ALB; total bilirubin (TBIL)

Table 1. Baseline characteristics of the subjects included in this study

	Control	HBsAg (+) HBeAg (+) Anti-HBc (+)	HBsAg (+) Anti-HBe (+) Anti-HBc (+)	*P*-value
Subjects, n	21	28	42	
Demographics				
Age, years	30 (26, 33)	32 (25, 45)	35 (27, 47)	0.272
Sex, % male	29%	50%	60%	0.070
Liver function indices				
ALT, U/L	12.80 (8.85, 16.85)	25.85 (19.60, 47.65)***	22.70 (15.40, 32.30)***	<0.001
AST, U/L	16.80 (14.95, 20.25)	23.20 (18.10, 37.93)**	20.50 (17.00, 25.85)*	0.002
TP, g/L	74.10 (71.45, 76.30)	70.25 (66.80, 73.50)*	67.55 (62.78, 71.10)***	<0.001
ALB, g/L	46.20 (43.70, 48.00)	43.40 (38.88, 45.08)**	39.55 (36.05, 43.00)***	<0.001
GLO, g/L	28.20 (26.25, 29.15)	27.50 (25.38, 31.13)	27.10 (25.18, 29.48)	0.618
TBIL, μmol/L	8.70 (6.70, 12.50)	10.20 (8.53, 11.85)	9.25 (7.08, 13.03)	0.600
DBIL, μmol/L	3.00 (1.90, 3.85)	3.40 (2.85, 3.78)	3.00 (2.30, 4.15)	0.384
TBA, μmol/L	3.00 (1.95, 5.35)	5.70 (2.58, 8.90)	4.45 (2.78, 7.58)	0.058

Data are presented as median with interquartile range. *P<0.05, **P<0.01, ***P<0.001, HBsAg (+) HBeAg (+) Anti-HBc (+) group and HBsAg (+) Anti-HBe (+) Anti-HBc (+) group compared to the control group respectively. AST = alanine aminotransferase; ALT = aspartate transaminase; TP = total protein; ALB = albumin; GLO = globulin, TBIL = total bilirubin; DBIL = direct bilirubin; TBA = Total bile acid.

and direct bilirubin (DBIL) were determined by diazotization method. Total bile acid (TBA) was determined by enzymatic cycling method, which all use a HITACHI 7600-210 (HITACHI, Japan).

Liver fibrosis index assays

Fibrosis-4 score (FIB-4) and aspartate aminotransferase platelet ratio index (APRI) were solved by the formula:

$$FIB\text{-}4 = [Age(years) \times AST(U/L)] / [PLT(10^9/L)\, LT\sqrt{ALT(U/L)}];$$

APRI=[AST(U/L)÷ULN×100]/PLT(10^9/L), ULN refers to the upper limit of normal AST.

Levels of serum of collagen type IV protein (C IV), hyaluronic acid (HA), laminin (LN), and procollagen type III peptide (PIIINP) were determined by chemiluminescence method using a CL6000i (Mindray, China); and the concentration of serum chitinase-3-like protein-1 (CHI3L1) was determined by chemiluminescence method using an iFlash 3000-H (YHLO, China).

Serum S1P assays

The concentration of serum S1P was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an API5500 (AB SIENX, USA).

Statistical analyses

Continuous data are expressed as median (interquartile range). Relationships between continuous variables were tested by Spearman's rank correlation coefficient analysis. Median differences were compared by Kruskal Wallis test. Statistical analyses were performed using SPSS 25.0 (SPSS Statistics, USA) or GraphPad Prism 8.0 (GraphPad Software, USA) software. Two-tailed *P*-values <0.05 were considered significant.

Results

Baseline characteristics of the subjects included in this study

70 patients with hepatitis B were selected according to the principle of the method, and 42 hepatitis B patients with HBsAg (+) HBeAg (+) Anti-HBc (+) and 28 patients with HBsAg (+) Anti-HBe (+) Anti-HBc (+) were selected. In addition, among the 40 HBsAg (-) Anti-HBs (-) HBeAg (-) Anti-HBe (-) Anti-HBc (-) control groups, 8 subjects in the control group had abnormal liver function and 11 subjects refused to participate in study. Therefore, 21 subjects were included in the control group of this study (**Figure 1**). The clinical data of the subjects are shown in **Table 1**. There was no difference in age and gender among the three groups.

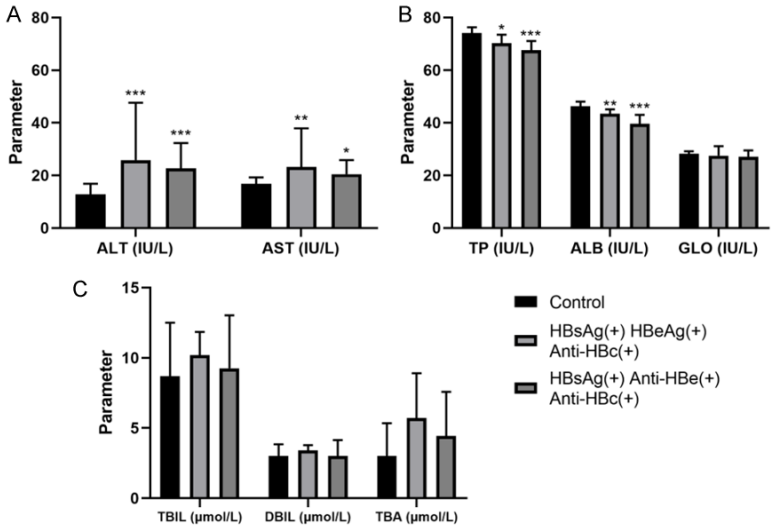

Figure 2. Serum concentrations of liver function indices in the control group and hepatitis B patients. A. The concentrations of ALT and AST in the HBsAg (+) HBeAg (+) Anti-HBc (+) group and the HBsAg (+) Anti-HBe (+) Anti-HBc (+) group were higher than in the control group; B. The concentrations of TP and ALB in the HBsAg (+) HBeAg (+) Anti-HBc (+) group and the HBsAg (+) Anti-HBe (+) Anti-HBc (+) group were lower than in the control group, while the difference of GLO between the three groups was not significant; C. The difference of TBIL, DBIL and TBA between the three groups were not significant. Data are the median with interquartile range. *P<0.05, **P<0.01, ***P<0.001, HBsAg (+) HBeAg (+) Anti-HBc (+) group and HBsAg (+) Anti-HBe (+) Anti-HBc (+) group compared to the control group respectively. (AST = alanine aminotransferase; ALT = aspartate transaminase; TP = total protein; ALB = albumin; GLO = globulin; TBIL = total bilirubin; DBIL = direct bilirubin; TBA = Total bile acid).

Changes in liver function indicators of HBeAg (+) hepatitis B patients, Anti-HBe (+) hepatitis B patients, and control group

As shown in **Table 1** and **Figure 2**, serum ALT level in the HBeAg (+) group (P<0.001), Anti-HBe (+) group (P=0.001) was significantly higher than that of the control group. At the same time, the serum AST level in HBeAg (+) group (P=0.002) and Anti-HBe (+) group (P=0.038) were also higher than that in the control group. In addition, serum TP (P= 0.05) and ALB (P=0.005) were decreased in HBeAg (+) group compared with the control group; Compared to the control group, serum TP (P<0.001) and ALB (P<0.001) in the Anti-HBe (+) group also decreased. However, there was no significant difference in liver function indices between the HBeAg (+) group and Anti-HBe (+) group, and no significant difference was observed in serum GLO, TBIL, DBIL, and TBA among the three groups (P>0.05).

Changes in liver fibrosis indicators among HBeAg (+) hepatitis B patients, Anti-HBe (+) hepatitis B patients, and control group

There was no difference in liver fibrosis index FIB-4 among the three groups (P> 0.05). Compared to the control group, the liver fibrosis indices APRI [0.36 (0.22, 0.54) vs. 0.19 (0.16, 0.25); P<0.001], CHI3L1 [74.70 (43.39, 104.85) vs. 37.76 (30.25, 41.62) ng/ml; P= 0.007] in the HBeAg (+) group were higher, while there was no significant difference in C IV, HA, LN and PIIINP (P>0.05). However, in the Anti-HBe (+) group, liver fibrosis indices APRI [0.24 (0.20, 0.37) vs. 0.19 (0.16, 0.25); P=0.026], C IV [72.91 (57.49, 98.57) vs. 51.46 (41.52, 61.64) ng/ml; P<0.001], HA [50.54 (26.33, 98.85) vs. 16.12 (13.63, 23.17) ng/ml; P<0.001], LN [30.37 (25.08, 36.23) vs. 25.80 (21.43, 28.72) ng/ml; P=0.021], PIIINP [10.99 (8.72, 18.03) vs. 7.12 (5.96, 8.75) ng/ml; P<0.001] and CHI3L1 [128.55 (67.96, 246.59) vs. 37.76 (30.25, 41.62) ng/ml; P<0.001] were higher than those in the control group. In addition, the indices of liver fibrosis C IV (P=0.038), HA (P=0.001), CHI3L1 (P=0.027) in Anti-HBe (+) group were higher than those in HBeAg (+) group (**Figure 3**).

Anti-HBe (+) hepatitis B patients with elevated serum S1P

Figure 4 shows that the serum S1P concentration of the Anti-HBe (+) group was higher than that of the control group [297.07 (245.18, 314.25) vs. 314.90 (279.03, 376.38) ng/ml; P=0.017], and there was no significant difference in serum S1P concentration between the control group and HBeAg (+) group (P>0.05). In addition, serum S1P concentration in the Anti-HBe (+) group was higher than that in HBeAg (+) group [297.07 (245.18, 314.25) vs. 285.01 (240.61, 307.90) ng/ml; P=0.007].

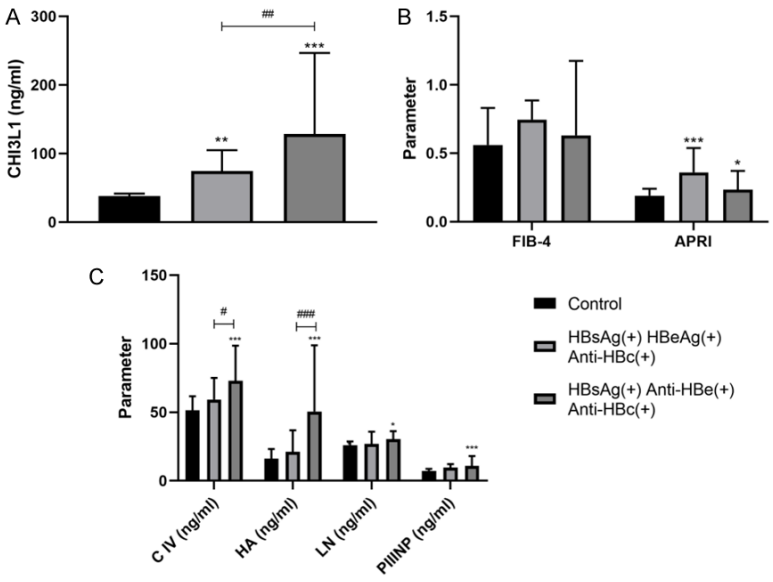

Figure 3. Serum concentrations of liver fibrosis indices in control group and hepatitis B patients. A. CHI3L1 of both hepatitis B groups were higher than the control group, and higher CHI3L1 levels in HBsAg (+) Anti-HBe (+) Anti-HBc (+) group than the HBsAg (+) HBeAg (+) Anti-HBc (+) group; B. The level of APRI in the HBsAg (+) HBeAg (+) Anti-HBc (+) group and the HBsAg (+) Anti-HBe (+) Anti-HBc (+) group were higher than in the control group, while the difference of FIB-4 between the three groups was not significant; C. Only the concentrations of C IV, HA, LN and PIIINP in the HBsAg (+) Anti-HBe (+) Anti-HBc (+) group were higher than in the control group, while the difference of C IV, HA, LN and PIIINP between the HBsAg (+) HBeAg (+) Anti-HBc (+) group and the control group were not significant Higher C IV and HA levels were noted in the HBsAg (+) Anti-HBe (+) Anti-HBc (+) group than in the HBsAg (+) HBeAg (+) Anti-HBc (+) group. Data are the median with interquartile range. *P<0.05, **P<0.01, ***P<0.001, HBsAg (+) HBeAg (+) Anti-HBc (+) group and the HBsAg (+) Anti-HBe (+) Anti-HBc (+) group compared to the control group respectively. $^{\#}$P<0.05, $^{\#\#\#}$P<0.001, the HBsAg (+) HBeAg (+) Anti-HBc (+) group compared to the HBsAg (+) Anti-HBe (+) Anti-HBc (+) group. (CHI3L1 = chitinase-3-like protein-1; C IV = collagen type IV protein; HA = hyaluronic acid; LN = laminin; PIIINP = procollagen type III peptide; FIB-4 = fibrosis-4 score; APRI = aspartate aminotransferase platelet ratio index).

Correlation between serum S1P concentration and liver function and liver fibrosis indices

As shown in **Table 2**, we further analyzed the correlation between serum S1P concentration and the degree of liver function injury and liver fibrosis. S1P was positively correlated with liver fibrosis indices C IV (r=0.264; P=0.011) and CHI3L1 (r=0.295; P=0.004) (**Figure 5**). There was no correlation (P>0.05) between the serum S1P concentration and all the liver function indices (**Figure 6**).

S1P promotes the activation of LX-2 cells

To further explore the target cells of S1P, we investigated the role of S1P on L02 and LX-2 cells, which are the pivotal cells involved in the development of liver fibrosis. The previous results demonstrated that 0.2 μM S1P was the optimal concentration to study liver fibrosis. As shown in **Figure 7**, we treated L02 and LX-2 cells with the same concentration of S1P and found that the indicators of liver fibrosis matrix metalloproteinase-9 (MMP9) and fibronectin (FN) in L02 cells showed no difference but were upregulated in LX-2 cells. These results suggested that S1P can activate LX-2 cells while showing little effect on L02 cells, indicating that LX-2 cells may exert an essential role in the profibrogenic effect of S1P.

Discussions

Although the hepatitis B vaccine and antiviral treatment have been promoted, the infection of hepatitis B virus still seriously affects global public health. It is known that CHB induced liver injury can lead to liver fibrosis, cirrhosis, and even liver cancer [13]. Our results further verify the theory that the liver dysfunction of hepatitis B patients is more evident than that of the control group, with a worse manifestation of liver functional indices presented in hepatitis B patients. In addition, the liver fibrosis indices of the hepatitis B group were also higher than in the control group.

S1P is a sphingolipid substance with biological functions, mainly derived from red blood cells and platelets [14]. At present, S1P is mainly detected by ELISA and LC-MS/MS. However, ELISA possesses the disadvantages of limited sensitivity and easy distortion: it belongs to the antigen-antibody reaction and has a cross-reaction, especially when the specimen may contain analogs of S1P, exhibiting the same antigenic determinant with S1P, leading to false

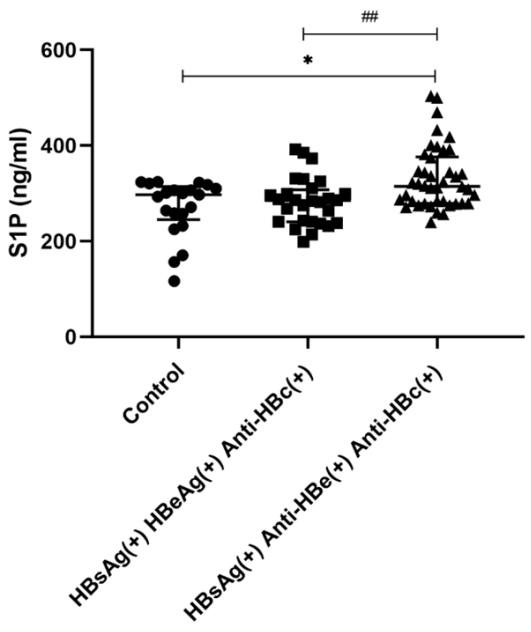

Figure 4. Serum concentrations of S1P in the control group and hepatitis B patients. S1P of HBsAg (+) Anti-HBe (+) Anti-HBc (+) group was higher than the control group and the HBsAg (+) HBeAg (+) Anti-HBc (+) group, while the difference in S1P between the HBsAg (+) HBeAg (+) Anti-HBc (+) group and control group was not significant. Data are the median with interquartile range. *P<0.05, HBsAg (+) Anti-HBe (+) Anti-HBc (+) group compared to the control group. ##P<0.01, HBsAg (+) Anti-HBe (+) Anti-HBc (+) group compared to the HBsAg (+) HBeAg (+) Anti-HBc (+) group. (S1P = sphingosine-1 phosphate).

Table 2. Correlation between serum levels of S1P and liver function and liver fibrosis indices

	Spearman's rho	*P*-value
ALT	0.019	0.855
AST	0.086	0.976
TP	-0.022	0.837
ALB	-0.098	0.357
GLO	0.117	0.268
TBIL	0.004	0.240
DBIL	0.006	0.105
TBA	-0.052	0.625
FIB-4	-0.029	0.783
APRI	0.001	0.636
C IV	0.295	0.011*
HA	0.137	0.195
LN	0.003	0.979
PIIINP	0.195	0.064
CHI3L1	0.264	0.004**

*P<0.05, **P<0.01; Liver function indices: AST = alanine aminotransferase; ALT = aspartate transaminase; TP = total protein; ALB = albumin; GLO = globulin, TBIL = total bilirubin; DBIL = direct bilirubin; TBA = Total bile acid; Liver fibrosis indices: FIB-4 = fibrosis-4 score; APRI = aspartate aminotransferase platelet ratio index; C IV = collagen type IV protein; HA = hyaluronic acid; LN = laminin; PIIINP = procollagen type III peptide; CHI3L1 = chitinase-3-like protein-1.

increase in detection results. By comparison, LC-MS/MS is one of the most widely used methods for metabolomics research. It is characterized by high accuracy, high resolution, high sensitivity, and small sample size [15]. Sakai et al. [16] established a detection system for S1P in the cerebrospinal fluid (CSF) of cancer meningitis based on LC-MS/MS. The detection limit of S1P in CSF was 0.01 ng/mL, and the variable coefficient (CV) was lower than 20%. Tang [17] et al. used LC-MS/MS to monitor the changes of serum S1P level in lung cancer patients, and the lower limit of S1P in serum samples was 25 ng/mL, with intra-batch and inter-batch precisions and accuracy lower than 10%. In addition, Mirzaian et al. [18] used (13) C5 C18-S1P or C17-S1P as internal standards to determine the content of S1P by LC-MS/MS in normal human plasma, whose CV was 3.9% and 3.5%, respectively. This indicates that LC-MS/MS is appropriate for detection of S1P in our study. Moreover, S1P regulates the development of inflammatory diseases by binding S1P receptors (S1PRs) on the effector cell membrane [14]. For example, in S1PR2 knockout THP-1 cells, the phosphorylation of Janus activated kinase (JAK) 1, JAK2, and signal transducer and activator of transcription 6 (STAT6) induced by inflammatory factors IL-4 and IL-13 was inhibited [19]. S1PR1 deficient heterozygous mice can weaken hyperoxia (HO) - induced bronchopulmonary dysplasia (BPD) and reduce inflammatory markers in bronchoalveolar lavage fluid [20]. Inhibition of S1P can protect mice against chondrocyte catabolism and alleviate osteoarthritis [21]. Moreover, our results also showed that serum S1P levels were higher in hepatitis B patients than healthy controls, which is consistent with the up-regulation of S1P in most inflammatory diseases.

However, our study showed that S1P increased only in serum of Anti-HBe (+) hepatitis B patients. As mentioned above, S1P originates mainly from red blood cells and platelets [14]. Studies has demonstrated that inflammatory

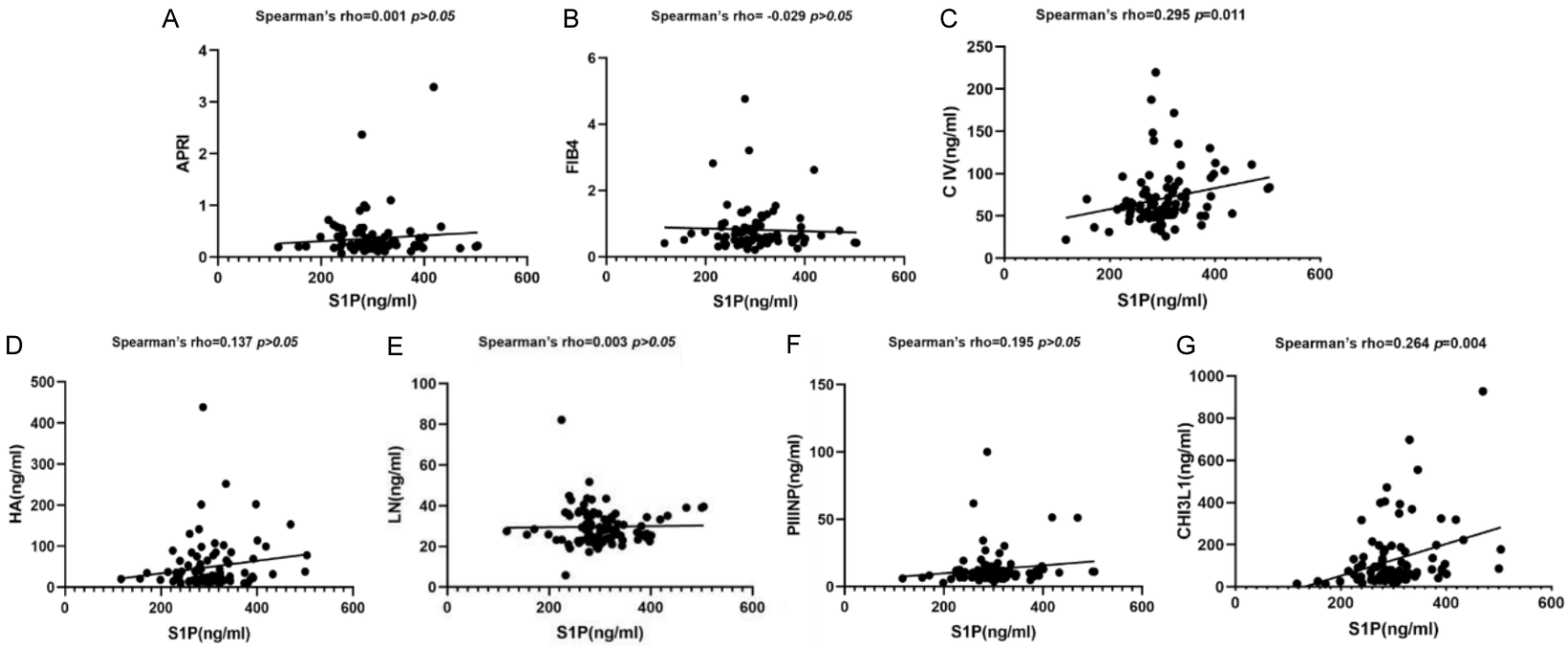

Figure 5. Correlation between serum levels of S1P and the liver fibrosis indices. (A) APRI, (B) FIB-4, (D) HA, (E) LN and (F) PIIINP were uncorrelated with S1P, while (C) C IV (r=0.295, P=0.011) and (G) CHI3L1 (r=0.264, P=0.004) were positively correlated with S1P. (CHI3L1 = chitinase-3-like protein-1; C IV = collagen type IV protein; HA = hyaluronic acid; LN = laminin; PIIINP = procollagen type III peptide; FIB-4 = fibrosis-4 score; APRI = aspartate aminotransferase platelet ratio index; S1P = sphingosine-1 phosphate).

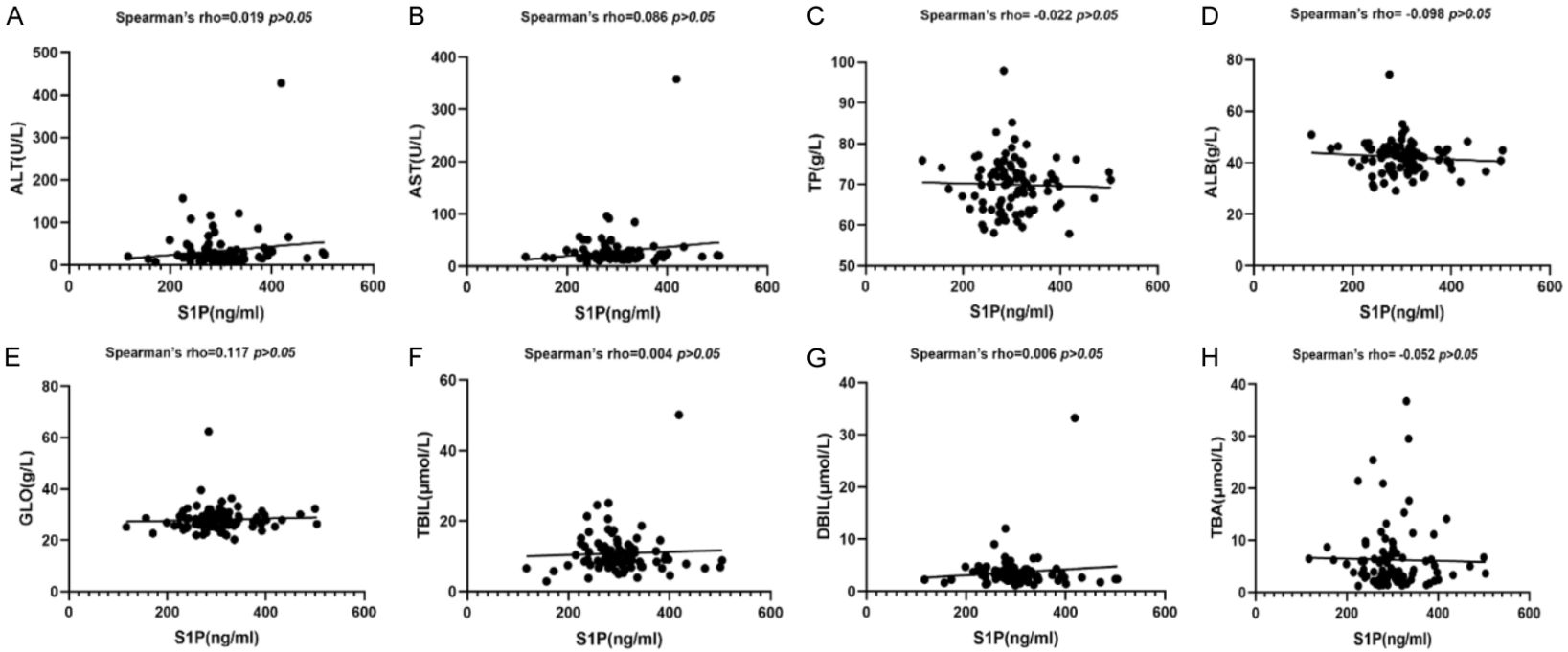

Figure 6. Correlation between serum levels of S1P and liver function indices. (A) ALT, (B) AST, (C) TP, (D) ALB, (E) GLO, (F) TIBL, (G) DBIL and (H) TBA were uncorrelated with S1P. (AST = alanine aminotransferase; ALT = aspartate transaminase; TP = total protein; ALB = albumin; GLO = globulin; TBIL = total bilirubin; DBIL = direct bilirubin; TBA = Total bile acid; S1P = sphingosine-1 phosphate).

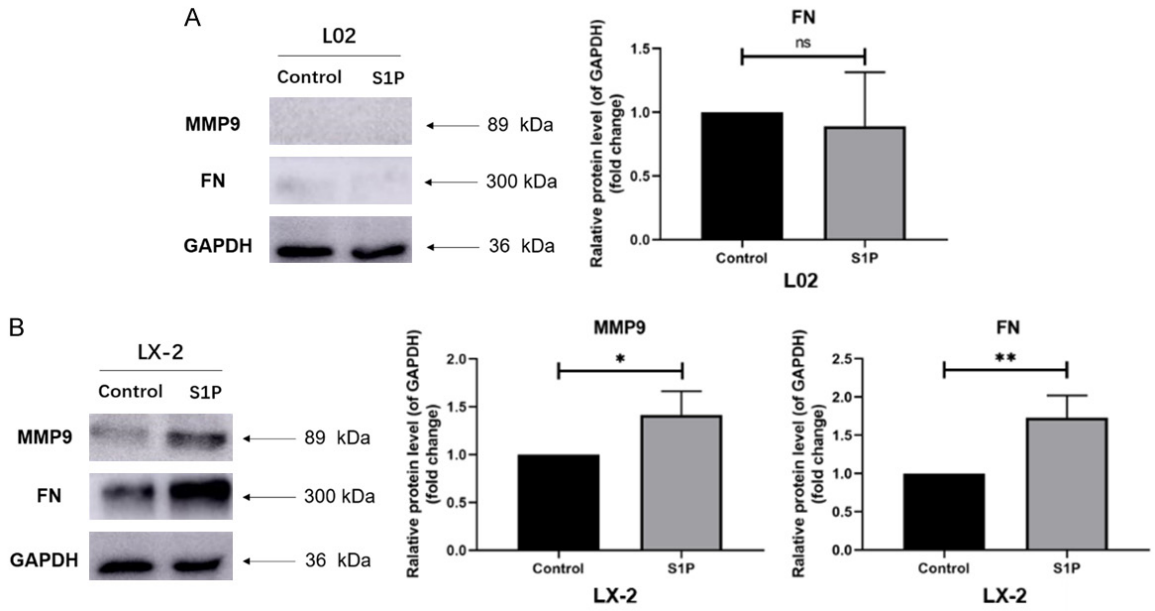

Figure 7. Variation of liver fibrosis indices in L02 and LX-2 cells after S1P treatment. A. No difference in MMP9 and FN in L02 cells between control group and S1P; B. The levels of MMP9 and FN were increased in LX-2 cells after S1P treatment compared with control group. *P<0.05, **P<0.01, control group compared to the S1P group. (MMP9 = matrix metalloproteinase-9; FN = fibronectin; S1P = sphingosine-1 phosphate).

response is closely associated with the promotion of angiogenesis [22]. Inflammatory response in Anti-HBe (+) hepatitis B patients promote angiogenesis, which in turn enhances the ability of red blood cells and platelets to generate S1P. It did not increase in HBsAg (+) hepatitis B patients, which was also consistent with the previous results of our research group: ApoM was the primary carrier of S1P in vivo, and serum ApoM increased in HBeAg (-) CHB patients and positively correlated with the level of HBV DNA, while in HBeAg (+) CHB patients, there was no correlation with HBV DNA level [23, 24]. The explanations are that HBV replication can induce liver metabolism disturbance and hematopoietic dysfunction [25-27]; HBeAg is an important indicator of clinical replication of hepatitis B virus in vivo, representing the active degree of HBV replication in hepatitis B patients [28]; Lower S1P concentration in HBeAg (+) patients may be the result of more active HBV replication than Anti-HBe (+) patients, which presents a stronger ability in HBeAg (+) group to impact the liver lipid metabolism and destroy red blood cells, causing the perturbation of S1P concentration.

At present, the gold standard for diagnosing of liver fibrosis is still liver biopsy [29]. However, there are some shortcomings with liver biopsy: on the one hand, it is invasive and expensive, which leads to the poor compliance of patients and limits its clinical application; and on the other hand, 10% to 30% have missed diagnosis in first biopsy results due to the small size of biopsy samples and the heterogeneity of pathological features among the patients [30]. At the same time, there are some non-invasive indices in the clinical diagnosis of liver fibrosis, including the direct and indirect markers. The commonly used direct indices are the four indicators of liver fibrosis: collagen type IV protein (C IV), hyaluronic acid (HA), laminin (LN) and procollagen type III peptide (PIIINP), which are positively correlated with the severity of fibrosis and can be used as an indicator of early fibrosis and severity of fibrosis [31]. Most of them are mainly detected by chemiluminescence, and widely used in the clinic. The indirect markers based on the liver function indices, such as liver fibrosis indices fibrosis-4 score (FIB-4) [32] and aspartate aminotransferase platelet ratio index (APRI), rely on the age and sex to improve its accuracy. Investigators found a new marker, chitinase-3-like protein-1 (CHI3L1), to assist the diagnosis of liver fibrosis [33]. The expression of CHI3L1 was relatively high in liver tissue, and it was mainly distributed in hepatic

stellate cells [34, 35]. Several studies have also shown that it is an excellent non-invasive diagnostic indicator of liver fibrosis [33, 36, 37]. Therefore, we chose those non-invasive markers to measure liver fibrosis in our study. CHI3L1 is mainly detected by ELISA [33, 38, 39]. However, as mentioned above, ELISA can cause instability of detection results. Therefore, chemiluminescence, a more sensitive methodology, was used to detect CHI3L1 in the study. The relative accuracy deviation of CHI3L1 results is lower than ±10.0%, and the detection limit is 1.50 ng/mL, and the CV of repeatability coefficient was no more than 8.0%.

In addition, S1P has also been reported to be related to various types of fibrosis: S1PR2 antagonist GLPG2938 can alleviate bleomycin induced pulmonary fibrosis in mice [40]; Exogenous S1P can induce the activation of cardiac fibroblasts and the secretion of α-smooth muscle actin (α-SMA) and collagen, promoting myocardial fibrosis [41]; Salidroside can inhibit the activation and migration of hepatic stellate cells and alleviate hepatic fibrosis by inhibiting the expression of SphK1 and down-regulating SphK/S1P/S1PR pathway [42]; LncRNA H19 is significantly elevated in human fibrosis, and knockout of lncRNA H19 can weaken the degree of liver fibrosis and reduce the expression of S1PR2 in cholestasis mice [43]. These references are compatible with the results of a positive correlation between serum S1P concentration and liver fibrosis indices C IV and CHI3L1. In addition, we found that under the same treatment of S1P, L02 cells had little changes while LX-2 cells were activated obviously. Therefore, it is assumed that S1P also has the prospect of being a non-invasive diagnostic indicator of liver fibrosis.

However, our research has limitations. First, the control group and hepatitis B patients group samples were limited. Strict inclusion and exclusion criteria (**Figure 1**), such as the virological assessment tests of the control group, greatly excluded the possible but not suitable candidates. Also, from analysis of the clinical data, the confirmed HBeAg (+) group is limited, which restricted the samples. There is a need to expand the sample size to validate our research results. Second, under the context that the patients undergoing liver biopsy are those who advanced to liver cirrhosis or liver cancer, a worse liver condition surpassing the stage of liver fibrosis, the simple liver fibrosis is hard to diagnose. Therefore, FIB-4, APRI, C IV, HA, LN, PIIINP, CHI3L1, and other non-invasive liver fibrosis indicators were applied to assess the extent of liver fibrosis of the subjects. Third, studies have found that a low serum S1P level means increased mortality of cirrhosis, and the expression of S1P is reduced in HCC patients [44]. However, there are studies demonstrating that S1P did not change in the liver tissue of liver cancer patients compared with healthy controls, while the levels of SphK1, S1P transporter spinster homolog 2 (SPNS2) and S1PR2 mRNA were increased [45]. The reason for the inconsistent changes of S1P in liver tissue and serum of liver cancer patients may be that 1) Elevated SK1 in HCC patients can promote the active production of S1P, which then is transported to the outside of cell through SPNS2, resulting the S1P in liver tissue does not increase; 2) The S1P transferred outside cells then binds to S1PRs in human liver to play a profibrotic role, resulting the dissociative S1P in serum was decreased. However, our study found that the level of serum S1P is increased in hepatitis B, indicating that the expression of S1P may be up-regulated first and later have a drop in the pathological stage which contains hepatitis B, liver fibrosis, cirrhosis, and liver cancer. There may be a certain threshold of S1P indicating the occurrence of liver fibrosis. Therefore, expanding the sample size and monitoring the range of changes in S1P at different stages of liver disease are also the direction we need to proceed on. Fourth, the research on the mechanism in the study is limited. We found that LX-2 may be the target cells of S1P, which is consistent with the theory that LX-2 is the core cell involved in liver fibrosis. The number of the research on the mechanism of S1P promoting liver fibrosis was less, which was mostly focused on the role of proinflammatory response and the activation of the SphK/S1P/S1PRs signaling pathway [8, 46, 47]. Therefore, it is possible that the increased S1P in hepatitis B patients promotes the expression of S1PRs in LX-2 cells, and S1P binds to S1PRs, inducing the activation of LX-2 cells.

In conclusion, our study found that serum S1P concentration in HBsAg (+) Anti-HBe (+) Anti-HBc (+) patients increased compared with that

of the control group and that HBsAg (+) Anti-HBe (+) Anti-HBc (+), and positively correlated with C IV and CHI3L1 of liver fibrosis markers. This provides new ideas for finding diagnosis or treatment indicators to reduce hepatitis B-related liver fibrosis incidence rate.

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Disclosure of conflict of interest

None.

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