The utility of M30 and M65 antigen concentration levels for predicting degree of hepatic injury in patients with chronic hepatitis B infection

Kronik hepatit B enfeksiyonununda hepatik hasar düzeyinin öngörülmesinde M30 ve M65 antijen düzeylerinin etkinliği

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Background and Aims: *We aimed to determine the utility of M30 and M65 antigen concentration levels for predicting hepatic injury in chronic hepatitis B disease.* ***Materials and Methods:*** *This study compared concentration levels of M30 and M65 antigens between patients with hepatitis B e-antigen negative chronic hepatitis B and healthy subjects. Furthermore, the correlations between either M30 or M65 antigen levels and aspartate aminotransferase, alanine aminotransferase, HBV-DNA, histological activity index and fibrosis were evaluated in the patient group.* ***Results:*** *A total of 81 subjects were included in the study; 50 patients with HBeAg negative chronic hepatitis B and 31 healthy subjects. The concentration of the M30 antigen was significantly higher in the chronic hepatitis B patient group than in the healthy subject group (p <0.05). However, there was no difference in M65 antigen concentration values between the two groups (p >0.05). Correlation analysis performed in the patient group revealed a significant correlation between M30 antigen concentration levels and aspartate aminotransferase levels (r: 0.207, p <0.05), and between M65 antigen concentration levels and HBV-DNA levels (r: 0.204, p <0.05). There was no significant correlation observed between M30 or M65 antigen concentration levels and both the histological activity index and fibrosis.* ***Discussion:*** *The presence of high M30 antigen levels in HBeAg negative chronic hepatitis B patients may suggest that M30 antigen concentration might be beneficial in disease monitoring and evaluation of treatment efficacy. This observation must be tested further in more comprehensive studies. However, the absence of a significant correlation between the concentration levels of either antigen or both the histological activity index and fibrosis suggests that pathological examination is unique in detecting hepatic injury.*

Key words: *Chronic hepatitis B, M30 antigen, M65 antigen, hepatic injury*

Giriş ve Amaç: *Tüm hepatit türlerinde inflamatuvar olay nekroz ve apopitozu içeren farklı mekanizmalar ile hepatosit ölümüne sebep olur. M30-antijen apoptozis esnasında kaspazlar tarafından parçalanmış CK18 düzeylerini ölçmede, M65-antijen ise nekroza giden hücrelerden salgılanan total CK18 düzeylerini ölçmede kullanılır. Bu iki marker'ın kronik hepatit B enfeksiyonunda kullanımı ile ilgili sınırlı sayıda çalışma mevcuttur. Biz bu çalışmada M30 ve M65 antijen düzeylerinin, kronik hepatit B hastalığında karaciğer hasarını göstermede kullanılabilir olup olmadığını göstermeyi amaçladık.* ***Gereç ve Yöntem:*** *Bu çalışmada, Katip Çelebi Üniversitesi, İzmir Atatürk Eğitim ve Araştırma Hastanesi, Gastroenteroloji polikliniğine başvuran kronik hepatit B enfeksiyonlu hastalar ve sağlıklı kontrol grubunda, M30 ve M65 antijen düzeyleri ölçüldü ve çalışma sonunda elde edilen veriler karşılaştırıldı. Kronik aktif hepatit B enfeksiyonlu hastalarda ve sağlıklı kontrol grubunda M30 ve M65 antijen düzeyleri karşılaştırıldı. Ayrıca hasta grupta M30-M65 düzeyleri ile aspartat aminotransferaz, alanin aminotransferaz, hepatit B virus DNA, histoloji aktivite indeksi ve fibrozis düzeyleri arasındaki ilişki değerlendirildi.* ***Bulgular:*** *Çalışmaya 50 kronik aktif hepatit B hastası ve 31 sağlıklı kontrol olmak üzere toplam 81 hasta alındı. Hepatit B hasta grubunda M30-antijen düzeyi kontrol grubuna göre anlamlı düzeyde yüksek bulundu (p<0.05), ancak iki grup arasında M65-antijen düzeyleri açısından anlamlı fark saptanmadı (p>0.05). Hasta grupta yapılan korelasyon analizinde M30-antijen ile aspartat aminotransferaz arasında (r:0.207, p<0.05), M65-antijen ile hepatit B virus DNA arasında anlamlı ilişki saptandı (r:0.204, p<0.05). M30-M65-antijen ile histoloji aktivite indeksi ve fibrozis arasında anlamlı ilişki saptanmadı.* ***Tartışma:*** *Apopitoz belirteci olan M30 düzeylerinin kronik aktif hepatit B hastalarında yüksek saptanması bize M30 düzeylerinin daha kapsamlı çalışmalar ile hastalık takibinde, hatta hastalara verilen tedavinin etkinliğinin değerlendirilmesinde faydalı bilgiler sağlayabileceğini düşündürdü. Ancak M30-M65 düzeyleri ile histoloji aktivite indeksi ve fibrozis arasında anlamlı ilişki olmamasının, patolojinin karaciğer hasarı tespitinde rakipsiz olduğunu bir kez daha ispatladığını düşünüyoruz.*

Anahtar kelimeler: *Kronik hepatit B, M30-M65 antijen, hepatik hasar*

INTRODUCTION

Approximately 350 million people worldwide are infected with the Hepatitis B virus (HBV). The disease spectrum and natural history of chronic HBV infection shows diversity and variety, ranging from a low-viremic inactive carrier to chronic hepatitis, capable of progression to cirrhosis and hepatocellular carcinoma (HCC). HBV-associa-

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ted end-stage liver disease or HCC causes 1 million deaths per year and is currently responsible for 5-10% of liver transplants (1-4).

Inflammatory events cause hepatocyte death through different mechanisms including necrosis and apoptosis in all types of hepatitis (5-7). Although genetically programmed apoptosis is controlled by intrinsic factors, extrinsic signals may also stimulate cell suicide via receptors in the cell membrane (8,9). Necrosis is an unintended process that results from harmful extracellular stimulants such as hypoxia, physical injury, hyperthermia, complement activation, and ultraviolet light (10). Necrosis is a pathological process, whereas apoptosis may occur either with physiological or pathological stimulants (11).

Cytokeratins are cytoskeletal components of epithelial cells (12). Each cytokeratin molecule is associated with an epithelium (13). Human cytokeratin 18 (CK18) is the first cytokeratin expressed during embryogenesis. In adults, it is secreted from the bladder epithelium, small intestine, colon mucosa, hepatocytes, eccrine glands, fallopian tubes, pancreatic acinar cells, cervix uteri and endometrium (14).

CK18 is the major cytoplasmic intermediate filament protein in hepatocytes. It is secreted into the circulation both during necrosis and apoptosis of the hepatocyte. Total (uncleaved) CK18 (the M65 antigen) is secreted into the circulation when cells die during necrosis. However, broken (caspase-cleaved) CK18 exists during apoptosis and is secreted into the circulation as cells progress to secondary necrosis (15). In particular, M30 monoclonal antibody recognizes CK18 fragments that are broken at aspartate 396 (the M30 antigen).

A correlation has been demonstrated between hepatic fibrosis and apoptotic CK18 concentrations in the circulation. In patients with successful hepatitis C virus (HCV) clearance, disease progression and fibrosis are diminished because of a significant reduction in circulating CK18 during hepatocellular apoptosis (16,17).

Serum aminotransferase activity is a marker for hepatocellular necrosis, and serum CK18 concentration is a marker for apoptosis. No correlation has been observed between serum aminotransferase concentration and caspase activity in liver biopsies. Bantel et al found a significant relationship between CK18 concentration levels and liver injury in 59 patients with chronic viral hepatitis C infection (17). There is a limited number of existing studies on the use of these two markers in chronic hepatitis B (CHB).

In this study, we aimed to demonstrate whether M30 and M65 antigen concentration levels were useful as markers in hepatitis B e-antigen (HBeAg) negative CHB. Moreover, we aimed to demonstrate the relationship between both M30 and M65 antigen concentration levels and alanine aminotransferase (ALT), aspartate aminotransferase (AST), HBV-DNA, hepatic activity index (HAI), and fibrosis in CHB infection. It is expected that this study will result in guiding outcomes for determining disease activity and predicting liver injury before biopsy.

MATERIALS and METHODS

In this study, M30 and M65 antigen concentration levels were measured in patients with HBeAg negative chronic hepatitis B who attended the Katip Çelebi University, Izmir Atatürk Training and Research Hospital, Gastroenterology Polyclinic. A healthy control group was also included in this study. These two groups were compared.

A total of 81 subjects, comprising 50 patients with HBeAg negative CHB and 31 healthy controls, were included in the study. At the time of admission, age, sex, AST, ALT, and HBV-DNA values were measured. Patients with HBeAg negative CHB were identified using the European Association for the Study of the Liver (EASL) 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection published by Journal of Hepatology (18). Patients with hepatitis B surface antigen (HBsAg) positivity for more than six months, with serum HBV-DNA concentration >2000 IU/mL, and with abnormally high ALT levels at least twice within six months were assigned to the CHB group and underwent liver biopsy (19). All patients were treatment naïve. Patients with hepatitis C infection, delta hepatitis or human immunodeficiency virus (HIV) co-infection, alcohol consumption >40 g/day, and patients with a known malignancy were not included in the study. Furthermore, HAI and degree of fibrosis detected in liver biopsies were recorded. Healthy subjects without any known disease, with negative HBsAg, immunoglobulin M (IgM) antibody to hepatitis B core antigen (Anti-Hbc IgG), anti-HCV, and anti-HIV viral markers and with normal hepatic function tests, normal body mass index (BMI), fasting blood glucose, and lipid parameters were assigned to the control group.

The study was carried out prospectively and informed consent forms were obtained from the patients. After serum samples were centrifuged, they were stored at -70°C until required for analysis. M30 and M65 antigen concentration levels were compared between the patient and healthy control group. The relationship between M30 and M65 antigen concentrations and AST, ALT,

HBV-DNA, HAI, and fibrosis was investigated in HBeAg negative CHB.

Furthermore, through regression analysis, the most significant determinant of M30 and M65 antigen levels was evaluated from AST, ALT, HBV-DNA, HAI, and fibrosis.

Measurement of M30 and M65-antigen concentration

Serum M30 antigen concentrations were measured using Human Cytokeratin 18-M30 (CK 18-M30) an enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Diagnostics, Wuhan, China), which had a measuring range between 15.6 mIU/mL-1000 mIU/mL. Serum M65 antigen concentrations were measured using a Human Cytokeratin 18-M65 (CK 18-M65) ELISA kit (Cusabio Diagnostics, Wuhan, China), which had a measuring range between 9.38 mIU/mL-600 mIU/mL. An antibody specific for CK 18-M30 or CK 18-M65 was pre-coated on to a microplate. Standards and samples were pipetted into the wells and any present CK 18-M30 or CK 18-M65 was bound by the immobilized antibody. After removing remaining unbound substances, a biotin-conjugated antibody specific to CK 18-M30 or CK 18-M65 was added to the wells. After washing, horseradish heroxidase-conjugated avidin (HRP) was added to the wells. Following a wash to remove any unbound enzyme-avidin reagent, a substrate solution was added to the wells and color was developed in proportion to the amount of CK 18-M30 or CK 18-M65 bound in the initial step. The color development was stopped, and the intensity of the color was measured.

Histologic evaluation of necroinflammation

Ultrasound-guided liver biopsies were performed under local anesthesia using an 18-gauge Tru-cut needle. Pathologic specimens were evaluated by an experienced pathologist, who had no clinical information about the patients, using the Ishak-scoring system.

Statistics

The Kolmogorov-Smirnov test was used to evaluate the normality of data. The two groups were compared between using the Mann-Whitney U test because the serum M30 antigen concentrations did not show normal distribution. The relationship between categorical variables was evaluated using either Chi-square or Fisher's exact test. The relationships between M30 and M65 antigen concentrations and AST, ALT, HAI, fibrosis, and HBV-DNA were analyzed using Pearson's correlation analysis. The linear relationship between both M30 and M65 antigen concentrations and AST, ALT, HAI, fibrosis, and HBV-DNA were evaluated using multiple linear regression models. Binary logistic regression analysis was performed. M30 and M65 levels were accepted as dependent variables. AST, ALT, HBV-DNA, HAI, and fibrosis levels were accepted as independent variables. HAI and fibrosis were marked as categorical variables. HAI levels were recorded from 1 to 18 and fibrosis levels were recorded from 1 to 6 as numerical values. Zero was marked as an initial value for both HAI and fibrosis.

A P value <0.05 was considered statistically significant. All analyses were two-tailed and were performed using SPSS 17.0 version.

RESULTS

The study comprised a total of 81 subjects; 31 healthy subjects which made up the control group (Group 1) and 50 subjects who had HBeAg negative CHB which made up the patient group (Group 2). AST ($p < 0.001$), ALT ($p < 0.001$) and M30 antigen ($p < 0.05$) concentration levels were significantly higher in the patient group than in the control group. However, no statistically significant differences in age, sex and M65 antigen concentration level were found between the two groups ($p > 0.05$) (Table 1).

Table 1. Comparison of the patient and the control groups in terms of age, gender, AST, ALT, M30-antigen and M65-antigen concentrations.

	Control Group (n=31)	Patient Group (n=50)	*P* Value
Age	32.3±9.1	35.1±10.8	>0.05
Sex, M/F	14/17	27/23	>0.05
AST	17.9±5.8	50±34	<0.001
ALT	20.8±18.0	92±33	<0.001
M30-antigen	56.0±37.2	69.7±103.6	<0.05
M65-antigen	19.1±9.2	20.1±6.3	>0.05

M: Male, F: Female, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase.

Tablo 2. Correlation analysis between M30-M65-antigen concentrations and AST, ALT, HBV-DNA, HAI, fibrosis in the patient group.

	M30	M65
AST	r: 0.207, p <0.05	r: 0.032, p >0.05
ALT	r: 0.101, p >0.05	r: 0.007, p >0.05
HBV-DNA	r: 0.099, p >0.05	r: 0.204, p <0.05
HAI	r: -0.70, p >0.05	r: -0.195, p >0.05
Fibrosis	r: -0.110, p >0.05	r: 0.143, p >0.05
M30		r: 0.141, p >0.05

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, HB: Hepatitis B virüs, HAI: Hepatic activity index.

Tablo 3. Regression analysis between M30/M65 antigen concentration and HBV-DNA, AST, ALT, HAI, fibrosis.

Independent Predictor	M30		M65	
	β	P	β	P
HBV-DNA	-0.45	0.709	0.283	0.026
AST	0.636	0.004	0.15	0.947
ALT	0.642	0.007	-0.127	0.596
HAI	-0.225	0.186	-0.136	0.437
Fibrosis	0.142	0.360	0.045	0.778

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, HB: Hepatitis B virüs, HAI: Hepatic activity index.

Subsequently, the relationships between both M30 and M65 antigen levels and AST, ALT, HBV-DNA, HAI, fibrosis levels were evaluated in the patient group (Table 2). A significant correlation was found between M30 antigen and AST levels (r: 0.207, P <0.05), and between M65 antigen and HBV-DNA levels (r: 0.204, p <0.05). No significant correlation was found between M30 and M65 antigen concentration levels and HAI, fibrosis and ALT (Table 2). Significant correlation was found between HAI and AST levels (r: 0.489, p <0.001), ALT levels (r: 0.573, p <0.001), fibrosis (r: 0.667, p <0.001). There was significant correlation between fibrosis and HAI (r: 0.667, p <0.001), ALT (r: 0.376, P < 0.001).

Regression analysis revealed that AST and ALT concentrations were the primary determinants of M30 antigen concentration levels. HBV-DNA was found to be the primary determinant of M65 antigen concentration levels (Table 3).

DISCUSSION

Chronic hepatitis B is an important factor that causes progressive liver disease through hepatic necroinflammation. Although serum ALT is used in routine clinical practice as the surrogate marker for hepatic inflammation, CHB may cause liver cirrhosis and hepatocellular carcinoma, even in the absence of ALT elevation (16,20,21). Cell death during apoptosis occurs without membrane damage (22). Massive apoptosis, along with liver injury, may explain the development of cirrhosis and HCC without ALT elevation in patients with chronic hepatitis B.

Farnik H et al demonstrated a correlation between the levels of ALT and M65-necrosis in CHB patients but determined no significant correlation between the levels of ALT and M30-apoptosis. In the same study, the authors demonstrated a significant correlation between fibrosis and concentrations of M30 and M65 in CHB patients, despite the absence of a significant correlation between ALT levels and fibrosis (23). Another study demonstrated that M30 antigen concentration was elevated in patients with CHB compared to active carriers. However, the same study failed to demonstrate a significant correlation between M30 antigen concentrations levels and either severity of fibrosis or HAI in patients with CHB (24).

In this study, we found that M30 antigen concentration levels were significantly higher in patients with CHB versus the control group, but there was no significant difference in M65 antigen concentrations between both groups. This suggested that apoptosis might play a more dominant role in cell death than necrosis in CHB infection. Furthermore, although we determined a significant correlation between HAI and both AST and ALT levels, as well as between fibrosis and ALT levels, we found no significant correlation between both M30 and M65 antigen concentration levels and ALT levels, HAI, and fibrosis. The varying results from previous studies may be attributed to several reasons. Criteria alone, which are used in the Ishak-scoring system to calculate level of HAI and fibrosis, are unable to define apoptosis and necrosis. Furthermore, some apoptotic cells are removed before destruction of the cell wall, i.e. before appearance of broken C18. Therefore, the true level of apoptosis may be higher than what is detected with the measurement of M30 antigen concentrations (25).

It has been demonstrated that M30 and M65 antigen concentration levels are significantly higher in patients with non-alcoholic fatty liver disease (NAFLD) versus healthy controls. In addition, a strong correlation was found between both antigen concentrations and AST and ALT levels (26). In this study, we found a significant correlation between M30 antigen levels and AST levels, and between M65 levels and HBV-DNA. However, linear regression analysis revealed that AST and ALT levels were the determinants of the M30 antigen concentration.

The high M30 antigen levels in patients with chronic active hepatitis B suggest that more comprehensive studies would provide beneficial information on the use of M30 levels in disease monitoring, and perhaps in evaluation of treatment efficacy. However, the absence of a significant correlation between both M30 and M65 antigen concentrations levels and HAI and fibrosis may suggest that pathologic examination is unique in detecting liver injury.

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