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Original Article

The Role of Cytokeratin 18 and Quantitative HBsAg Levels in Hepatitis B Infection

Hepatit B Enfeksiyonunda Sitokeratin 18 ve Kantitatif HBsAg'nin Rolü

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ABSTRACT

Objective: We aimed to investigate the use of serum quantitative hepatitis B surface antigen (qHBsAg) and cytokeratin 18 (CK18) levels as noninvasive markers to differentiate inactive HBsAg carriers from hepatitis B e-antigen (HBeAg) negative chronic hepatitis B (CHB) patients.
Method: Sixty randomly selected treatment-naive patients with CHB and 25 healthy volunteers as control group were included in the study. Virological, serological and biochemical test results were assessed. Levels of M30 antigen which is the active form of CK18 were measured by M30-Apoptosense enzyme linked immunosorbent assays (ELISA). Liver biopsy specimens were assessed according to the modified Ishak scoring.
Results: Forty-eight (56.5%) participants were female. There was no significant difference between three groups in terms of age, gender, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin (T.Bil), direct bilirubin (D.Bil), prothrombin time (PT), international normalized ratio (INR), alfa-fetoprotein (AFP) and CK18 values. HBVDNA and qHBsAg levels were found to be significantly lower in inactive carriers compared with HbeAg-negative chronic HBV patients. The diagnostic efficacy of CK18 in differentiating inactive carriers from HbeAg-negative CHB patients was not statistically significant.
Conclusion: In conclusion, when used along with HBVDNA, qHBsAg may be an important parameter to differentiate inactive HBsAg carriers from HbeAg-negative chronic HBV patients. However, as one of the parameters targeted in this study, serum CK18 levels can not be used as a noninvasive marker.

Keywords: Cytokeratin 18, hepatitis B, non-invasive markers

ÖZ

Amaç: İnaktif hepatit B yüzey antijeni (HBsAg) taşıyıcılarını hepatit B e antijen (HBeAg) negatif kronik hepatit B (KHB) hastalarından ayırmak için serum kantitatif HBsAg (qHBsAg) ve sitokeratin 18 (CK18) düzeylerinin invaziv olmayan belirteçler olarak kullanımını araştırmayı amaçladık.
Yöntem: KHB ile takip edilen tedavi almamış 84 hastadan rastgele seçilen 60 hasta ve kontrol grubu olarak çalışmaya katılmayı kabul eden 25 sağlıklı gönüllü dahil edildi. Virolojik, serolojik ve biyokimyasal test sonuçları değerlendirildi. CK18'in aktif formu olan M30 antijen seviyeleri enzyme linked immunosorbent assay (ELISA) testi ile ölçüldü. Karaciğer biyopsi örnekleri, modifiye Ishak skorlamasına göre değerlendirildi.
Bulgular: Katılımcıların 48'i (% 56,5) kadındı. Üç grup arasında yaş, cinsiyet dağılımı, alkalen fosfataz (ALP), gama glutamil transferaz (GGT), direkt bilirubin, protrombin zamanı (PZ), uluslararası standardize oran (INR), alfa fetoprotein (AFP) ve CK18 değerleri açısından anlamlı fark yoktu. İnaktif taşıyıcılarda HBV DNA ve qHBsAg seviyelerinin HBeAg negatif kronik HBV hastalarına göre anlamlı derecede düşük olduğu bulunmuştur. İnaktif taşıyıcıları ve HBeAg negatif KHB hastalarını ayırt etmede CK18'in tanısal etkinliği istatistiksel olarak anlamlı değildi.
Sonuç: Sonuç olarak, qHBsAg, HBV DNA ile birlikte kullanıldığında, inaktif HBsAg taşıyıcılarını HBeAg negatif kronik HBV hastalarından ayırt etmek için önemli bir parametre olabilir. Bu çalışmada hedeflenen parametrelerden biri olan serum CK18 ise invaziv olmayan bir belirteç olarak saptanmadı.

Anahtar kelimeler: Sitokeratin, hepatit B, invaziv olmayan belirteçler

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INTRODUCTION

It is considered that there are 240 million people worldwide infected with chronic hepatitis B virus (HBV) with endemicity rates ranging between 2, and 8% (1,2). Totally, 57% of all cases of liver cirrhosis and 78% of primary liver cancers develop as a result of chronic HBV and hepatitis C virus infections (1).

Liver biopsy is still the gold standard in order to identify early stage of fibrosis and selection of proper treatment. However, liver biopsy has disadvantages such as patients' unwilingness to the invasive procedure, requirement of recurrent biyopsies due to retrieval of insufficient tissue samples, variations among pathologists and increased morbidity and mortality (3-5).

Contrary to invasive liver biopsy, noninvasive methods have been investigated so far to determine liver damage. Quantitative HBsAg (qHBsAg) and cytokeratin 18 (CK18) are two of the markers investigated for this purpose. Covalently closed circular DNA (cccDNA) is an intrahepatic reservoir for HBV. Theoretically, HBsAg titer reflects the transcriptional activity of cccDNA. Although it varies according to clinical phase of disease, qHBsAg was shown to be correlated with serum HBVDNA and intrahepatic cccDNA levels (6). CK18 is the major intermediary fibrillar protein in the liver. During apoptosis of the hepatocytes, CK18 fragments cleaved by caspases are released into the bloodstream. Based on this knowledge, recently, the number of studies which investigated the use of serum/plasma levels of CK18 fragments as noninvasive markers in chronic liver diseases for diagnosis and staging, have increased (7). During apoptosis, CK18 is cleaved at two different points by caspases as aspartate 238 (Asp238) and aspartate 396 (Asp396). M30 monoclonal antibody, a selective biological marker of apoptosis, is produced against the epitope mapped to positions 387–396 of CK18 and it specifically recognizes M30 antigen (M30 ag), the fragment cleaved from Asp396 point. M30 antigen, identified as, the active form of CK18 is used to quantitatively measure the serum levels of CK18 cleaved by the caspases responsible for apoptosis (7,8).

This study aims to investigate the use of serum CK18 and qHBsAg levels as noninvasive markers to differentiate inactive HBsAg carriers from HbeAg-negative chronic HBV patients.

MATERIALS AND METHODS

Sixty randomly selected treatment-naive patients with CHB, and 25 healthy volunteers aged >16 years as the control group were included in the study. Each participant and the families of the participants under the age of 18 were informed about the study and documented his/her consent on the "Informed Consent Form".

The stages of chronic HBV infection:
HBeAg-positive chronic HBV infection is characterized by the presence of serum HBeAg, high HBVDNA and normal alanine aminotransferase (ALT) levels but minimal or no liver necroinflammation. HBeAg-positive CHB is characterized by the presence of serum HBeAg, high levels of HBVDNA and elevated ALT and moderate or severe liver necroinflammation. HBeAg-negative chronic HBV infection is characterized by the lack of serum HBeAg and presence of serum anti-HBe, undetectable or low HBVDNA and normal ALT levels and minimal or no liver necroinflammation. HbeAg-negative CHB is characterized by the lack of serum HBeAg and presence of serum anti-HBe, high levels of serum HBVDNA, moderate or severe liver necroinflammation.

Patients diagnosed with chronic hepatitis C virus (HCV), hepatitis D, hemochromatosis, autoimmune disorders, Wilson's disease, alpha-1-antitrypsin deficiency, human immunodeficiency virus (HIV) infection, liver cancer or decompensated liver cirrhosis, and who had an history of alcohol consumption >20 grams per day, and previously received or already receiving oral antiviral and immunomodulatory treatment despite viral etiology were excluded.

Serum HBsAg, anti HBs, HBeAg, anti-HBe, anti-HDV, anti-HCV and anti-HIV levels were analyzed

by Enzyme Immunoassay (EIA, Liason, Diasory, Italy) method. Serum HBVDNA and qHBsAg levels were analyzed by quantitative Polymerase Chain Reaction (PCR) (Architect HBsAg Reagent kit, Sligo, Ireland) method. Complete blood counts, liver function tests (aspartate aminotransferase (AST), ALT, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin (T.Bil), direct bilirubin (D.Bil), prothorombin time (PT), international normalized ratio (INR) and alpha-fetoprotein (AFP) levels of each subject were also measured.

Totally 5 cc of venous blood sample was obtained from each patient and healthy controls. Blood samples were maintained at room temperature for 30-60 minutes and then centrifuged for 10 minutes at 4000 RPM, and the separated serum samples were stored in a deep-freezer at -80°C until CK18 analyses were performed. Serum samples were only thawed once for the assay. The levels of M30 antigen were measured by M30-Apoptosense enzyme-linked immunosorbent assays (ELISA) (PEVIVA, Sundbyberg, Sweden) for quantitative measurement of serum levels of CK18 cleaved by the apoptotic caspases. As per the manufacturer's instructions, M30-antigen concentrations were expressed in units per liter (U/L).

Statistical Package for the Social Sciences (SPSS) 21 program was used for data analysis. P values <0.05 were considered statistically significant.

The study protocol was approved by the Ethics Committee of Health Sciences, Tepecik Education and Research Hospital on April 24, 2013 (Ethics committee registration number:15)

RESULTS

Of 60 cases, 30 patients had HbeAg-negative chronic infection, and 30 had HbeAg-negative CHB, while the control group consisted of 25 (29.4%) healthy volunteers. Forty-eight cases consisted of female patients. There was no significant difference between three groups in terms of age, gender distribution, ALP, GGT, direct bilirubin, PT, INR, AFP and CK18 levels (Table 1). Platelet (PLT) counts were significantly lower in HbeAg-negative chronic infection group, compared to the control group (p=0.014). ALT and AST levels were significantly lower in the control group compared to the HbeAg-negative CHB group (p=0.003), (p<0.001).

Serum HBVDNA (p<0.001) and qHBsAg (p=0.011) levels were found to be significantly lower in HBeAg-negative chronic infection group

Table.1. The demographic and laboratory parameters of the groups.

	GROUPS			P VALUES
	HbeAg-negative chronic infection (n=30)	HBeAg-negative CHB (n=30)	Healthy Controls (n=25)	
Gender, women (%)	15 (50)	15 (50)	18 (72)	0.216
Age (year)	39.3±9.5	38.7±8.8	39.4±8.5	NS
qHBsAg, IU/ml	1688.8±3463.5	3818.3±3896.3	-	0.011
HBV DNA, IU/ml	179.5±416	6630±22370	-	<0.001
AFP, IU/ml	1.5±1.9	1.3±0.9	-	NS
ALT, U/L	19.5±11.0	24±18	16±10	0.003
AST, U/L	22±7	25.5±7	17±4	<0.001
Total bilirubin, mg/dl	0.8±0.5	0.8±0.3	0.5±0.2	<0.001
Prothrombin Time, sec	11.1±1.0	11.1±0.7	-	NS
INR	1±0.1	1±0.1	-	NS
PLT $\times 10^3/\mu L$	247.9±63.6	257.5±73.5	307.6±83.6	0.014

(NS; Not-significant, qHBsAg:quantitative hepatitis B surface antigen, HBV:hepatitis B virus, DNA:deoxyribonucleic acid, AFP:Alpha-fetoprotein, ALT: alanine aminotransferase, AST:aspartate aminotransferase, Tbil:total bilirubin, PT: prothrombin time, INR: international normalized ratio, Dbil: direct bilirubin, PLT: platelet)

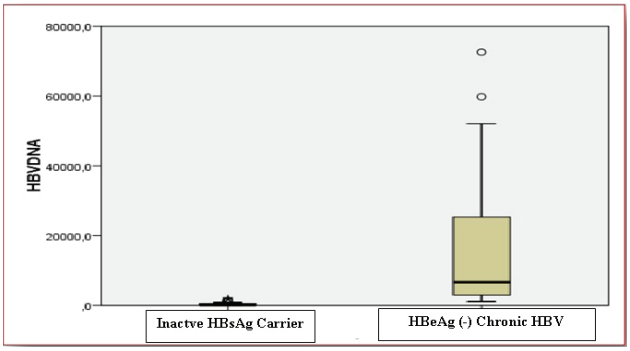

Figure 1

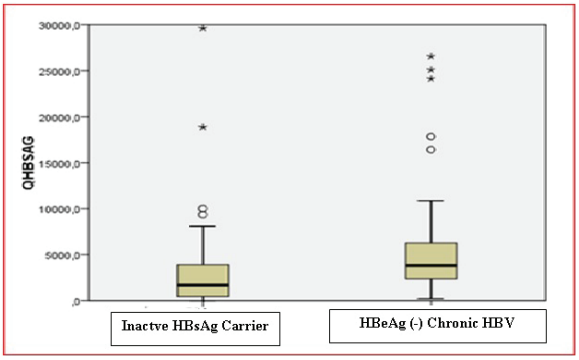

Figure 2

compared to the HBeAg-negative CHB group. HBVDNA and qHBsAg levels for all three groups are shown in Figures 1 and 2, respectively.

A receiver operating characteristic (ROC) analysis was performed in order to evaluate diagnostic value of serum qHBsAg and CK18 levels in differentiating between inactive HBsAg carriers and HBeAg-negative chronic HBV patients. The analysis showed that the cut-off value for qHBsAg was 2114.7 IU/ml. Therefore, patients with qHBsAg levels below 2114.7 IU/ml were most likely defined as HBeAg-negative chronic infection group (specificity 56.6%, negative likelihood ratio 73.9%) and patients with higher qHBsAg levels were defined as HBeAg-negative CHB group (sensitivity 80%, positive likelihood ratio 64.9 [AUC (Area under the ROC curve): 0.689±0.069] (p=0.012) (Figure 3).

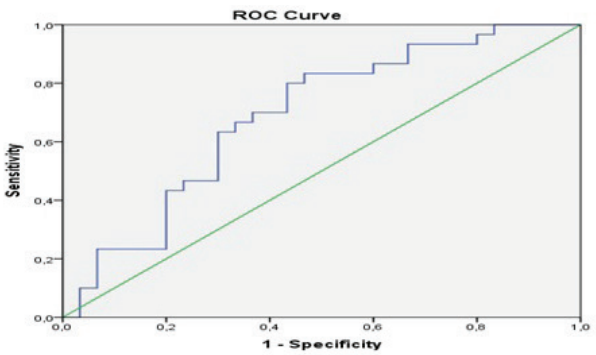

Figure 3

On the other hand, the diagnostic value of CK18 in differentiating HBeAg-negative chronic infection group from HBeAg-negative CHB group (AUC: 0.474±0.076), HBeAg-negative chronic infection group from healthy controls (AUC: 0.611±0.077), HBeAg-negative CHB group from healthy controls (AUC:0.581±0.078) was not statistically significant (p>0.05).

DISCUSSION

Reports based on long-term patient follow-ups (mean:18 years) have indicated that HBeAg-negative chronic infection phase can progress into permanent biochemical remission and carry a very low risk of HCC and cirrhosis (9,10). On the other hand, HBeAg-negative CHB leads a progressive course associated with variable and frequently exacerbating aminotransferase levels, possibly fluctuating viral load despite lower HBVDNA levels compared to HBeAg-positive patients. Therefore, these patients suffer from moderate to severe necroinflammation of the liver and carry a higher risk of cirrhosis and HCC (11). Studies have shown that the risk of HCC and cirrhosis are directly associated with HBVDNA levels. As a result, guidelines recommend that the serum HBVDNA threshold level to define inactive HBV carriers should be 2000 IU/mL.

In the present study ALT, AST and T.Bil levels of healthy controls were significantly lower than HBeAg-negative CHB patients. Elevated liver enzymes of patients with chronic HBV infection indicates necroinflammation, which develops as a result of cellular cytotoxicity against HBV-infected hepatocytes. Meanwhile, ongoing HBV replication triggers immune response and results in liver damage. Sayan et al.[12] reported that ALT levels were significantly higher in HbeAg-negative CHB group having HBVDNA>10^5copies/mL compared to the HBeAg-negative chronic infection with HBVDNA <10^5copies/mL, and concluded that ALT levels rise along with the increase in HBVDNA load. In a study performed by Chu CM et al.[13] on 250 inactive HBsAg carriers with normal ALT levels persisting for more than 10 years and 90 age-matched

HBeAg-negative chronic HBV patients, HBVDNA levels in the inactive HBsAg carrier group were found to be significantly lower than HBeAg-negative chronic HBV patients ($p<0.0001$). The present study similarly showed significantly lower HBVDNA levels in inactive HBsAg carrier group compared to HBeAg-negative chronic HBV patients.

Noninvasive tests that are routinely used to differentiate inactive HBsAg carriers from HBeAg-negative chronic HBV patients can be inadequate on several occasions. Sonnoveld MJ et al.[14] reported that qHBsAg can be used together with long-term ALT and HBVDNA monitoring to differentiate HBeAg-negative chronic HBV patients with low qHBsAg levels from the inactive carriers. Serum qHBsAg levels were found positively correlated with intrahepatic cccDNA, so it was emphasized that in order to detect cccDNA, measuring serum qHBsAg levels is more practical rather than an invasive procedure like liver biopsy (15,16). In the present study, qHBsAg levels were significantly higher in HBeAg-negative chronic HBV patients compared to the inactive HBsAg carriers. To indicate the potential use of qHBsAg to differentiate between these two patient groups, ROC analyses were performed which showed that the cut-off value of 2114.7 IU/ml for qHBsAg had a predictive value. In the study of Chen CH et al.[17] which also supported these findings, cut-off value of 1600 IU/mL for qHBsAg had a predictive value for differentiating HBVDNA negative patients from other patient groups having detectable and higher HBVDNA levels, with 69.4% sensitivity and 66.7% specificity. Recent studies have demonstrated that hepatic apoptosis plays a crucial role in the pathogenesis of chronic liver disease (18). Apoptosis reflects a specific morphology of cell death, and it is also referred as programmed cell death (19). During apoptotic cell death of the hepatocytes, CK18 fragments cleaved by caspases are released into the bloodstream. Recently, the number of studies which investigated the use of serum/plasma levels of CK18 fragments as noninvasive markers in chronic liver diseases for diagnosis and staging, have increased (7).

Serum CK18 levels were investigated in a study performed by Papatheodoridis et al.[5] with 115 patients with chronic HBV (53 inactive HBsAg carriers, 62 HBeAg-negative chronic HBV patients) and 30 healthy controls and and significantly higher serum CK18 levels were found in HBeAg -negative chronic HBV patients compared to inactive carriers. The authors concluded that serum CK18 is a useful biomarker to differentiate between these two groups, with a cut-off value of 240 U/L with 60% predictive value, 60% sensitivity, 100% specificity and positive predictive value. In addition, CK18 levels in healthy controls were significantly lower than in inactive carriers and HBeAg-negative chronic HBV patients. In a study performed by Bae et al.[20] with 339 chronic HBV patients who had undergone liver biopsy, serum CK18 levels were reported to be significantly elevated in HBeAg-negative chronic HBV patients compared to the inactive carriers. The authors concluded that the serum level of CK18 can be used as a biomarker to differentiate between these two groups.

CONCLUSION

In the present study, a statistically significant difference was not detected between the mean serum CK18 levels of inactive HBsAg carriers, HBeAg-negative chronic HBV group and healthy controls. Therefore, the diagnostic value and possible predictive value of serum CK18 levels to differentiate chronic HBV patients from healthy controls and inactive carriers from HBeAg-negative chronic HBV patients could not be demonstrated. We believe that these findings can be explained by the limited number of cases included in this study and the lack of biopsy-confirmed data of all cases, as opposed to the previous studies. Moreover, studies performed so far have reported different predictive values; thus, additional prospective studies including larger patient populations are required in order to generalize the findings of previous studies.

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