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ORIGINAL ARTICLE

The Measurement of 25-Hydroxyvitamin-D in Chronic HBV Patients Using LC-MS/MS

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SUMMARY

Background: **Vitamin D deficiency is universal among patients with chronic liver disease. Vitamin D may be involved in the regulation of immune function of chronic hepatitis B and related to disease progression.**
Methods: **The study was a cross-sectional study. The level of vitamin 25(OH)D was detected in patients with chronic hepatitis B, hepatitis B cirrhosis, hepatitis B cancer, and healthy groups by isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS). At the same time, the clinical data, biochemical indexes, and T lymphocyte subsets were collected to study the relationship between vitamin 25(OH)D deficiency and clinical indexes of hepatitis B patients.**
Results: **The prevalence of vitamin D deficiency (< 20 ng/mL) was higher in patients with liver cancer group (96.97%, 10.59 ± 3.06 ng/mL) and cirrhosis group (93.18%, 11.85 ± 2.66 ng/mL) than in the healthy group (76.92%, 16.38 ± 5.53 ng/mL) and chronic hepatitis B group (77.83%, 15.06 ± 4.91 ng/mL). There were significant differences in vitamin 25(OH)D levels between the cirrhosis groups and the healthy groups, the liver cancer groups and the healthy groups, the hepatitis B cirrhosis groups and the chronic hepatitis B groups, the liver cancer groups and the chronic hepatitis B groups ($p < 0.05$). There was no significant difference in vitamin 25 (OH)D level between liver cancer group and hepatitis B cirrhosis group, healthy group and chronic hepatitis B group ($p > 0.05$). Vitamin 25(OH)D level was correlated with age ($r = -0.24$, $p = 0.015$), lymphocyte ($r = 0.24$, $p = 0.015$), hemoglobin ($r = 0.28$, $p = 0.005$), platelet ($r = 0.27$, $p = 0.006$), PTA ($r = 0.33$, $p = 0.001$), albumin ($r = 0.30$, $p = 0.002$), prealbumin ($r = 0.39$, $p = 0.001$), cholinesterase ($r = 0.29$, $p = 0.003$), CD_3^+ ($r = 0.20$, $p = 0.04$), CD_3^+ CD_8^+ ($r = 0.20$, $p = 0.04$), CD_{45}^+ ($r = 0.24$, $p = 0.017$), but none correlated with liver function and HBV-DNA.**
Conclusions: **Vitamin D deficiency existed in patients with hepatitis B, which was related to the clinical progress of hepatitis B and may be involved in the regulation of immune function in patients with chronic HBV infection.**
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KEY WORDS

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INTRODUCTION

Vitamin D deficiency is universal among patients with chronic liver disease, and at least one-third of them suffer from severe vitamin D deficiency [1,2]. Previous studies reported that vitamin D based treatment had better therapeutic effect in liver diseases [3,4]. However,

studies have shown that there were no causal relationships between serum vitamin D level and viral load, liver dysfunction, and liver fibrosis in patients with chronic HBV infection [5,6].

There are many reasons for vitamin D deficiency, including reduced intake or absorption, less sunshine time, increased liver catabolism, and reduced endogenous synthesis. The clinical manifestations of vitamin D deficiency depend on the severity and duration of the deficiency. Although vitamin D mainly plays its biological role through vitamin 1, $25(OH)_2D_3$, the half-life of vitamin 1, $25(OH)_2D_3$ is short, which is not suitable to be used as an index to reflect the body's vitamin D level. The content of vitamin $25(OH)D_3$ in blood is relatively high, and the half-life is 12 - 20 days. Detecting the concentration of serum vitamin $25(OH)D_3$ is the most reliable way to evaluate the state of vitamin D in peripheral blood [7-9].

At present, there are many methods for detecting serum vitamin $25(OH)D_3$, but all the methods have limitations and shortcomings. Enzyme linked immunosorbent assay (ELISA) has the advantages of simple equipment and operation, no pollution, but poor sensitivity and specificity [10]. Radioimmunoassay (RIA) has high sensitivity, strong specificity, good precision and low requirements for instruments and equipment. It is the main means for the determination of ultra trace substances in grass-roots units, but there is a potential risk of radiation pollution. Chemiluminescence immunoassay (CLIA) has the advantages of simple and rapid operation, no isotope pollution, and easy automation, but its detection efficiency is lower than that of liquid chromatography tandem mass spectrometry [11]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) has high sensitivity and specificity. The uncertainty of serum vitamin $25(OH)D_3$ is relatively small, but the operation procedure is complex and requires more expensive special instruments [12].

The normal range and intake standard of vitamin D levels have been controversial [13]. The consensus on clinical application of vitamin D and its analogues in 2019 [14] pointed out that serum vitamin 25(OH)D < 20 μg/L (50 nmol/L) is vitamin D deficiency, 20 - 30 μg/L (50 - 75 nmol/L) is vitamin D deficiency, > 30 μg/L (75 nmol/L) is vitamin D sufficient, < 10 μg/L (25 nmol/L) is a serious deficiency. The optimal vitamin 25(OH)D level is uncertain in patients with chronic liver disease.

Vitamin D may be involved in the regulation of immune function of chronic hepatitis B and related to disease progression, but there are few relevant studies. In this study, isotope diluted liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to explore the relationship between vitamin D deficiency and clinical indexes of patients with HBV infection, and further explore the value of vitamin 25(OH)D in the treatment of patients with chronic HBV infection.

MATERIALS AND METHODS

Study population

Overall, 91 HBV-related patients were enrolled in this study. Blood samples were obtained from 14 CHB patients, 44 HBV-cirrhosis patients, 33 HBV-cancer patients and 13 age- and gender-matched healthy donors as the healthy group from May 2020 to December 2020. The diagnosis of CHB infection was based on the recommendations of the American Association for the Study of Liver Diseases [15]. The diagnosis of cirrhosis was based on the medical history, physical examination, biochemical findings, and imaging techniques (ultrasound and/or computed tomography). The diagnosis of liver cancer was based on the standard for diagnosis and treatment of primary liver cancer [16]. The included controls were all negative for hepatitis B surface antigen/antibody, hepatitis B e antigen/e antibody, and hepatitis B core antibody, and had normal alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. The exclusion criteria were: (1) Hepatitis C infection, (2) Alcoholic liver injury, (3) Drug induced liver injury, (4) HIV infection, (5) Parathyroid diseases, (6) Immune related diseases, (7) Pregnant and lactating women, (8) Unable to cooperate with treatment monitoring, incomplete clinical data, and refusing to sign informed consent, (9) Patients with other liver diseases and using vitamin D, hormones or immunosuppressants. This study was approved by the Ethical Committee at Youan Hospital in the Capital Medical University (No. of Ethics Committee: JYKLY2021-315), and all the patients provided written informed consent.

Reagents and instruments

$25(OH)D_3$ reference standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). 25-OH VD_3 (D6) stable isotope standard was purchased from Cambridge Isotope Laboratories. Formic acid (FA), acetonitrile, methanol, n-hexane (LC-MS grade), anhydrous ethanol (HPLC grade), and ZnSO4•7H2O were purchased from Fisher (USA). Waters Acquity UPLC™ with a triple quadrupole mass detector (Xevo TQ-S) system in positive electrospray ionization (ESI) mode (USA).

Sample preparation

First, 160 μL of sample or serum sample was placed in a 5 mL centrifuge tube, and 160 μL internal standard ($25OH\text{-}VD_3\text{-}D_6$ 50 ng/mL dissolved in methanol) was added to the centrifuge tube, which was vortexed for 10 seconds. Then, 0.2 mmol/L aqueous zinc sulfate solution was added, followed by 240 μL of methanol and vortexed for 30 seconds, then 800 μL of n-hexane was added, vortexed, and extracted for 10 minutes. The sample was centrifuged at 13,200 r/minute for 10 minutes, 40 μL supernatant was transferred to a 5 mL centrifuge tube, which was dried with nitrogen at room temperature and then redissolved with 120 μL solution (methanol:water 7:3), centrifuged at 13,200 r/minute for 5 min-

utes. The supernatant was transferred into 250 µL LC Vial Inserts for testing.

Biochemical parameters

For every patient, blood specimens were collected at 6 am on the second morning of admission. Then, serum was separated by centrifugation at 3,000 g for 10 minutes to determine the blood chemistry profile. Biochemistry parameters including liver function test and renal function test were measured in a clinical laboratory using a Roche Cobas c701 automated biochemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA). Whole blood cell parameters such as white blood cells (WBCs), hemoglobin (HB), lymphocytes (LyMs), and platelets (PLTs) were measured with a Symex XN-2000 (Sysmex, Kobe, Japan) automated blood cell analyzer at the Capital Medicine University Youan Hospital, Beijing, China.

25(OH)D measurement by LC–MS/MS was performed on a Waters Acquity UPLC™ with a triple quadrupole mass detector (Xevo TQ-S) system with positive electrospray ionization (ESI).

LC-MS/MS conditions

Chromatography was performed with a Waters UPLC system. The Waters column HSS T_3 was 2.1 mm x 150 mm C_{18} reversed phrase column balanced in buffer A (H_2O, 0.1% formic acid). The serum was loaded onto the Waters column HSS T_3, then buffer B (acetonitrile) was separated at a flow rate of 0.3 mL/minute. Initially, the mobile phase composition was 20% A and 80% B and was maintained for 2.0 minutes. Then, mobile phase B was increased to 100% from 2.5 minutes to 4 minutes. The gradient was maintained at 20% A and 80% B until 6.0 minutes (Table 1). The temperature of the autosampler was set at 45°C, and the injection volume was 5 µL.

A Waters Acquity UPLC™ with a triple quadrupole mass detector (Xevo TQ-S) system in positive electrospray ionization (ESI) mode was used for MS analysis. The dwell time was 52 ms for multiple reaction monitoring (MRM) mode. The transitions and conditions for the Waters Acquity Xevo TQ-S were listed in Table 2. The optimized instrumental settings were capillary voltage (3.0 kV), desolvation temperature (500°C), source temperature (150°C), desolvation gas flow (1,000 L/hr), cone gas flow (10 L/hr), nebulizer (7 bar), and collision gas flow (0.14 mL/minute).

Data analysis and statistical methods

Demographic and anthropometric statistics were expressed as the mean ± SD when appropriate. Non-normal distribution data were represented by median and quartile [M(P_{25}-P_{75})]. Groups were formed on the basis of patient groups and season. Demographic data were analyzed using descriptive statistical tests performed with SPSS software package, version 23.0, GraphPad Prism 5, and Microsoft Excel 2007 (Microsoft, Redmond, WA, USA). The correlations were analyzed between two groups using the Spearman's r test. The difference comparisons between 2 groups were performed with the unpaired *t*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Demographic and anthropometric data

A total of 104 subjects were included, including 80 males and 24 females, with a median age of 53 years (Table 3).

Quantitative standard curve of 25-OH VD_3

According to the concentration of each standard point and the response value of each concentration point in the mass spectrum after computer detection, the standard curve was fitted after isotope internal standard correction to obtain the quantitative standard curve of 25-OH VD3 (Figure 1), $y = 0.0196957x - 0.0106847$, $R^2 = 0.999505$ (Figure 1).

Comparison of vitamin D levels in patients with liver disease in different seasons

There was no significant difference in serum vitamin 25(OH)D levels among the four groups in different seasons ($p > 0.05$). See Table 4.

Comparison of vitamin D levels in different hepatitis B patients

The prevalence of vitamin D deficiency (< 20 ng/mL) was higher in the patients with liver cancer group (96.97%) and cirrhosis group (93.18%) than in the control group (76.92%) and chronic hepatitis B group (77.83%) (See Table 4). Comparison of vitamin 25(OH)D levels in different hepatitis B patients: (1) There was significant difference in vitamin 25(OH)D level between hepatitis B cirrhosis group and healthy group ($p < 0.05$). (2) There was significant difference in vitamin 25(OH)D level between liver cancer group and healthy group ($p < 0.05$). (3) There was significant difference in vitamin 25(OH)D level between chronic hepatitis B group and hepatitis B cirrhosis group and it was statistically significant ($p < 0.05$). (4) There was significant difference in vitamin 25(OH)D level between liver cancer group and chronic hepatitis B group ($p < 0.05$). (5) There was no significant difference in vitamin 25(OH)D level between healthy group and chronic hepatitis B group ($p > 0.05$). (6) There was no significant difference in vitamin 25(OH)D level between HBV cirrhosis group and HCC group ($p > 0.05$). See Figure 2.

Correlation analysis between vitamin D level and laboratory parameters

Vitamin 25(OH)D levels were correlated with age, lymphocytes, hemoglobin, platelets, PTA, albumin, prealbumin, cholinesterase, CD_3^+, CD_3^+ CD_8^+, as well as CD_{45}^+. (1) Vitamin 25(OH)D level was negatively correlated with age ($r = -0.24$, $p = 0.015$). (2) Vitamin

Table 1. Gradient elution procedure.

Time (minutes)	A (v%)	B (v%)
0	20	80
2	10	90
2.5	0	100
4	0	100
4.1	20	80
6	20	80

Table 2. Conditions of Triple Quadrupole MS.

	Compound	Average mass	MRM parameters					
			Parent Ion	Product Ion	DP [V]	CE [V]	EP [V]	CXP [V]
1	25OH-VD3	383.5	383.5	257.5	90	25	10	13
2	25OH-VD3-D6	389.6	389.6	263.5	90	15	10	13

Table 3. Comparison of data of subjects in different patient groups.

Items	Groups			
	Control group	CHB	Cirrhosis	HCC
Case	13	14	44	33
Age	37.4 ± 11	42.8 ± 13.6	54.1 ± 11.1	58.1 ± 9.5
Gender	8:5	11:3	33:11	28:5
WBC	5.41 ± 11.66	5.09 ± 1.45	4.6 ± 2.86	5.06 ± 2.43
LYMPH	1.59 ± 0.45	1.62 ± 0.46	1.22 ± 0.86	0.97 ± 0.55
Hb	132.46 ± 24.13	132 ± 24.48	109.5 ± 26.6	119 ± 26.9
PLT	228.77 ± 82.5	192.5 ± 67.9	83 (51, 158)	85 (65, 124)
NH3	29.79 ± 6.01	56 ± 26.8	70 (54, 85.5)	57 (38, 73)
PTA	98.07 ± 6.81	82.6 ± 22.76	67.6 ± 19	71.6 ± 18.6
ALT	20.7 ± 8.28	113.2 (32.7, 342)	26 (16, 42)	30 (24, 37.8)
AST	20.28 ± 5.77	117 (36.7, 413)	33 (24, 52.9)	36.5 (27.5, 45.4)
TbiL	9.7 ± 3.59	30 (16.25, 115.3)	25.7(14.2, 34.8)	29.5 (20.1, 57.6)
DBiL	2.4 ± 1.28	16.3 (11.1, 81.7	10 (6.15, 17.8)	11.5 (10.4, 31.4)
ALB	41.95 ± 6.68	36.9 (36.4, 40.2)	29.8 ± 6.08	32.5 ± 6.8
GGT	19.1 ± 6.13	57.8 (36.7, 171	43.1 (20.2, 99.1)	50.7 (20, 149)
ALP	66.61 ± 16.62	129.7 ± 46.6	105 (75, 148)	109 (84, 170)
PALB	247.1 ± 20.6	149.5 ± 94.5	86 (61, 125.7)	112.2 (53.4, 165)
ChE	6,674.9 ± 1,425.5	5,897.8 ± 1,550	3,183 (2,310; 3,966)	3,870 (1,854; 6,002)
TG (mmol/L)	1.76 ± 1.41	1.64 ± 0.71	1.13 ± 0.56	1.36 ± 0.73
CHOL (mmol/L)	4.08 (3.9,4.2)	3.97 ± 1.02	3.45 ± 1.09	3.58 ± 1.21
HDL (mmol/L)	1.17 ± 0.29	0.89 ± 0.43	0.93 ± 0.38	0.82 ± 0.36
LDL (mmol/L)	2.02 (1.84,2.62)	2.14 ± 0.72	1.8 ± 0.89	2.14 ± 1.21
APOA1 (g/L)	1.46 ± 0.09	1.04 ± 0.40	1.04 ± 0.32	1.2 (0.66, 1.33)
APOB1 (g/L)	0.74 ± 0.14	0.86 ± 0.19	0.6 (0.5, 0.88)	0.67 (0.51, 0.78)
A/B	0.48 ± 0.23	1.3 ± 0.6	11.8 ± 2.66	1.63 ± 0.50

Table 4. Vitamin 2 (OH)D levels in patients with liver disease in different seasons.

Groups	Vitamin 25(OH)D levels (ng/mL)		p-value	Difference 95% CI	
	Spring-summer	Autumn-winter		Lower limit	Upper limit
Healthy	19.65 ± 4.54	14.94 ± 6.98	0.86	-3.767	13.186
CHB	17.22 ± 7.41	18.34 ± 14.30	0.26	-14.628	14.725
Cirrohsis	9.10 ± 4.02	10.23 ± 6.70	0.25	-5.463	3.202
Liver cancer	10.43 ± 3.82	10.19 ± 7.36	0.42	-4.291	4.761

Table 5. Vitamin 25(OH)D levels in different groups.

Groups	Vitamin 25(OH)D level (ng/mL)		
	Mean ± SD	Rang	Vitamin D deficiency (%)
Healthy	16.38 ± 5.53	8.69 - 32.12	76.92
CHB	15.06 ± 4.91	6.77 - 22.32	77.43
Cirrhosis	11.85 ± 2.66	8.06 - 17.57	93.18
HCC	10.59 ± 3.06	5.56 - 15.16	96.97

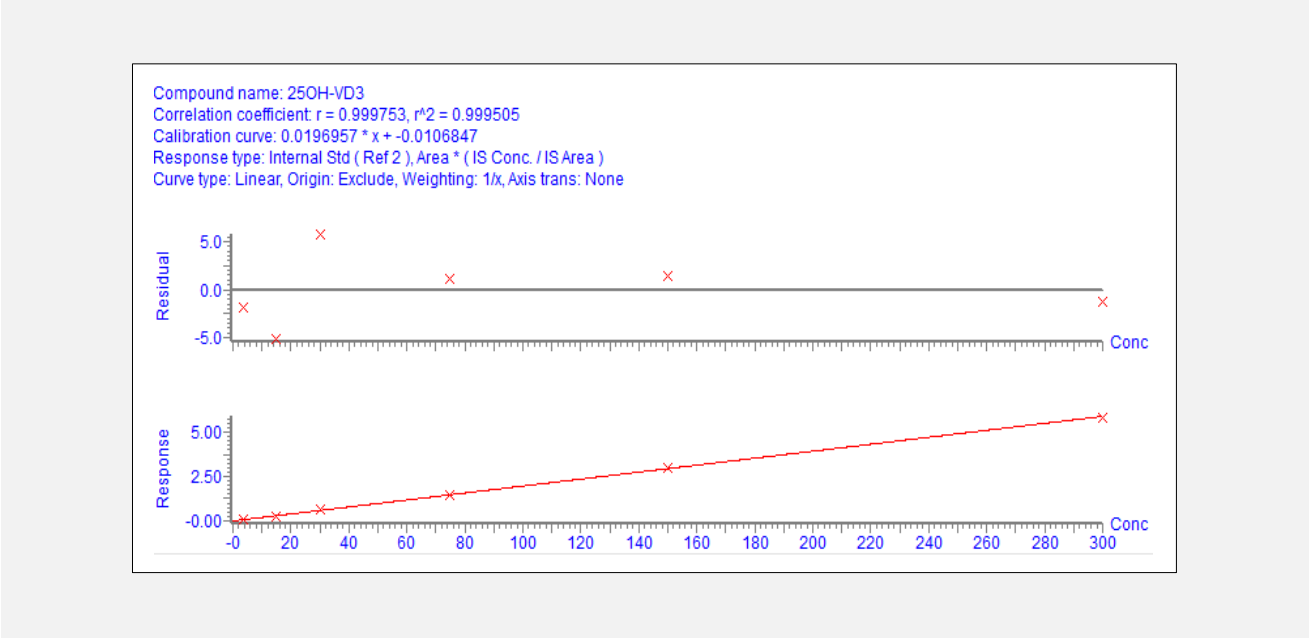

Figure 1.

25(OH)D levels were positively correlated with lymphocytes (r = 0.24, p = 0.015), hemoglobin (r = 0.28, p = 0.005), platelets (r = 0.27, p = 0.006), PTA (r = 0.33, p = 0.001), albumin (r = 0.30, p = 0.002), prealbumin (r = 0.39, p = 0.001), cholinesterase (r = 0.29, p = 0.003), CD_3^+ (r = 0.20, p = 0.04), $CD_3^+CD_8^+$ (r = 0.20, p = 0.04), CD_{45}^+ (r = 0.24, p = 0.017). (3) There was no correlation between vitamin 25(OH)D level and liver function and HBV-DNA (p > 0.05).

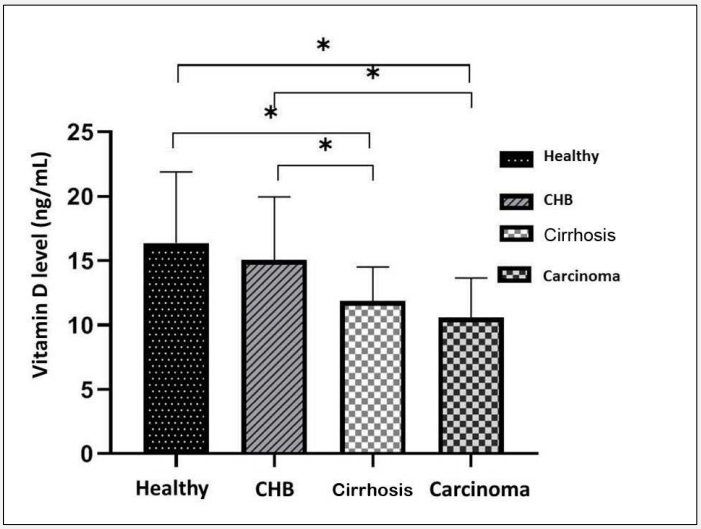

Figure 2. Comparison of vitamin D levels in patients with hepatitis B (* $p < 0.05$).

DISCUSSION

Vitamin D has a potential adjuvant therapy for the treatment of primary liver cancer and viral hepatitis [17]. Vitamin D supplementation could effectively improve the levels of platelets and albumin in circulating blood of patients with liver cirrhosis, improve liver function, and maintain the dynamic balance of bone salt metabolism [18], delay the progression of liver cirrhosis, and reduce complications. In addition, vitamin D deficiency was also associated with advanced hepatocellular carcinoma and poor prognosis [19].

Wong [20] conducted a long-term prospective cohort study on the adverse effect of vitamin D deficiency on the clinical outcome of patients with chronic hepatitis B and found that vitamin D deficiency was very common (82%) in patients with chronic hepatitis B. In this study, vitamin D deficiency was 77.43% in CHB patients. Hepatitis B cirrhosis patients generally had vitamin D deficiency with 86% incidence rate [21], and 30% of patients with serum vitamin D level < 7 ng/mL [22]. The degree of vitamin D deficiency was related to the severity of liver function damage [23].

This study found that there was no significant difference in vitamin 25(OH)D level between chronic hepatitis B patients and healthy groups, while vitamin 25(OH)D levels in patients with hepatitis B cirrhosis and hepatitis B liver cancer were significantly different from those in healthy groups and chronic hepatitis B group, and their vitamin 25(OH)D levels were significantly lower than those in healthy groups and chronic hepatitis B groups. It was suggested that the vitamin 25(OH)D level of chronic HBV infection related diseases decrease with the progress of the disease course, the decrease of vitamin 25(OH)D level could reflect the severity of liver diseases, which was consistent with previous study [24]. This study also found that the prevalence rates of vitamin 25(OH)D in healthy group, chronic hepatitis B, hepatitis B cirrhosis, and liver cancer at the same latitude were higher than those in the same season. This study also found that the level of vitamin 25(OH)D was related to lymphocytes, hemoglobin, platelets, PTA, albumin, prealbumin, cholinesterase, T lymphocyte subsets (CD_3^+, $CD_3^+CD_8^+$, CD_{45}^+), and vitamin D was related to liver synthetic function, immune state or immune abnormalities. This study also found that serum 25(OH)D had no significant correlation with liver function and HBV-DNA level.

This study was a current study, which could not eliminate some confounding factors, such as abnormal liver function, so it could not get a clear causal relationship. There was mutual cause and effect between vitamin D deficiency and chronic liver disease. On the one hand, the hydroxylation of vitamin D precursor in the liver is an important intermediate link in the process of vitamin D metabolism. In case of liver dysfunction, the activity of 25 hydroxylase decreases, the synthesis of $25(OH)D_3$ would decrease in the liver, and the absorption of vitamin D also decrease, resulting in the decline of vitamin D level in the body. On the other hand, with the decrease of vitamin 25(OH)D level in patients with chronic liver disease, the normal physiological role of vitamin D in the liver was affected, which aggravated the damage of liver function and further caused the decrease of

vitamin D level in the body. The two cause and effect form a vicious circle [25].

There were some problems in this study, such as small sample, lack of vitamin D intervention research and cytokine and protein expression, which need to further verify the role and mechanism of vitamin D. As a new immunomodulator, there were almost no toxic and side effects of immunosuppression and immune damage. Vitamin D would have a broad research prospect in the treatment of liver cirrhosis. Further research is needed to evaluate and manage the vitamin D status of patients with chronic hepatitis B infection.

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Declaration of Interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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