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RESEARCH ARTICLE

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The importance of serum biglycan levels as a fibrosis marker in patients with chronic hepatitis B

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Background: Liver biopsy is recommended in the majority of patients with chronic viral hepatitis for fibrosis evaluation. Because of the potential risks of liver biopsy, many studies related to non-invasive biomarkers of hepatic fibrosis have been performed. We aimed to assess the diagnostic value of serum biglycan as a non-invasive fibrosis marker in chronic hepatitis B patients.

Methods: This study included 120 patients with biopsy-proven hepatitis B patients and 60 healthy controls. Fibrosis stage and necroinflammatory activity were assessed in liver biopsy specimens. Biglycan level was measured using an ELISA assay.

Results: Serum biglycan levels of chronic hepatitis B patients were found to be significantly higher than those of healthy controls (337.3±363.0 pg/mL vs 189.1±61.9 pg/mL, respectively, $P<.001$). There was a statistically significant positive correlation between serum biglycan level and fibrosis stage ($P=.004$; $r=.213$). Besides, a statistically significant positive correlation was found between serum biglycan level and necroinflammatory activity ($P<.001$; $r=.271$). The AUROC of BGN levels was 0.702 for fibrosis stage, differentiating patients from healthy controls with statistical significance ($P<.001$). The AUROC of BGN levels was 0.632 for necroinflammatory activity score, differentiating patients from healthy controls with statistical significance ($P=.004$).

Conclusions: Serum biglycan might be used as a non-invasive marker of liver fibrosis. Further studies are needed to evaluate the usefulness of this marker.

KEYWORDS
biglycan, chronic hepatitis B, liver biopsy, liver fibrosis, non-invasive marker

1 | BACKGROUND

Chronic hepatitis B infection, chronic hepatitis C infection, and non-alcoholic steatohepatitis are the most common chronic liver diseases all over the world.[1] Hepatitis B virus infection is a global public health problem. It is estimated that there are 350 million HBV carriers and nearly 1 million patients die from HBV-related liver disease annually.[2] HBV infection can cause acute or chronic infection, liver cirrhosis, or hepatocellular carcinoma.[3] HBV infection is the most common cause of hepatocellular carcinoma. Liver biopsy is the gold standard method for the diagnosis of many liver diseases such as the non-alcoholic steatohepatitis, assessment of liver fibrosis, and for determining the prognosis of chronic hepatitis infection. However, liver biopsy has some disadvantages: It is an invasive procedure and is prone to variation in the length and size of tissue specimen. Therefore, the development of non-invasive biomarkers of fibrogenesis is important for the staging of fibrosis and monitoring of chronic liver disease. Many tests have been evaluated in specific populations.[4,5]

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under the receiver operator curve; BGN, biglycan; ECM, extracelullar matrix; ECMR, extracellular matrix remodeling; GGT, gamma-glutamyl transpeptidase; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HIV, human immunodeficiency virus; INR, international normalized ratio; NIA, necroinflammatory activity index; SD, standard deviation; SLRP, small leucine-rich proteoglycan family.

Institutional review board statement: The study was approved by the ethics committee of Gazi University Faculty of Medicine, Ankara, Turkey.

Informed consent statement: All patients gave informed consent.

Fibrosis occurs in region where injury is most severe, particularly in chronic liver diseases due to alcohol or viral infection. Extracellular matrix (ECM) proteins play major roles in fibrotic diseases, metastasis, and tumor progression.[6] ECM represents a group of macromolecules, including collagen, non-collagen glycoprotein, glycosaminoglycans, proteoglycans, and matrix proteins. Biglycan (BGN) is a secreted proteoglycan; it is involved in collagen fibril assembly while its fragmentation is likely to be associated with collagen turnover during the pathogenesis of diseases which involve dysregulated extracellular matrix remodeling (ECMR), such as liver fibrosis.[7,8] BGN has a role in fibrogenesis. It belongs to a small leucine-rich proteoglycan (SLRP) family. BGN regulates cytokine activity in the pathogenesis of fibrosis. Based on its capacity to bind TGF-β and TNFα, BGN is known to be involved in regulating cytokine activity.[9] Numerous attempts have been made to identify the non-invasive markers that are capable of providing accurate information about the extent of fibrosis in liver. There are some studies in rats about BGN and the extent of liver fibrosis. To the best of our knowledge, there is no human study about the association of serum BGN level and the extent of liver fibrosis.

Therefore, this study is the first one to investigate any correlation between serum BGN level and the extent of liver fibrosis in chronic hepatitis B patients. We aimed to assess the diagnostic value of serum BGN as a non-invasive fibrosis marker to predict liver fibrosis in treatment-naive chronic hepatitis B patients.

2 | OBJECTIVES

We aimed to assess the diagnostic value of serum biglycan as a non-invasive fibrosis marker in chronic hepatitis B patients.

3 | MATERIALS AND METHODS

3.1 | Study subjects

This prospective study involved 120 patients diagnosed with chronic hepatitis B infection, as defined by positive hepatitis B surface antigen (HBsAg) for at least 6 months, and 60 age- and gender-matched healthy controls. Peripheral venous blood samples were evaluated for the presence of HBV surface antigen; as well as levels of ALT, AST, hemoglobin, and albumin. Platelet count and prothrombin time were also determined. The patients' liver biopsies were performed in the Department of Gastroenterology at Gazi University Hospital between December 2012 and January 2015. Liver biopsy was performed for the assessment of the severity of liver fibrosis and inflammation prior to treatment of the underlying liver disease. Exclusion criteria included co-infection with hepatitis C, hepatitis D virus, and HIV, chronic liver disease due to other causes, non-alcoholic steatohepatitis, and alcoholic liver disease. As for 60 healthy controls, there was no indication for liver biopsy; their liver and renal function tests were normal, they had negative serology for HBV, HCV, and HIV, their abdominal ultrasonography examinations were within normal limits. These healthy controls had never suffered from any form of hepatitis, had not been exposed to hepatotoxic drugs, and had no history of alcohol abuse.

Laboratory tests, including hemoglobin, platelet count, ALT, AST, prothrombin time, and albumin were evaluated in all patients on the day of the liver biopsy. The markers of hepatitis virus including HBsAg and HBV DNA concentration were recorded.

The study protocol was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. All patients provided written informed consents for their participation in the study.

3.2 | Measurement of serum biglycan levels

For each participant, 5 mL of peripheral venous blood sample was drawn using Vacutainer system and centrifuged at 1600 g for 15 minutes in order to separate serum. All serum samples were kept at −80°C until the assay date.

In order to measure the BGN level in serum samples, a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Human Biglycan ELISA Kit, Catalog number: EK1357, Boster Immunoleader, Boster Biological Technology Co., Ltd., Pleasanton, CA, USA) was used and the assay was performed according to manufacturer's instructions. The test principle for biglycan ELISA assay can be summarized as follows: A monoclonal antibody from mouse specific for Biglycan has been precoated onto 96-well plates. Standards and test samples were added to the wells; a biotinylated detection polyclonal antibody from goat specific for Biglycan was added subsequently and then followed by washing with PBS or TBS buffer. Avidin-biotin-peroxidase complex was added and unbound conjugates were washed away with PBS or TBS buffer. HRP substrate TMB was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The assay range is 156 pg/mL-10 000 pg/mL. The specificity of the assay has been defined as natural and recombinant human biglycan, whereas its sensitivity is <10 pg/mL. No detectable cross-reactivity with other relevant proteins has been defined. The average coefficient of variation (CV) regarding intra-assay precision is 4.66%; and the average CV regarding inter-assay precision is 5.36%.

After the steps of ELISA assay were completed, the optical density (OD) values were read spectrophotometrically at 450 nm. BGN levels in serum samples were calculated using OD values of standards with known concentrations by regression–correlation analysis using CurveExpert Basic (Version 1.4; CurveExpert, Hixson, TN, USA) statistical software package. Producer of the software program is Mr. Daniel Hyams of 1698 Chadwick Court, Hixson, TN 37343. CurveExpert is validated against the Statistical Reference Datasets Project of the National Institute of Standards and Technology. http://www.curveexpert.net/products/curveexpert-basic/

3.3 | Histological assessment

Liver biopsies were performed employing ultrasonography guidance with 16 F true- cut biopsy needles. Specimens of minimum 10 mm

length were immediately fixed in %10 formalin and routinely embedded in paraffin. The tissue sections were stained with hematoxylin-eosin, Masson trichrome and reticular fiber staining. All specimens were at least 20 mm in length with a minimum of 11 portal tracts. All biopsies were assessed by experienced pathologists who were blinded to the results of serum BGN levels, and were scored according to the Ishak's scoring ([10]): F0, no fibrosis; F1, fibrous expansion of some portal areas, with or without short fibrous septa; F2, fibrous expansion of most portal areas, with or without short fibrous septa; F3, fibrous expansion of most portal areas, with occasional portal to portal bridging; F4, fibrous expansion of portal areas with marked bridging (portal to portal) as well as portal to central; F5, marked bridging (portal to portal and/or portal to central) with occasional nodules (incomplete cirrhosis); F6, cirrhosis, probable or definite.

3.4 | Statistics

Baseline characteristics were presented as mean ± standard deviation, median (interquartile range), and categorical variables as number (percentage). Statistical analyses were carried out using SPSS v.15 (SPSS, Chicago, IL, USA) software statistical package. Normality of distribution was assessed using Kolmogorov-Smirnov test. Statistical analyses were performed using *t* test and Mann-Whitney *U* test for comparisons between patient and healthy control groups. Correlations were calculated using Spearman rank test and Kendall's Tau-b test. A value of $P<.05$ was considered statistically significant. The predictive accuracy of each non-invasive index was assessed by calculating the AUROC. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of biglycan was calculated using the receiver operator curves (ROC).

4 | RESULTS

4.1 | Patient characteristics

One hundred and twenty patients with chronic hepatitis B and 60 healthy controls were enrolled in this study. Their age and gender characteristics are given in Table 1. The mean age of chronic hepatitis B patients was 46.8±13.3 years, and 56 patients were female while 64 were male. The mean age of 60 healthy controls was 45.4±13.0 years, and 32 were female while 28 were male. There was no significant difference between the patient group and healthy controls either in age ($P=.233$) or in sex distribution ($P=.312$).

The fibrosis stages were identified pathologically upon liver biopsy: Fibrosis stage was F0 in 22 patients (%18.3), F 1-2-3 in 84 patients (%70), and F4-5-6 in 14 patients (%11.7) as seen in Table 1.

The mean levels of AST and ALT were significantly higher in the patient group than in healthy controls ($P<.001$). The mean platelet count was significantly lower in the patient group than in healthy controls ($P<.001$; Table 1). There were no statistically significant differences in the mean values of other parameters between the two groups. The mean level of serum BGN was significantly higher in the patient group when compared to that in healthy controls (337.3±363.0 pg/mL vs 189.1±61.9 pg/mL, respectively, $P<.001$; Table 1).

4.2 | Correlation between laboratory tests and stages of liver fibrosis

The mean levels of serum AST ($r=.332$; $P<.001$) and ALT ($r=.262$; $P=.004$) increase as the fibrosis score increases from F0 to F4 or higher: The differences were statistically significant (Table 2).

TABLE 1 The baseline characteristics of patients and healthy controls

	Patient group (n:120)		Healthy controls (n:60)		
	Mean±SD	Median (min–max)	Mean±SD	Median (min–max)	*P*
Sex (female/male)	56 (46.7%)/64 (53.3%)		32 (53.3)/28 (46.7)		.399
Age (y)	46.8±13.3	50 (18-79)	45.4±13.0	43 (24-75)	.233
Hb (g/dL)	14.1±1.6	14.3 (9.6-17.2)	13.6±1.1	13.7 (11.3-17.8)	.021
Plt (e^3/UL)	215.1±61.3	246.0 (146.0-457.0)	262.7±61.3	264.0 (146-457.0)	**<.001**
AST (U/L)	33.1±26.6	20.0 (12-221)	20.9±5.9	19.5	**<.001**
ALT (U/L)	44±38	26 (5-257)	23.0±7.8	19.5 (9-40)	**<.001**
Albumin (g/dL)	4.2±0.3	4.3 (3.4-5.3)	4.3±0.3	4.3 (3.4-5.3)	.704
INR	0.9±0.08	0.9 (0.8-1.4)	0.9±0.06	0.9 (0.8-1.05)	.320
Biglycan (pg/mL)	337.3±363.0	222.0 (110.8-2330.3)	189.1±61.9	182.4 (95.9-427.4)	**<.001**
Fibrosis stage (n/%)					
F0	22 (18.3%) patients				
F1-3	84 (70.0%) patients				
F4-6	14 (11.7%) patients				

Hb, hemoglobin; plt, platelet number; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; INR, international normalized ratio.
Data are expressed as mean±standard deviation.
Bolded text indicates statistical significance at $P<.05$.

TABLE 2 Correlation between laboratory studies and stage of fibrosis

Fibrosis	*r*	*P*
Hb (g/dL)	.100	.277
Plt (e3/UL)	−.235	**.010**
AST (U/L)	.332	**<.001**
ALT (U/L)	.262	**.004**
INR	−.056	.548
Alb (g/dL)	−.59	.432
NIA	.406	**<.001**
Biglycan (pg/mL)	.213	**.004**
HBV DNA copies/ML	.320	−.092

Hb, hemoglobin; plt, platelet number; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; INR, international normalized ratio; NIA, necroinflammatory activity index; SD, standard deviation; *r*, correlation coefficient.

TABLE 3 Serum biglycan levels according to the stage of fibrosis and necroinflammatory activity index

	Mean±SD	Median (min–max)
F0	200.1±90.7	180.7 (95.9-687.3)
F1-3	243.1±110.7	214.1 (110.3-869.7)
F4-6	2294.1±2094.4	853.9 (141.0-4982.0)
NIA0	208.1±101.6	186.0 (95.9-687.3)
NIA (1-4)	208.5±67.2	196.7 (110.1-428.1)
NIA (5-8)	300.2±183.1	224.3 (110.8-953.9)
NIA (9-12)	1569.9±2050.6	278.4 (151.2-4982.0)
NIA (13-16)	—	—

NIA, necroinflammatory activity index; SD, standard deviation.

Moreover, there was a statistically significant positive correlation between necroinflammatory activity and fibrosis score (*r*=.406; *P*<.001), and there was a statistically significant negative correlation between platelet count and fibrosis stage (*P*=.010; *r*=−.235; Table 2).

There was a statistically significant positive correlation between serum BGN level and fibrosis stage (*P*=.004; *r*=.213). The mean level of serum BGN was significantly higher in patients with F4-6 than in patients with F1-3 (2294.1±2094.4 vs 243.1±110.7 pg/mL, respectively, *P*<.001). Besides, a statistically significant positive correlation was found between serum BGN level and necroinflammatory activity (*P*<.001; *r*=.271). The mean level of serum BGN was significantly higher in patients with NIA 9-12 than in patients with NIA 5-8 (1569.9±2050.6 vs 300.2±183.1 pg/mL, respectively, *P*=.004; Table 3).

There was no significant correlation between serum BGN level and HBV DNA level (*P*=.320; *r*=−.092).

4.3 | ROC

We assessed the diagnostic performance of serum BGN levels and fibrosis stages using a ROC. The AUROC of BGN levels was 0.702 for fibrosis stage, differentiating patients from healthy controls with statistical significance (*P*<.001). The AUROC of BGN levels was 0.632 for necroinflammatory activity score, differentiating patients from healthy controls with statistical significance (*P*=.004; Figure 1). The AUROC of BGN levels was 0.524 for F0-F3 vs F4-F6 (Figure 2).

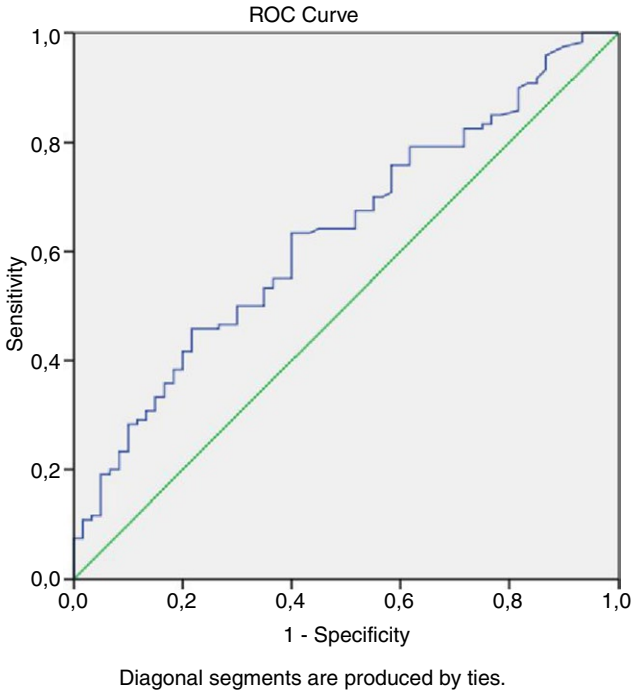

FIGURE 1 Receiver operator curves for necroinflammatory activity

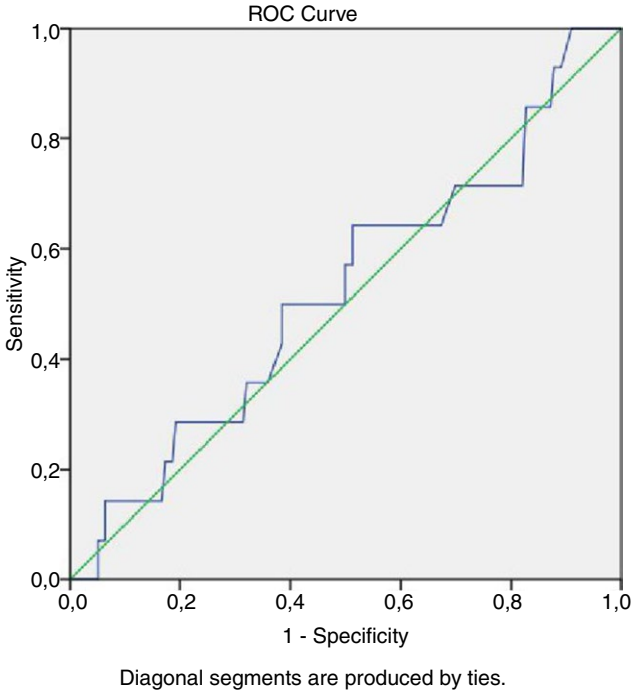

FIGURE 2 Receiver operator curves for having clinical significant fibrosis vs not (F0-F3 vs F4-F6)

5 | DISCUSSION

Liver fibrosis is a serious medical condition that occurs as a result of chronic viral hepatitis infection, alcoholic liver disease, non-alcoholic steatohepatitis, drug abuse, and autoimmune hepatitis.[11] Liver fibrosis is the final common pathway of most chronic liver diseases, and it is characterized by increasing accumulation of collagen in the matrix resulting in scar tissue.[12] Liver biopsy is the gold standard diagnostic method for grading fibrosis. Nevertheless, it is an invasive procedure and has some disadvantages.[13] Therefore, many studies have been performed in order to find out new non-invasive biomarkers of liver fibrosis, and to investigate the roles of these biomarkers to predict liver fibrosis. BGN belongs to the small leucine-rich family of proteoglycans.[9] Decorin, BGN, lumican, and fibromodulin are the key regulators of collagen fibers.[14]

Aim of this study was to determine whether serum BGN level could be a potential serological marker of pathological extracellular matrix remodeling in chronic HBV infection. Previously, some animal studies have sought the answer to this question; however, ours is the first human study on this subject.

In a study of Leeming et al., C3M, BGM, C4M, P4NP-7S, ELM were studied in 249 HIV-infected patients and 55 controls. The serum levels of MMP-degraded collagen type III (C3M), biglycan (BGM), elastin (ELM), as well as the formation marker 7S (P4NP-7S), and MMP-degraded collagen type IV (C4M) were determined using specific ELISAs. Sixty-eight patients underwent a follow-up visit 3 years later including assessment of ECM markers and fibrosis using transient elastography (Fibroscan). These markers were found to be significantly higher in HIV-infected patients than in the control group; and in patients with high viral load compared to those with low viral load. A negative correlation was found between the duration of antiretroviral therapy and the levels of these markers. This study has shown that BGM level measurements are effective in monitoring the hepatic effects of antiretroviral therapy.[15]

In another study that involved 94 alcoholic cirrhosis patients and 20 control subjects, the levels of type 1 collagen, C3M, PROC3, C6M, P4NP-7S, C5M, biglycan, and elastin denoted the degree of hepatic dysfunction. A significant correlation was found between these markers and both hepatic venous pressure gradient and portal hypertension. By this study, new non-invasive markers were found to spot clinically important portal hypertension.[16]

Genovese et al., established a hepatic fibrosis model in rats by employing CCL4 and bile duct ligation. Biglycan that is cleaved by matrix metalloproteinases was found to be correlated with pathological extracellular matrix remodeling. Hence, a significant positive correlation was shown between the degree of hepatic fibrosis and serum biglycan level in an animal study.[17]

In a study performed by Jia et al., 30 rats were employed. In 20 of them, hepatocellular carcinoma (HCC) model was established by administering N-diethylnitrosamine. Aggrecan (chondroitin sulfate proteoglycan 1), biglycan, and decorin (proteoglycan core protein) levels were found to be significantly higher in hepatic tissues of these rats.[18] It is not surprising that decorin and biglycan levels were high in hepatic fibrosis because they are members of the small leucine-rich proteoglycan (SLRP) family.[11] Interestingly, in HCC tissue, aggrecan, biglycan, and decorin spread into cytoplasm, pericellular matrix, and cellular membrane in contrast to the control group in which they are found exclusively on hepatocyte membrane and pericellular matrix.[19]

In another study, decorin, biglycan, proteoglycan 100, and proteoglycan colony stimulating factor were studied in both normal and fibrotic human livers. In HBsAg-positive chronic active hepatitis patients, decorin and biglycan showed strong immunoreactivity in fibrotic areas while they are clearly seen in the space of Disse in normal hepatic tissue.[20]

In an animal study performed on 52 rats, a hepatic fibrosis model was established by administering CCL4. Biomarkers including type 1 collagen, type 3 collagen, type 4 collagen, type 6 collagen, citrulline, vimentin, biglycan, PN3P, P4NP-7S, and PCP5 were shown to correlate with the degree of hepatic fibrosis induced by CCL4.[21]

In a study by Nielsen et al., Pro-C3 and C3M levels were measured by ELISA in plasma obtained from CHC patients (n=194) who had been enrolled in a prior phase II antifibrotic trial. Pro-C3 levels were significantly higher in CHC patients in Ishak stage 4 compared to those in stage 2 ($P<.001$) or in stage 3 ($P<.01$). Pro-C3 could significantly distinguish moderate fibrosis (stage 4) from mild fibrosis (stage 2/3) (AUC=0.72, $P<.001$). Pro-C3 was a useful test to predict fibrogenesis and monitor disease progression. Moreover, it could differentiate mild from moderate disease.[22]

In this study, we aimed to investigate any association between serum biglycan levels and fibrotic stage and necroinflammatory activity in chronic HBV infection, because there was no previously published human study to show this association. Serum biglycan levels of 120 chronic hepatitis B patients were compared with 60 control subjects. In chronic hepatitis B patients, mean serum biglycan level was found to be significantly higher than that in the control group. When biglycan levels were assessed according to histopathological fibrotic stages in liver biopsy specimens, a positive correlation was shown, and histopathologically determined necroinflammatory activity increased as serum biglycan levels increased.

Significance of level is evaluated with ROC curve. Area under the ROC curve (AUROC), can be between values of 0.5 and 1.0 according to effectiveness. Significance is shown by the levels near 1 in chronic hepatitis B patients; serum biglycan proved to be a promising diagnostic test for the evaluation of hepatic fibrosis (0.702). Serum biglycan level was statistically meaningful to evaluate the presence of fibrosis. It also performed fairly as a diagnostic test to predict the liver necroinflammatory activity (0.632) and it was statistically significant also. The aim of our study was to investigate an alternative new screening method, regarding liver biopsy. In chronic hepatitis B patients, a significant correlation was found between serum biglycan level and the stage of fibrosis.

6 | CONCLUSION

As a result, we have shown a correlation between serum biglycan level and fibrotic and necroinflammatory stage in chronic hepatitis

B patients. Nevertheless, it should be noted that this correlation is modest. It was also found that serum biglycan levels of the patients and healthy control group showed statistically significant differences. Validity of this non-invasive method needs to be verified by performing further studies. On the other hand, liver biopsy is still the gold standard diagnostic method in the evaluation of hepatic fibrosis. This non-invasive marker may be beneficial in the circumstances when liver biopsy needs to be repeated or when it is contraindicated.

AUTHORS' CONTRIBUTIONS

Rafiye Ciftciler was responsible for collecting blood samples, informing volunteers, data collection, performed the majority of experiments, and wrote the article; Seren Ozenirler organized research and corrected the article; Aysegul Atak Yucel performed immunological analysis of the samples and corrected the article; Mustafa Cengiz and Erkan Buyukdemirci performed statistical analysis of data; Gulbanu Erkan served as scientific adviser, drafted the article, and revised it critically for important intellectual content; performed statistical analysis of data; Cemile Sönmez performed immunological analysis of the samples; Guldal Yılmaz Esendaglı evaluated liver tissue samples and determined fibrosis stage.

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