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The expression of serum M30 and M65 in chronic hepatitis B patients with non-alcoholic fatty liver disease

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Abstract. – OBJECTIVE: Chronic hepatitis B patients with fatty liver disease are gradually increasing. We aim to investigate the serum fragment level of cytokeratin 18 (CK-18), M30 and M65, in chronic hepatitis B (CHB) patients with and non-alcoholic fatty liver disease (NAFLD).

PATIENTS AND METHODS: Serum M30 and M65 levels were measured by ELISA assay in 46 CHB patients with NAFLD and 42 CHB patients without NAFLD. The association of serum M30 levels in 46 CHB patients with NAFLD and biochemistry and pathological indexes were investigated.

RESULTS: The serum M30 levels in CHB with NAFLD group were 614.48 ± 471.43 U/L, which were significantly higher than non NAFLD group (374.50 ± 231.04 U/L, $p < 0.01$). But there were no differences in serum M65 levels between NAFLD group (369.41 ± 262.21 U/L) and non-NAFLD group (296.50 ± 231.44 U/L, $p = 0.172$). We observed significantly higher serum M30 levels in CHB with NAFLD patients with positive HBV-DNA (752.36 ± 554.79 U/L) as compared with patients with negative HBV-DNA (400.0 ± 171.64 U/L, $p < 0.05$). While the M65 levels have no significant difference ($p = 0.285$). For CHB patient with NAFLD patients, the M30 level was positively correlated with ALT, AST, HBVDNA, TG, FBG, histology inflammation score, fibrosis score and steatosis ($p < 0.01$).

CONCLUSIONS: Serum M30 levels in CHB with NAFLD patients are significantly higher than CHB patients without NAFLD, especially for HBV-DNA positive patients. It could be a reference value for evaluating the inflammation degree of CHB with NAFLD.

Key Words:

Chronic hepatitis B, Non-alcoholic fatty liver disease, Serum fragmented cytokeratin 18, M65, M30, Inflammation in CHB.

Introduction

Hepatitis B (CHB) and non-alcohol fatty liver disease (NAFLD) are worldwide chronic liver disease with a high incidence[1,2]. CHB and NAFLD commonly cause cirrhosis and liver cancer[3]. Currently, chronic hepatitis B patients with fatty liver disease gradually increasing[4]. The existence of fatty liver may mask the morphology changes of liver in patients with chronic hepatitis B, it is hard to accurately evaluate the degree of inflammatory in liver cells, and subsequently affect the treatment strategies for hepatitis B virus.

Cytokeratin 18 (CK-18) is the major intermediate filament protein resulting in the characteristic structural changes of apoptosis of the liver. Caspase generated CK-18 fragments were increased in patients with NAFLD compared with healthy age-matched controls, and plasma levels of CK-18 is correlated with its expression levels in the liver[5,6]. Serum CK-18 fragments is increased in various acute and chronic liver diseases. After cleavage of the CK-18 caspase substrate, M30 antigen could be selectively recognized by ELISA methods, while M65 is composed by caspase-cleaved and uncleaved CK-18[7]. As serum cell death biomarkers, those two fragments are currently used in prognosis evaluating for some digestive system disease such as biliary cirrhosis, liver fibrosis, hepatocellular carcinoma and gastric carcinoma[7-10].

In this paper, we analyzed the clinical characteristics of CHB patients with non-alcoholic fatty liver disease (NAFLD). We also tested the level of M30 and M65 (CK18) in their serum, and analyzed their relationship with liver cell apoptosis and necrosis. We also explored the possible mechanism of fatty change in patients with chronic hepatitis B, and explored whether M65 and M30 could be used as a predictive indicator for liver inflammation in chronic hepatitis B patients with fatty liver.

Patients and Methods

Patients

The study was approved by the Institutional Review Board of the Third Center Hospital of

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Tianjin in 2013.Written informed consent was obtained from each patient for the use of their blood, liver biopsy and clinical information.

From September 2013 to April 2015, 88 patients confirmed having chronic hepatitis B was recruited consecutively; there were 66 males and 22 females, with average age of 39.3 years. Chronic hepatitis B was diagnosed according to the 2009 American Association for the Study of Liver Disease practice guidelines[11]. The non-alcoholic fatty liver disease was diagnosed according to Guidelines for the Diagnosis and Management of Nonalcoholic Fatty Liver Disease: update 2010: (published in Chinese on Chinese Journal of Hepatology 2010)[12]. According to whether they having fatty liver, patients were divided into chronic hepatitis B without NAFLD (N = 42) and CHB with NAFLD (N = 46). Abdominal B ultrasound and liver pathology were performed to these patients to confirm the existence of fatty liver.

The exclusion criteria including: (1) The alcohol fatty liver disease (in the past 5 years, intake of more than 40 g/d alcohol in the male or intake of more than 20 g/d alcohol in female); (2) patients having autoimmune hepatitis or primary biliary cholangitis; (3) drugs or toxic hepatitis; (4) genetic or metabolic liver disease such as Wilson's disease, hemochromatosis, anti-trypsin deficiency etc. (4) patients having the liver or biliary malignant tumor.

Blood Sampling and Laboratory Data Collecting

3 ml of fasting venous blood was drawn at the early of the morning, the blood was preserved in a blood collection tube containing no anticoagulant biochemical (with separation gel) (KHB Biotech, Shanghai, China). The tube was centrifuged for 15 min at 3 000 R/min within 2 hours and the serum was separated, then stored at –80° refrigerator for testing. The M30 and M65 concentrations in serum were determined by ELISA Kit (PEVIVA, Bromma, Sweden) according to the manufacture's instruction.

Laboratory parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), r-glutamine total (rGT), total bilirubin (TBil), triglyceride (TG), total cholesterol (TC), and fasting blood-glucose (FBG) were analyzed by an automatic biochemistry analyzer (PUZS-300, PERLONG MEDICAL, Nanjing, China). HBV-DNA content was analyzed by DNA amplification (ABI 7500, Applied Biosystems, Foster City, CA, USA). In this study, the HBV DNA content of $> 10^3$ cp/ml was considered as HBV-DNA positive.

Liver Biopsy and Grading of Pathological Changes

Liver biopsy was performed underwent B-mode ultrasonography using a 16 g bard disposable biopsy needle. The liver tissue was acquired through 1-seconds quick puncture biopsy, the obtained liver tissue specimens should have length of greater or equal to 1.5 cm with the number no less 7 in number of liver portal area. Specimens were fixed with 10% poly formaldehyde solution and prepared as paraffin sections, then stained by hematoxylin and eosin (HE) staining.

Their liver inflammation degree were graded according to Scheuer grade criteria[13], the inflammation activities were divided into G (1-4) stage and S (1-4) stage (Figure 1, A-B) by 2 indepen-

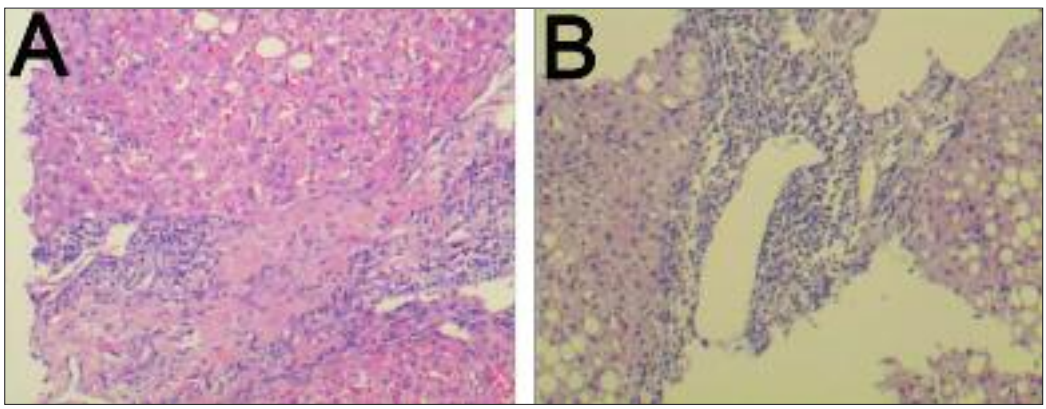

Figure 1. Grading of liver pathological changes after hematoxylin eosin (HE) staining. ***A,*** Grade of F1G2S2, patient has mild steatosis, portal inflammation and fibrosis (×100). ***B,*** Grade of F3G3, patient has severe steatosis and portal inflammation (×100).

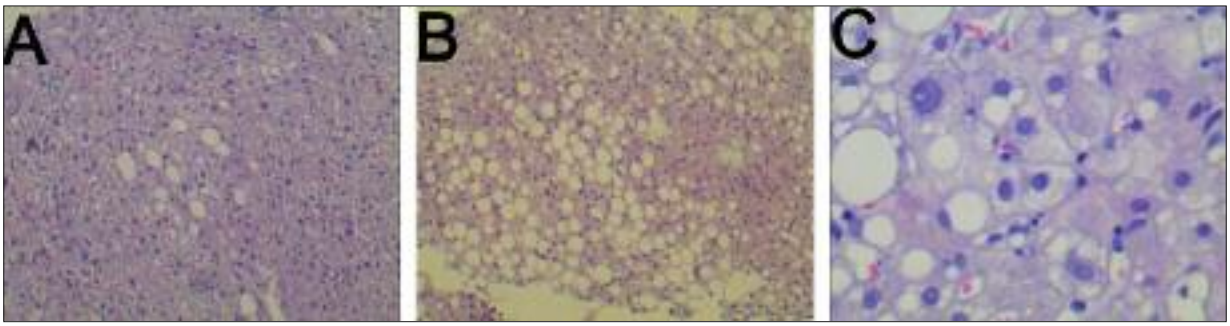

Figure 2. The fatty degeneration of liver grading after hematoxylin eosin (HE) staining. ***A,*** Grade of F1, mild steatosis (×100). ***B,*** Grade of F3, patient has severe steatosis (×100). ***C,*** Grade of F3, balloon like change could be seen (×400).

dent experienced pathologists by blinded evaluating. The fatty degeneration of liver was evaluated according to scoring system designed by Kleiner et al[14]: F1 (5% ≤ liver fatty degeneration percentage < 33%); F2 (33% ≤ liver fatty degeneration percentage < 66%) and F3 (liver fatty degeneration percentage ≥ 66%) (Figure 2, A-C).

Statistical Analysis

Measured data was recorded as mean ± SD. The HBV-DNA levels were converted in the logarithm to base 10 and calculated as measurement data. Statistical analysis was performed using SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA). The measurement data in two groups were compared by two independent samples t test; The count data in two groups were compared with chi-square test; correlation analysis was performed using Pearson method; Partial correlation analysis was used for correlation analysis between M30 and HBV-DNA; the correlation between liver pathological changes and M30, M65 (CK18) levels was using Spearman rank correlation analysis. $p < 0.05$ was considered as statistically significant.

Results

Demographic Data Comparison

There were 42 patients were divided into CHB without NAFLD group and 46 patients were divided into CHB with NAFLD group. Their demographic data are listed in Table I, as we can see: there was no statistical difference in age, gender distribution, ALT, AST, rGT, TBiL, HBV-DNA between the 2 groups; but we observed higher level of triglyceride (TG), fasting blood glucose (FBG) and M30 existed in CHB with NAFLD group with significant difference ($p < 0.01$) when compared with CHB without NAFLD group. However, we didn't observed statistical difference in M65 level between the 2 groups.

Table I. Demographic data comparison in 2 groups.

	CHB without NAFLD	CHB with NAFLD	*p*-value
Male/Female	35/11	31/11	0.758
Age (yr)	39.28 ± 10.78	37.65 ± 11.77	0.497
ALT (u/l)	75.59 ± 64.92	65.96 ± 66.17	0.493
AST (u/l)	30.39 ± 28.59	27.79 ± 22.93	0.640
rGT (u/l)	43.11 ± 19.37	35.00 ± 22.25	0.071
TBil (umol/l)	15.65 ± 5.71	13.67 ± 6.11	0.118
LG10 (HBV-DNA)	4.81 ± 2.10	5.21 ± 2.28	0.405
TC (mmol/l)	4.43 ± 1.13	4.13 ± 0.69	0.219
TG (mmol/l)	1.75 ± 0.61	1.40 ± 0.40	0.002**
FBG (mmol/l)	5.45 ± 0.75	4.75 ± 0.70	0.000**
M30 (μ/l)	614.48 ± 471.43	374.50 ± 231.04	0.004**
M65 (μ/l)	369.41 ± 262.21	296.50 ± 231.44	0.172

**$p < 0.01$.

HBV-DNA Levels

In these CHB patients with NAFLD, the HBV-DNA content of > 10^3 cp/ml was considered as HBV DNA positive. According to their HBV-DNA level, they were dividend to HBV-DNA positive and negative group. Results showed the M30 level in HBV-DNA positive group was significantly higher than that in HBV-DNA negative group ($p < 0.05$), but the M65 levels have no statistical difference in the two groups ($p>0.05$) (Table II). Interestingly, we found same tendency in HBeAg positive patients and negative patients: the M30 level in HBeAg positive group was significantly higher than that in HBeAg positive group ($p < 0.05$), but the M65 levels have no statistical difference in the two groups ($p>0.05$) (Table II).

Correlation Analysis

Correlation analysis was conducted in CHB patients with NAFLD, M30 level was considered as independent variable, HBV-DNA level, ALT, AST, rGT, TBiL, TC, TG, FBG, inflammatory activity score (G), fibrosis score (S), and fatty grading (F) in liver tissues were considered as dependent variables. Results showed the M30 level was positively correlated with ALT, AST, and TG levels (Table III). Spearman rank correlation analysis indicated that the M30 level was positively correlated with the degree of liver inflammation activity score, fibrosis score and liver fatty degeneration (Table IV).

Discussions

China is a country with large number of chronic hepatitis B patients, the hepatitis B virus plagued more than 93,000,000 people[15]. With the changes of the living standards and dietary structure in recent years, the incidence of non-alcoholic fatty liver disease has gradually increased[16,17]. NAFLD and CHB are the two major causes of liver enzyme elevation; both of them cause the progression of chronic liver disease. Mi et al[18] performed a survey and they found the prevalence of fatty degeneration of liver in 1263 patients with chronic hepatitis B was 33.4%. Corey[19] indicated that NAFLD has become the most common cause of liver disease in the United States, and also become one of the comorbidities in liver disease. However, it has not been clearly demonstrated whether hepatic steatosis could affect patients with chronic hepatitis B; and there has big controversy on the relationship between hepatitis B virus and liver steatosis[20,21]. It is still not clear whether hepatic steatosis could aggravate the inflammation of the liver cells and promote the fibrosis.

Research has demonstrated that hepatitis C virus could promote the formation of fatty liver[22,23], while whether the hepatitis B virus may promote fatty liver has no clear conclusion; there was report indicated hepatitis B virus involved in fatty degeneration of liver cells through interfering protein SREBP-1c, a hepatocyte sterol regulatory element[20]. In this study, there was no statistical difference in age, gender and aspartate aminotransferase levels between hepatitis B patients with fatty liver and hepatitis B patients with non-fatty liver; however, CHB patients with NAFLD have significantly higher level of TG, FBP and M30 but not HBV-DNA level, these data suggest that the occurrence of fatty change in hepatitis B patients may related with metabolic factors such as glucose metabolism, TG and insulin resistance of these patients. However, there is no sufficient evidence in hepatitis B virus replication effect on fatty degeneration.

As a member of the intermediate filament protein family, a large number of CK proteins express in the liver cells[24,25], and the main function of CK protein is to maintain the normal structure of liver cells and protect them from mechanical and non-mechanical damage[26,27]. Damage to the integrity of the cell membrane occurred in the

Table II. Comparisons of serum M65 and M30 levels in CHB patients with NAFLD.

	M30 (u/l)	*p*-value	M65 (u/l)	*p*-value
HBV-DNA positive (n = 28)	752.36 ± 554.79	0.012*	394.75 ± 329.77	0.285
HBV-DNA negative (n = 18)	400.0 ± 171.64		308.33 ± 91.47	
HBeAg positive (n = 18)	875.67 ± 651.85	0.002**	457.67 ± 384.27	0.067
HBeAg negative (n = 28)	446.57 ± 185.67		312.68 ± 114.41	

*$p < 0.05$, **$p < 0.01$.

Table III. Correlation analysis of serum M30 level and biochemical parameters in CHB patients with NAFLD.

	M30	
	R-value	p-value
ALT (u/l)	0.507	0.000**
AST (u/l)	0.369	0.012*
rGT (u/l)	0.243	0.104
TBil (umol/l)	0.027	0.857
TC (mmo/l)	0.110	0.468
TG (mmol/l)	0.456	0.001**
FBG (mmol/l)	0.384	0.008**
LG10 (HBV-DNA)	0.423	0.005**

**$p < 0.01$.

early stage of liver injury, as a result, CK18 is released into the cell, then decomposed by caspase and exposed its specific Asp396 binding sites of the protein fragment; the released fragment is stable in the physiological concentration of the blood, the CK18-M30 antibody can identify the protein fragment. In the process of autophagy and necrosis, CK18 protein is not decomposed directly into the blood, this complete fragment of CK18 can be identified by CK18-M65 antibody. M30 can reflect the level of hepatocyte apoptosis, and M65 can reflect all the cell death caused by various reasons. The significantly incensement of the two fragments indicate the liver function failure[28].

Elevated M30 has been found in many chronic liver diseases such as viral hepatitis, drug-induced hepatitis, toxic hepatitis and liver damage of bile[29], especially in non-alcoholic fatty liver and chronic viral hepatitis. The level of CK18 fragment is related to the severity of the fatty hepatitis, CK 18 fragment could also be used as a noninvasive index to distinguish non-alcoholic steatohepatitis (NASH)[5,30]. In addition, M30 can be used as a predicting marker to distinguish non active carrier and HBeAg negative in chronic hepatitis B patients; and used to evaluate liver inflammatory activity for normal and mild abnormal liver function in the early stage of liver damage[31], it can predict liver damage before liver enzymes elevating. Without considering the triggering factor of liver damage, M30 can reflect the level of apoptosis, and the extent of liver cell inflammation damage in viral hepatitis and fatty hepatitis[32]. In this study, serum M30 level was significantly higher in CHB patients with NAFLD than non NAFLD, which indicated that the apoptosis and inflammation in CHB patients with fatty liver were more evident than those without NAFLD.

Table IV. Correlation analysis of serum M30 and pathological parameters in CHB patients with NAFLD.

		M30	
		R-value	p-value
G1/G2/G3/S4	5/35/6/0	0.551	0.000**
S1/S2/S3/S4	29/16/1/0	0.458	0.001**
F1/F2/F3	22/20/4	0.457	0.001**

**$p < 0.01$.

We found that M30 levels were significantly higher in patients with positive HBV-DNA and HBeAg, this indicate that the replication of hepatitis B virus may aggravate the apoptosis and damage of liver cells with the existence of fatty liver. We found that serum M30 levels in patients with hepatitis B and fatty liver were positively correlated with triglyceride, blood glucose and liver fatty grading, which was positively correlated with HBV-DNA loading, this further confirmed that M30 was not only related to lipid, blood glucose and liver fatty grading, but also related with active replication of hepatitis B virus. Although there is no definite conclusion about whether the hepatitis B virus is associated with the formation of fatty, but it can be concluded the synergistic effect of hepatitis B virus and fatty liver aggravate the liver cell inflammation.

In this study, we didn't find difference in M65 levels between the 2 groups, and we didn't find difference existed in M65 level whether HBV-DNA or HBeAg were positive. A former research[33] proposed M65 can be found in liver cells as a large number of apoptotic products, while cell necrosis cause non-specific cell death, which can be influenced by many factors, as a results, the accuracy and specificity of M65 are not high. At the same time, liver damage of the two groups may be only limited in apoptosis and inflammation level, not yet reached the serious level of cell damage and necrosis.

Conclusions

Currently, AST and ALT are the most commonly used clinical indicators for liver inflammation. The level of M30 in CHB patients with fatty liver is positively correlated with ALT and

AST, and it is positively correlated with the score of liver inflammation and fibrosis score, we believe that the M30 level could better reflect the degree of inflammation in CHB patients with fatty liver.

The deficiency of this study is lack of a larger sample, and further follow-up data are still needed to enrich the mechanism of hepatitis B virus combining fatty liver disease. The preliminary results of this study suggest that CHB patients with non-alcoholic fatty liver could increase the apoptosis and damage of their liver cell, the impact factors including glucose and lipid metabolism and hepatitis B virus replication levels. As a biological marker of liver cell apoptosis, M30 could be used for evaluating the degree of inflammation of liver cells in hepatitis B patients with fatty liver.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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