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Original Article

Serum β-klotho is a potential biomarker for the progression of hepatitis B virus-related liver diseases

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Abstract

Introduction: Hepatitis B virus (HBV) infection is a global epidemic that can lead to several liver diseases, seriously affecting people's health. This study aimed to investigate the clinical potential of serum β-klotho (KLB) as a promising biomarker in HBV-related liver diseases.

Methodology: This study enrolled 30 patients with chronic hepatitis B (CHB), 35 with HBV-related cirrhosis, 66 with HBV-related hepatocellular carcinoma (HCC), and 48 healthy individuals. ELISA measured the levels of serum KLB in the four groups. We then compared the differences in serum KLB levels among the groups and analyzed the relationship between serum KLB and routine clinical parameters.

Results: The concentrations of serum KLB levels were increased sequentially among the healthy subjects, the HBV-related CHB group, the HBV-related cirrhosis group, and the HBV-related HCC group ($p < 0.05$). Expression of KLB was positively correlated with alpha-fetoprotein (AFP), total bilirubin, direct bilirubin, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl-transferase, alkaline phosphatase, total bile acid, serum markers for liver fibrosis, ascites, cirrhosis, splenomegaly, and model for end-stage liver disease sodium, while negatively correlated with platelet count, albumin, and prothrombin activity ($p < 0.05$). In addition, serum KLB has better sensitivity in diagnosing HCC than AFP, and serum KLB combined with AFP has higher sensitivity and specificity than AFP alone in diagnosing HCC.

Conclusions: Serum KLB level is associated with the severity of HBV-related liver diseases and has important diagnostic value for HCC. Therefore, it could be a predictive biomarker for monitoring disease progression.

Key words: β-Klotho (KLB); hepatitis B virus (HBV); cirrhosis; hepatocellular carcinoma.

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Introduction

Hepatitis B virus (HBV) is a small enveloped DNA virus belonging to the hepadnaviridae family [1]. Although universal vaccination programs exist, HBV infection affects about 250 million people worldwide [1]. After HBV invades the human body, covalently closed ring DNA (cccDNA) forms in the liver cells' nucleus. HBV cccDNA is central to the maintenance of chronic infection [1]. Chronic HBV infection may progress to HBV-related liver diseases, including hepatitis, liver cirrhosis, and liver failure, and is the primary cause of hepatocellular carcinoma (HCC) [2,3]. Although medical treatment constantly improves, cirrhosis and HCC are still challenges. According to studies on the burden of liver diseases worldwide, it is estimated that approximately 1 million people die each year from complications of cirrhosis and 1 million from viral hepatitis and HCC [4]. The lack of typical clinical symptoms is one of the reasons for the low rate of early HBV-related liver disease diagnosis, which generally limits treatment efficacy. Screening and monitoring the disease progression of HBV-related liver diseases has been a primary clinical concern for early treatment and lowering morbidity and mortality. Therefore, finding new biomarkers that more effectively screen and monitor disease progression in HBV-related liver diseases is crucial.

As one of the Klotho family members, β-Klotho (KLB) is a single-pass transmembrane protein composed of 1,043 amino acids, predominantly expressed in the liver, pancreas, and white adipose tissue [5,6]. KLB is usually a co-receptor with fibroblast growth factor receptor (FGFR) [7]. By mediating FGF21 and FGF19 binding to FGFR, KLB plays critical roles in regulating metabolism [8-10]. In addition, KLB was proven to play an essential clinical significance in nonalcoholic fatty liver disease and obesity [11,12]. Interestingly, KLB showed a more complex role in studies of multiple tumors, such as endometrial adenocarcinoma, cervical cancer, and thyroid cancer [13-15]. Furthermore, Zhou *et al*. [5] pointed out that serum KLB can predict the clinical prognosis of non-small cell lung cancer (NSCLC) and speculated that KLB has an antitumor effect in NSCLC. However, the function of KLB in liver cancer remains controversial. Some studies have suggested that KLB can promote the occurrence and development of liver cancer [16,17]. In contrast, other studies have found that KLB is a tumor suppressor in liver cancer [8,18]. Although KLB is linked to various diseases, the role of serum KLB in HBV-related liver diseases is still unclear. We thus aimed to investigate the functional role of serum KLB in HBV-related liver diseases and assess the value of serum KLB in acting as a new biomarker for monitoring disease progression.

Methodology

Clinical data

There were 179 serum samples, including 30 from CHB patients, 35 from HBV-related cirrhosis (HBV-cirrhosis) patients, 66 from HBV-related HCC (HBV-HCC) patients, and 48 from healthy individuals, were taken from Chongqing University Three Gorges Hospital between June 2019 and December 2021. Inclusion criteria include 1. Age is between 30 and 70 years (including 30 and 70) with no gender limitation; 2. CHB was defined as patients with positive HBsAg or HBV-DNA > 2000 IU/mL for at least six months; serological or histopathological evidence was also required, excluding cirrhosis and tumor; 3. HBV-cirrhosis was defined as HBV infection patients with liver cirrhosis identified by imageology or histological diagnosis; 4. HBV-HCC was described as HBV infection patients with a space-occupying lesion in the liver discovered by histopathological evidence or more than two clinical indicators such as alpha-fetoprotein (AFP), ultrasound, and CT. Exclusion criteria include 1. under 30 or over 70; 2. Autoimmune liver disease (ANA > 1/320), alcoholic hepatitis, drug, toxic liver injury, and other conditions can cause significant liver damage; 3. Hepatitis, cirrhosis, and liver cancer caused by other etiologies; 4. Acute cardio-cerebrovascular accident or extrahepatic end-stage diseases; 5. Pregnant, parturient, or lactating women; 6. Other conditions are considered unsuitable for inclusion by researchers. In addition, 48 healthy individuals were screened to exclude hypertension, diabetes, kidney disease, coronary heart disease, and other diseases. A cross-sectional study was used to collect laboratory results from patients. Furthermore, we calculated the Child-Pugh score of all patients with cirrhosis in this study. The Child-Pugh score combines five clinical measures of liver disease, including total bilirubin level (TBIL), albumin (ALB), prothrombin time (PT), ascites, and hepatic encephalopathy [19]. Within each index, a score of 1 to 3 is given depending on the severity of the abnormality. The final ordinal score then allows further classification into one of three Child-Pugh classes: Child-Pugh A (score 5-6), Child-Pugh B (score 7-9), and Child-Pugh C (score 10-15) [19]. The Model for End-Stage Liver Disease Sodium (MELD-Na) [20] score was calculated from all patients with HBV-related liver diseases in whom TBIL, international normalized ratio (INR), creatinine, and sodium levels were available. The privacy rights of human subjects are always observed. This study was conducted by the Declaration of Helsinki and was approved by the Ethics Committee of Chongqing University Three Gorges Hospital (dated 28/01/2022, issue no: No. 13, 2022). All participants present in the study have given their informed consent.

Serum KLB measurement

The serum specimens were collected after fasting for at least 8-10 hours. All samples in this study were frozen at −80 °C before laboratory testing. Serum KLB levels were measured with a commercially available ELISA kit (R&D Systems, Catalog Number: DY5889-05) according to the standard procedures of the reagent instructions. The Spectra Max M4 was used for detection (serial number: 21300111).

Statistical analysis

All analyses were performed using SPSS 26 for Windows. Categorical variables were presented as frequency counts, and chi-square tests were conducted to evaluate intergroup differences. The Kolmogorov-Smirnov test determined normality. Normally distributed measurement data are expressed as mean ± standard deviation (mean ± SD), while nonnormally distributed data are expressed as median with

interquartile range [M (Q1, Q3)]. The student's unpaired t-test was used to compare two groups; one-way ANOVA was used as appropriate for comparisons between groups, and nonparametric tests were used when normality or homogeneity of variance was not satisfied. A correlation analysis was done using Spearman rank correlation (r), Pearson correlation (rs), or Point Biserial correlation, depending on the data character. The accuracy of HCC prediction by serum KLB was evaluated using the area under the receiver operating characteristic (ROC) curve. The area under the curve (AUC) was presented with a 95% confidence interval (CI). All analyses were two-sided, and *p*-values < 0.05 were considered statistically significant.

Results

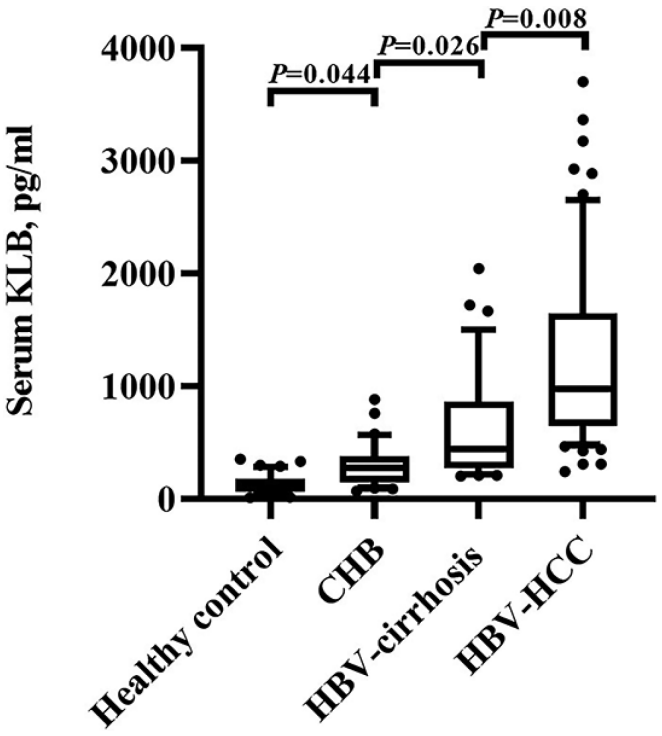
Comparison of clinical characteristics among the four groups

The biochemical indexes of liver damage in HBV-cirrhosis and HBV-HCC groups were significantly higher than in the control and CHB groups (Table 1).

Comparison of serum KLB levels among the four groups

Serum KLB levels increased sequentially among the healthy controls [111.9 (66.0, 180.2) pg/ml], the CHB group [273.3 (143.3, 381.0) pg/mL], the HBV-cirrhosis group [439.7 (268.4, 861.0) pg/mL], and the HBV-HCC group [976.0 (642.4, 1646.5) pg/mL] (all $p < 0.05$) (Figure 1).

Figure 1. Comparison of serum KLB levels among the four groups.



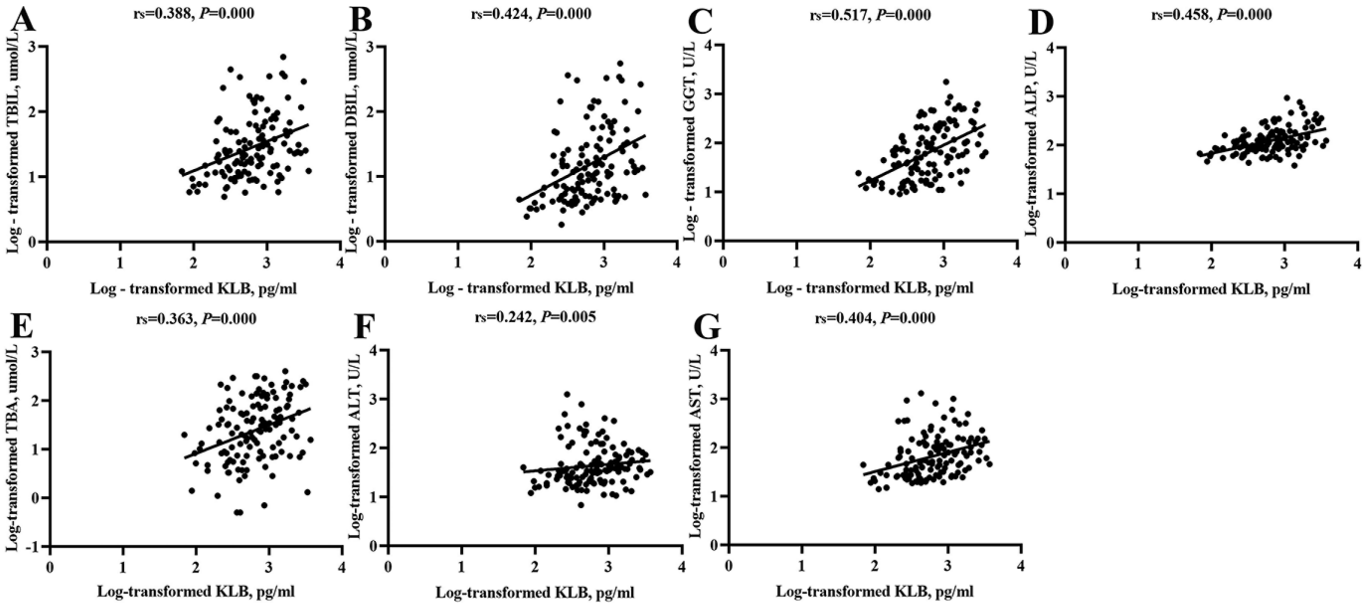
Data are shown as box-and-whisker plots. The horizontal line in the middle of each box indicates the median value. The top and bottom borders of the boxes represent the 75th and 25th percentiles, respectively; the whisker represents the 10th and 90th percentiles, respectively; and the dots represent the outliers.

Table 1. Clinical characteristics in the control, the CHB, the HBV-cirrhosis, and the HBV-HCC groups.

Variables	Control	CHB	HBV-cirrhosis	HBV-HCC
	N = 48	N = 30	N = 35	N = 66
Age (years)	50.0 (46.3, 56.0)	50.0 (47.0, 55.0)	51.0 (47.0, 62.0)	55.0 (49.8, 60.0)
Gender (M/F)	13/35	12/18	9/26	11/55
ALB (g/L)	46.1 (43.8, 47.4)	46.3 (44.1, 48.5)	34.1 (31.8, 39.6) *, #	33.5 (27.5, 40.5) *, #
TBIL (umol/L)	12.2 (8.3, 15.1)	11.7 (8.3, 16.2)	25.6 (15.5, 64.5) *, #	30.9 (15.8, 77.5) *, #
ALT (U/L)	13.7 (12.4, 19.0)	20.4 (17.0, 35.3) *	37.7 (25.5, 121.0) *	39.0 (25.2, 64.4) *, #
AST (U/L)	17.5 (15.9, 19.8)	24.5 (19.9, 40.4) *	61.5 (34.7, 101.3) *, #	80.4 (42.9, 151.5) *, #
GGT (U/L)	18.5 (13.0, 29.3)	16.5 (12.0, 27.8)	64.0 (29.0, 102.0) *, #	111.5 (47.5, 276.75) *, #
ALP (U/L)	63.0 (54.5, 75.0)	82.0 (59.0, 87.3)	106.0 (83.0, 145.0) *, #	132.5 (93.5, 263.8) *, #
TBA (umol/L)	1.8 (1.1, 3.4)	5.5 (3.3, 10.8)	35.0 (21.2, 75.9) *, #	39.5 (11.1, 125.3) *, #
HA (ug/L)	/	71.9 (59.8, 96.6)	319.4 (187.5, 622.7) #	225.9 (119.2, 491.3) #
CIV (ug/L)	/	19.1 (16.7, 28.8)	84.7 (58.4, 192.1) #	56.5 (40.9, 170.2) #
PIIINP (ug/L)	/	23.2 (18.8, 34.8)	75.6 (38.5, 138.6) #	66.7 (35.1, 185.4) #
LN (ug/L)	/	17.2 (15.4, 22.6)	65.8 (35.1, 182.7) #	110.8 (25.7, 205.3) #
CG (ug/L)	/	1.4 (1.1, 3.8)	15.5 (7.8, 30.4) #	10.3 (3.7, 33.2) #
AFP (ng/ml)	3.1 (2.3, 4.4)	3.5 (2.8, 4.3)	6.5 (3.3, 49.9) *	188.2 (19.1, 1200.0) *, #
LgHBV-DNA (IU/ml)	/	4.1 (3.5, 5.3)	3.2 (2.3, 4.9)	2.7 (2.3, 4.3) #

*$p < 0.05$ when compared to healthy control; #$p < 0.05$ when compared to CHB; ALB: albumin; TBIL: total bilirubin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: γ-glutamyl transpeptidase; ALP: alkaline phosphatase; TBA: total bile acid; HA: serum hyaluronic acid; CIV: type IV collagen; PIIINP: type III procollagen; LN: laminin; CG: cholyglycine; AFP: alpha fetoprotein; HBV: hepatitis B virus; LgHBV-DNA: log-transformed HBV-DNA load; CHB: chronic hepatitis B; HCC: hepatocellular carcinoma.

Figure 2. Serum KLB levels were positively correlated with TBIL (A), DBIL (B), GGT (C), ALP (D), TBA (E), ALT (F), AST (G) in HBV-related liver diseases.



TBIL: total bilirubin; DBIL: direct bilirubin; GGT: γ-glutamyl transferase; ALP: alkaline phosphatase; TBA: total bile acid; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

Serum KLB levels reflect the degree of cholestasis and hepatocyte injury

Serum levels of TBIL, direct bilirubin (DBIL), γ-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), and total bile acid (TBA) are typical indicators to assess the degree of cholestasis in patients. Serum KLB was positively correlated with serologic markers of cholestasis, as shown in Figure 2 A-E. In addition, serum transaminases can reflect the degree of liver cell injury and necrosis to a certain extent. In this study, we found that the serum KLB in patients with HBV-related liver diseases was also positively correlated with alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Figure 2 F-G).

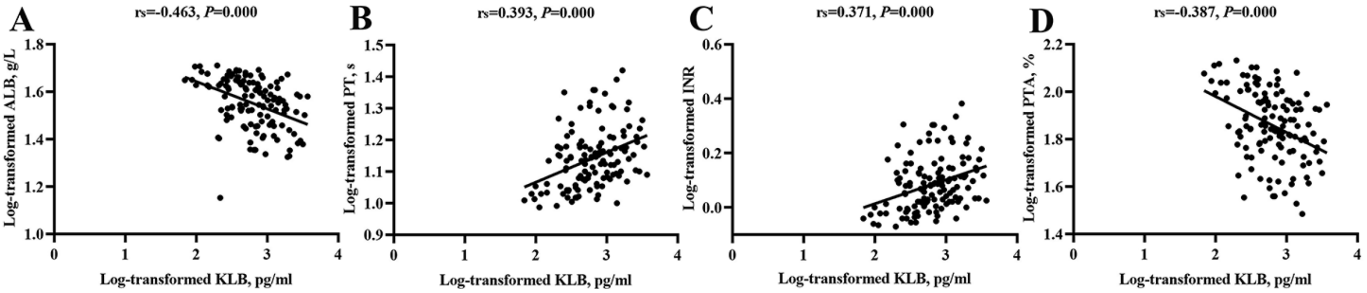
Serum KLB levels reflect the liver synthesis capacity

The liver is the only place to synthesize ALB and some coagulation factors, including coagulation factors II, VII, IX, and X. There may be hypoalbuminemia, prolonged PT, decreased prothrombin activity (PTA), and increased internationally normalized ratio (INR) if hepatocyte necrosis or liver synthesis function declines. In HBV-related liver diseases, serum KLB levels were negatively correlated with ALB and PTA but positively correlated with INR and PT (Figure 3).

Serum KLB levels reflect the degree of liver fibrosis

Serum markers of serum hyaluronic acid (HA), type IV collagen (CIV), N-terminal pro-peptide of type III procollagen (PIIINP), laminin (LN), cholyglycine (CG)

Figure 3. Serum KLB levels were negatively correlated with ALB (A) and PTA (D) while positively with PT (B) and INR (C) in HBV-related liver diseases.



ALB: albumin; PT: prothrombin time; INR: international normalized ratio; PTA: prothrombin activity.

can reflect liver fibrosis to a certain extent [21-23]. In this study, serum KLB levels were positively correlated with the above parameters (Figure 4 A-E). Furthermore, the expression of KLB was positively related to fibrosis index based on four factors (FIB-4), aspartate transaminase-to-platelet ratio index (APRI), King's score, and S-index (Table 2); notably, these serum markers are also used to quantify liver fibrosis in HBV-related liver diseases [24-26].

Relationship between serum KLB levels and other parameters

According to the correlation analysis, serum KLB was positively correlated with age, AFP, ascites, liver cirrhosis, splenomegaly, and MELD-Na score while negatively correlated with platelet count (PLT). However, no significant correlation was found between serum KLB level and HBV-DNA load in each group (Table 2).

Predictive value of serum KLB for HCC

Serum KLB in the HBV-related HCC group increased dramatically compared to the other three groups. What's more, KLB had a significantly positive correlation with AFP, the most commonly used serological indicator to monitor the occurrence of HCC. Given that, KLB elevation may provide crucial information on HCC onset. To compare the predictive value of KLB and AFP for HCC, we generated the area under the ROC curve in all HBV-related liver disease patients. The results are shown in Figure 5 and Table 3.

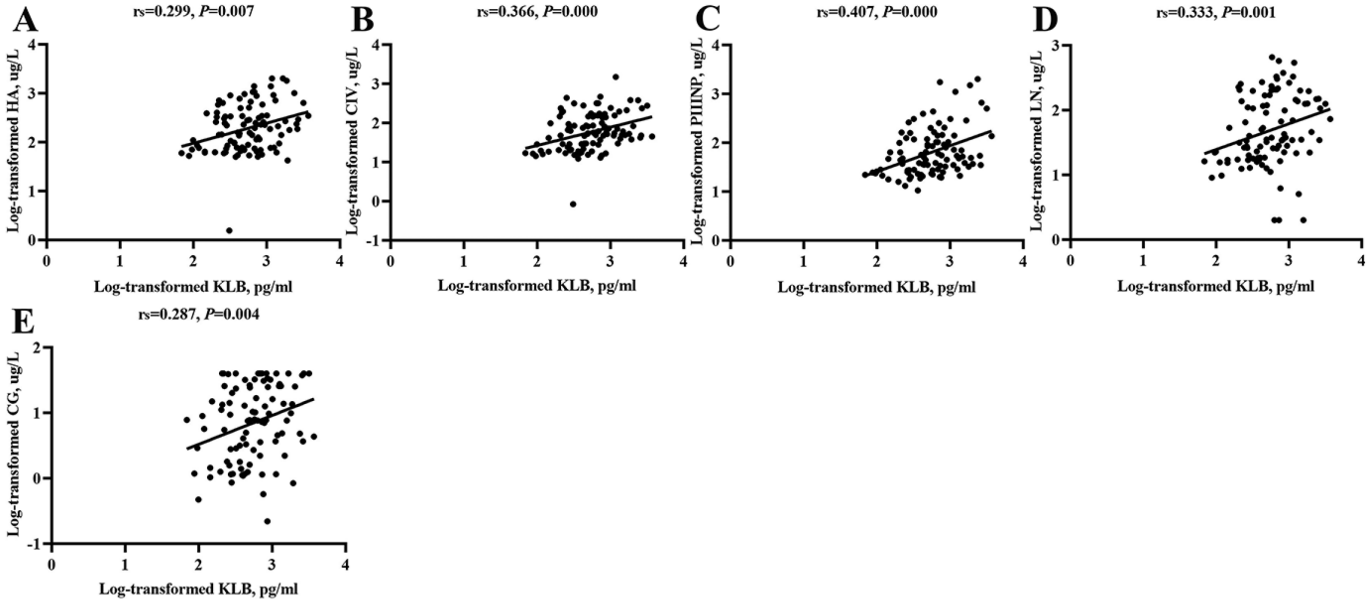
Table 2. The relationship between serum KLB with other clinical parameters in patients with HBV-related liver diseases.

Feature	Serum KLB	
	Correlation coefficient	*p* value
Age	0.327	0.000
Gender	-0.102	0.248
WBC	0.023	0.798
PLT	-0.187	0.032
CHOL	-0.151	0.130
TG	-0.186	0.062
HDL	-0.192	0.054
LDL	-0.194	0.052
GLU	-0.172	0.051
AFP	0.561	0.000
HBV-DNA load	-0.077	0.386
HBeAg (positive)	-0.053	0.578
Ascites	0.315	0.000
Liver cirrhosis	0.293	0.001
Splenomegalia	0.209	0.021
FIB-4	0.399	0.000
APRI	0.382	0.000
King's score	0.401	0.000
S-index	0.583	0.000
Child-Pugh classification	0.106	0.319
MELD-Na score	0.364	0.000

WBC: white blood cell count; PLT: platelet count; CHOL: cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; GLU: glucose; AFP: alpha-fetoprotein; FIB-4: Fibrosis index based on 4 factors; APRI: aspartate transaminase-to-platelet ratio index; MELD-Na: Model for End-Stage Liver Disease Sodium score.

The AUC of KLB was 0.862 (95% CI 0.790-0.916, $p < 0.0001$ compared with the reference line), with a sensitivity of 92.4% and specificity of 71.4% for the diagnosis of HCC. The AUC of AFP was 0.844 (95% CI 0.770-0.902, $p < 0.0001$ compared with reference line), with a sensitivity of 83.3% and specificity of 74.6%. Serum KLB had higher sensitivity but lower

Figure 4. Serum KLB levels were positively correlated with HA (A), CIV (B), PIIINP (C), LN (D), CG (E).



HA: serum hyaluronic acid; CIV: type IV collagen; PIIINP: N-terminal propeptide of type III procollagen; LN: laminin; CG: cholyglycine.

Table 3. Sensitivity and specificity for HCC prediction using serum KLB or/and AFP in HBV-related liver diseases patients.

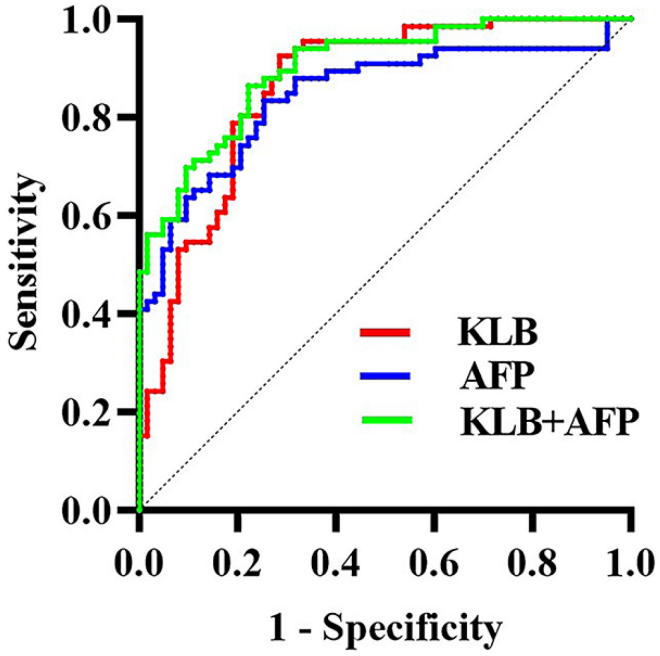
Serum marker	AUC	Sensitivity (%)	Specificity (%)	Cut-off (pg/mL)	Youden index
KLB	0.862	92.4	71.4	464.1	0.639
AFP	0.844	83.3	74.6	11325.0	0.579
KLB + AFP	0.899	86.4	77.8	/	0.641

specificity than AFP in diagnosing HCC in patients with HBV-related liver diseases. We then combined KLB with AFP to diagnose HCC, and it could be seen that the sensitivity and specificity for HCC diagnosis had been improved to a certain extent.

Discussion

The study demonstrated the association of serum KLB levels with HBV-related liver diseases for the first time. In this study, we found that serum KLB levels were increased sequentially among the healthy controls, the CHB group, the HBV-cirrhosis group, and the HBV-HCC group. These data demonstrate that elevated serum KLB levels may be positively associated with the progression of HBV-related liver diseases. However, Zhou *et al.* [5] pointed out that serum KLB levels were decreased in NSCLC and inversely associated with disease progression. This result implied that the clinical significance of serum KLB might vary with different diseases. We next analyzed the correlation of baseline serum KLB expression with routine clinical indicators. Notably, serum KLB levels reflected the degree of liver injury and impairment of liver synthetic function caused by HBV infection.

Figure 5. ROC curves for predicting HCC in patients with HBV-related liver diseases.



KLB: AUC = 0.862 (95% CI: 0.790-0.916); AFP: AUC = 0.844 (95% CI: 0.770-0.902); KLB vs. AFP: p = 0.667

Among the enzymes used to diagnose liver parenchymal injury, serum transaminase demonstrates the degree of hepatocyte injury and necrosis to a certain extent. When liver injury occurs, the ALT accumulated in the hepatocyte cytoplasm is released into the circulation, increasing serum concentrations [27]. The release of AST from hepatocyte mitochondria is evidence of hepatocyte necrosis [28]. We observed that serum KLB levels were positively correlated with both ALT and AST in HBV-related liver diseases. Still, the correlation with AST was stronger than ALT, indicating that KLB may be mainly regulated by mitochondrial stress and hepatocyte necrosis.

Moreover, this study found significant positive correlations between serum KLB and indicators of cholestasis, including TBIL, DBIL, GGT, ALP, and TBA. It has been demonstrated in past studies that chronic cholestatic disorders may lead to liver fibrosis and cirrhosis if left untreated [29]. As is well-known, HBV infection is one of the significant causes of cholestasis. Therefore, monitoring the degree of cholestasis in the progression of HBV-related liver diseases is of great clinical significance. Serum KLB might be purposed to fill this role. These findings are consistent with the positive correlation of KLB single nucleotide polymorphisms (SNPs) with biomarkers of liver injury observed in patients with non-alcoholic liver disease [30]. Furthermore, serum KLB levels were positively associated with the MELD-Na score, which assesses liver functional reserve and the severity of liver diseases [31,32]. Based on the above results, it is clear that serum KLB levels could reflect the degree of liver injury caused by HBV infection.

The liver is a vital organ involved in synthesis and metabolism. It is the only place for the synthesis of ALB and some coagulation factors, including coagulation factors II, VII, IX, and X. According to the relationship between serum KLB and ALB, PT, PTA, INR observed in this study, we speculated that serum KLB has an essential significance for the impairment of synthetic function caused by HBV infection. In addition, long-term chronic HBV infection can lead to liver fibrosis. Patients with CHB must undergo histological staging for liver fibrosis, not only for treatment decisions but also for prognosis [33]. Advanced fibrosis can progress

to cirrhosis, liver failure, and hepatocellular carcinoma [34]. In the past, some researchers have found that the KLB rs17618244 variant is associated with hepatic fibrosis and cirrhosis, mainly in obese patients with metabolic-associated fatty liver disease [11]. Furthermore, previous studies have reported that KLB plasma levels were lower in carriers of the rs17618244 minor A allele and were related to lobular inflammation, ballooning, and fibrosis [35]. It has also been noted that the inflammatory response during fibrogenesis suppresses KLB [36]. In this study, we found serum KLB levels were positively correlated with the multiple serum markers of fibrosis. Moreover, the serum KLB levels in the cirrhotic group were significantly higher than those in the non-cirrhotic group. Given that we speculated that serum KLB in patients with HBV-related liver diseases might be positively associated with the degree of liver fibrosis, the dynamic changes of serum KLB could be used to evaluate the efficacy of anti-fibrotic therapy. Interestingly, the study of Lee *et al.* [36] pointed out that KLB was proposed to inhibit liver fibrosis. Therefore, we hypothesized that elevated serum KLB in HBV-related liver diseases might lack biological activity, or its anti-fibrotic training may not be sufficient to reverse disease progression. Viral hepatitis is one of the causes of hypersplenism. When hypersplenism occurs, splenomegaly and one or more blood cell reductions will occur. We observed that serum KLB levels were negatively correlated with PLT and positively correlated with splenomegaly, indicating that serum KLB may be an essential indication for hypersplenism.

In addition, previous studies have found that KLB plays an essential role in several tumors. However, the expression of KLB in liver cancer and its function remain controversial. Our results showed that serum KLB levels were significantly elevated in patients who developed HCC, which indicated the potential role of KLB as a biomarker in monitoring tumorigenesis in patients with HBV-related liver diseases. AFP is currently the most widely used serological index for detecting HCC. Still, its specificity needs to be improved due to the nonspecific elevation of AFP in 15-58% of CHB patients and 11-47% of cirrhotic patients [27]. In our study, serum KLB levels were significantly correlated with AFP, and the specificities of serum KLB were higher than AFP. A combination of serum KLB and AFP might be more conducive to the early screening of HCC. Although KLB is not necessarily a substitute for existing markers, its flexible application in various aspects of HCC management and treatment may help improve the prognosis of HCC patients.

Notably, our results did not find a specific association between serum KLB and HBV-DNA levels, which indicated that serum KLB levels are more likely to be a response induced by immune injury but not affected directly by HBV infection.

Certain limitations of our study should be acknowledged. The first is the need for dynamic change of serum KLB since we only looked at a one-time point in each subject. Secondly, the pathological data of study subjects should be collected to make the results more reliable. Thirdly, the BMI was not matched in disease groups, which may have impacted the results. In addition, further studies are needed to elucidate the causal relationship between serum KLB and HBV-related disease progression.

Conclusions

In conclusion, we present the first clinical evidence revealing that KLB expression levels may be associated with disease progression in HBV-related liver diseases. Serum KLB levels were positively related to cholestasis, hepatocellular damage, liver fibrosis, and hypersplenism but negatively correlated with the liver functional reserve in HBV-related liver diseases. Furthermore, serum KLB has better sensitivity in diagnosing HCC in patients with HBV-related liver disease than AFP, and serum KLB combined with AFP has higher sensitivity and specificity than AFP alone in diagnosing HCC. In summary, serum KLB is negatively associated with liver damage in HBV-related liver diseases and might be a useful biomarker in monitoring disease progression. KLB has important implications in diagnosing HCC.

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Authors' Contributions

Concept and design of the study: Xin Miao, GuiCheng Wu; Methodological support: Xin Miao, ChuYan Peng, Xuan An, Fang Yan, LiNa Xia; Patient enrolment: Xin Miao, ChuYan Peng, Qiang Song; Experiments and procedures: Xin Miao, ChuYan Peng, LiNa Xia; Data analysis and Manuscript

writing: Xin Miao, GuiCheng Wu ; Manuscript reviewing: Xuan An, Fang Yan, XiQing Guo

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