

Cell death biomarker M65 is a useful indicator of liver inflammation and fibrosis in chronic hepatitis B

A cross-sectional study of diagnostic accuracy

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Abstract

Cell death markers, M65 and M30, have been suggested to be sensitive markers of liver inflammation and fibrosis in nonalcoholic fatty liver disease and chronic hepatitis C. Our aim was to investigate whether these markers were useful in diagnosing liver inflammation and fibrosis in chronic hepatitis B (CHB).

We examined 186 patients with CHB; 18 sex- and age-matched healthy subjects were controls. The blood samples were collected from CHB patients within 1 week before or after liver biopsy. According to METAVIR score system, liver inflammation was graded from A0 to A3, and fibrosis from F0 to F4.

Serum M65 and M30 levels were in parallel with the grades of liver inflammation. M65, not M30, increased significantly in patients with severe inflammation and normal alanine aminotransferase. M65 is one of the independent predictors of severe liver inflammation ($\geq A2$). The levels of M65 and M30 levels significantly increased in parallel with the degree of inflammation in F1 patients, whereas they showed no statistical difference between different stages of fibrosis in A1 patients.

Serum M65 is a useful indicator of liver inflammation in CHB patients. Serum M65, not M30, is valuable in the grading of liver fibrosis.

Abbreviations: ALT = alanine aminotransferase, AST = aspartate aminotransferase, AUC = area under the ROC, CHB = chronic hepatitis B, CHC = chronic hepatitis C, CI = confidence interval, CK-18 = cytokeratin-18, ELISA = enzyme-linked immunosorbent assay, HBV = hepatitis B virus, HSC = hepatic stellate cells, IQR = interquartile range, NAFLD = nonalcoholic fatty liver disease, PT = prothrombin time, ROC = receiver-operating characteristic, TC = total cholesterol, TMB = tetramethylbenzidine.

Keywords: chronic hepatitis B, fibrosis, inflammation, M30, M65

1. Introduction

Chronic hepatitis B (CHB) is the most common liver disease in the Asia Pacific region. Significant liver inflammation and fibrosis are the courses toward cirrhosis. Blocking the process of inflamma-

tion and fibrosis prevents the development of cirrhosis. However, the currently available noninvasive methods could not accurately diagnose the degree of liver inflammation or fibrosis. As the most common marker of liver injury, alanine aminotransferase (ALT) is unable to reflect the grade of cell death and liver inflammation in some patients.^[1-4] Although Forns Index,^[5] Fibrotest,^[6] aspartate aminotransferase/platelet ratio index,^[7] FibroIndex,^[8] and other noninvasive modalities have been in use, more accurate noninvasive markers of liver fibrosis are still urgently needed. The accuracy of Fibroscan,^[9] the best diagnostic method until now, is interfered by body weight, liver inflammation, and steatosis, which decreases its value in these patients.

Cytokeratin-18 (CK-18), a major intermediate filament protein in the liver cells,^[10] is cleaved by the activated caspases during hepatocyte apoptosis. CK-18 cleavage generates a neoepitope, which can be detected by the monoclonal antibody M30 and allows the specific assessment of apoptosis.^[11] Another assay, the M65 enzyme-linked immunosorbent assay (ELISA), can detect both cleaved and uncleaved CK-18, which is used as a marker of cell death in both necrosis and apoptosis.^[12] In nonalcoholic fatty liver disease (NAFLD)^[13,14] and chronic hepatitis C (CHC),^[15] both M65 and M30 were found to be correlated with the severity of liver inflammation and fibrosis. But few studies have been carried out in CHB and some results are inconsistent. The aim of this study was to evaluate the diagnostic significance of serum M65 and M30 in liver inflammation and fibrosis in CHB patients.

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Table 1**The clinical characteristics of the normal control and chronic hepatitis B.**

Groups	Control group (n=18)	A1F1 (n=105)	≥A2F2 (n=81)	P [*]
Male (n%)	13 (61.9%)	59 (55.6%)	62 (75.6%)	.005
Age, y	35.0 (20.4–52.6)	32.5 (27.8–40.2)	37.5 (32.0–47.0)	.001
ALT, U/L	18.0 (13.0–43.5)	34.3 (21.4–44.0)	31.7 (22.1–47.9)	.932
AST, U/L	22.0 (18.0–24.0)	26.7 (21.5–34.1)	29.0 (24.0–44.7)	.019
Total bilirubin, μmol/L	11.4 (4.3–18.8)	11.7 (8.8–15.9)	12.1 (9.3–16.8)	.395
ALB, g/L	45.3 (40.1–48.7)	46.5 (43.9–47.9)	46.1 (44.4–48.4)	.758
Platelet, ×10 ⁹ cells/L	230.0 (212.5–261.0)	200.0 (165.8–197.8)	174.5 (128.7–211.3)	.006
Cholinesterase, U/L	—	8825 (7286–10700)	8066 (6718–9290)	.008
TC, mmol/L	4.8 (3.7–5.4)	4.4 (3.9–5.0)	3.9 (3.7–4.8)	.181
PT, s	—	12.2 (11.5–12.6)	12.1 (11.7–13.0)	.205
HBV DNA (×10 ⁴ IU/mL)	—	1.1 (0.8–1.1)	0.05 (0.4–1.6)	.000
M30, U/L	208.8 (190.5–241.3)	238.5 (171.5–345.0)	260.5 (184.4–468.3)	.064
M65, U/L	144.5 (112.0–241.0)	189.4 (96.4–315.0)	248.5 (147.3–554.5)	.003

ALB=albumin, ALT=alanine aminotransferase, AST=aspartate aminotransferase, PT=prothrombin time, TC=total cholesterol.

^{*} P shows the difference between A1F1 and ≥A2F2 groups.

2. Patients and test methods

2.1. Participants

The protocol was approved by the ethics committee of Beijing Youan Hospital, Capital Medical University and was conformed to the guidelines of Helsinki Declaration. All patients provided a written informed consent. We recruited a consecutive series of 186 CHB patients with biopsy. Blood samples were collected within 1 week before or after liver biopsy. None of the included patients was treated with any antiviral drugs. Patients with steatosis and hepatitis C virus, hepatitis D virus, and human immunodeficiency virus coinfections were excluded from the study. Age and sex-matched 18 healthy individuals were served as controls.

2.2. Histological examination

Liver biopsies were performed with an 18-gauge Bard Magnum needle (length: 10–25 mm). Biopsy specimens were fixed with 10% formalin, paraffin-embedded, sectioned, and stained with hematoxylin-eosin, Masson trichrome, and reticulin stain. The specimens were examined by 2 experienced hepatopathologists who were blinded to each other and also blinded to the patients' biochemical results. The degree of liver inflammation and fibrosis was assessed according to METAVIR score system.^[16] Histologic activity was graded from A0 to A3 according to the presence of piecemeal necrosis and focal lobular necrosis. The fibrosis was staged from F0 to F4 according to the severity in the hepatic lobules and septae. Accordingly, A2-A3 were noted as severe inflammation and F2-F4 were noted as advanced fibrosis. Clinically, patients with severe inflammation or advanced fibrosis are candidates for antiviral therapy.^[17]

2.3. Serum M65/M30 assays

The blood samples were centrifuged at 2000 rpm for 10 minutes. The serum was collected and stored at –80°C. The serum levels of M65 and M30 were quantitatively measured using the M65 EpiDeath ELISA and the M30 Apoptosense ELISA kits (Peviva, Bromma, Sweden) according to the manufacturer's protocol. All reagents were allowed to reach room temperature before use. All the samples were tested in duplicates. Twenty-five microliter standards and serum samples were loaded first, then 75 μL of M65 EpiDeath Conjugate or M30 Horseradish peroxidase conjugate was added. The wells were sealed and incubated for

4 hours on a plate shaker (600 rpm). After washing for 5 times, 200 μL of tetramethylbenzidine (TMB) substrate was added. The wells were sealed again and incubated in darkness for approximately 20 minutes to develop the color. Finally, 50 μL of the stop solution was added. The plate was shaken for 5 to 10 seconds and incubated for 5 minutes to ensure complete mixing of the TMB substrate and the stop solution. The plate was read at 450 nm within 30 minutes. The serum levels were calculated according to the standard cubic spline.

2.4. Statistical methods

Statistical analysis was performed using SPSS 16.0 (SPSS Inc, Chicago, IL). All results were expressed as median (interquartile range, IQR). The comparison among different groups was analyzed by Kruskal-Wallis or Mann-Whitney test. Spearman correlations were applied. Logistic regression was performed with backward method for multivariable analysis (the entry probability for stepwise: $P < .05$, removal: $P > .10$). The diagnostic performance of an assay was assessed by the analysis of receiver-operating characteristic (ROC) curve. The assessment of sensitivity and specificity was determined using selected cutoff value. A $P < .05$ was considered statistically significant.

3. Results

3.1. Participants

We enrolled 186 CHB patients from February 2012 to June 2013. Among them, 119 were males and the median age was 36.5 years. One hundred and five were histologically classified as A1F1. The clinical characteristics of the control and CHB patients are shown in Table 1. The pathology results are shown in Table 2. As only 3 patients were classified as F4, we combined F4 and F3 and expressed as cF3.

3.2. Serum M65 and M30 in CHB and control group

The median serum levels of M65 and M30 in CHB patients were 210.9 U/L (112.2–375.9 U/L) and 249.9 U/L (173.7–414.6 U/L), respectively, which showed no significant difference in comparison with the controls (M65: median 144.5 U/L, IQR 112.0–241.0 U/L, $P = .109$; M30: median 208.8 U/L, IQR 190.5–241.3 U/L, $P = .129$).

Table 2**Pathology of the 186 chronic hepatitis B patients included in the study.**

Pathological diagnosis	A1 (n)	A2 (n)	A3 (n)	Total (n)
F1 (n)	105	7	1	113
F2 (n)	37	10	7	54
F3 (n)	6	6	4	16
F4 (n)	1	1	1	3
Total (n)	149	24	13	186

3.3. Correlation of serum M65 and M30 with laboratory results

We found a weak but statistically significant and positive correlation between serum M65 and M30 ($r=0.45$, $P<.001$), ALT ($r=0.24$, $P=.001$), and aspartate aminotransferase (AST) ($r=0.32$, $P<.001$), respectively. M30 was only weakly related with AST ($r=0.19$, $P=.006$). There was no relationship between hepatitis B virus (HBV) DNA level and M30 ($r=-0.054$, $P=.443$) or M65 ($r=0.006$, $P=.933$).

3.4. Correlation of serum M65 and M30 with liver inflammation

The median serum levels of M65 in A1, A2, and A3 groups were 195.3 U/L (104.7–345.3 U/L), 250.3 U/L (158.9–559.5 U/L), and 541.2 U/L (171.2–1166.0 U/L), respectively, whereas M30 levels were 236.5 U/L (171.4–366.0 U/L), 282.9 U/L (186.8–446.7 U/L), and 441.8 U/L (262.1–693.3 U/L), respectively. The differences of serum M65 and M30 in different groups were shown in Figure 1A and B.

The levels of M65 and M30 differed between A1 and $\geq A2$ group (A1 group: M65 median 195.3 U/L, IQR 104.7–345.3 U/L; M30 median 236.5 U/L, IQR 171.4–366.0 U/L; $\geq A2$ group: M65 median 251.7 U/L, IQR 171.2–672.3 U/L; M30 median 341.9 U/L, IQR

196.1–514.0 U/L; M65: $P=.002$, M30: $P=.007$; Fig. 1C and D). When analyzing the value of M65 and M30 in predicting the patients with $\geq A2$, the area under the ROC (AUC) was 0.656 (95% confidence interval [CI]: 0.560–0.752, $P=.003$) and 0.636 (95% CI: 0.541–0.731, $P=.011$), respectively.

Among the 124 patients with normal ALT levels (<40 U/L), 99 (79.8%) had mild histologic activity of A1, 25 (20.2%) had severe inflammation ($\geq A2$); the serum levels of M65 were higher in patients with severe inflammation (median 248.5 U/L, IQR 139.1–396.4 U/L) compared with those with mild activity (A1, median 187.0 U/L, IQR 100.0–309.1 U/L, $P=.022$). There was no difference of M30 levels between A1 and $\geq A2$ group (median 226.6 U/L, IQR 158.2–355.3 U/L vs. median 282.9 U/L, IQR 175.5–438.3 U/L, $P=.122$).

Univariate analysis showed that AST, total bilirubin, HBV DNA, platelet, and M65 were associated with severe liver inflammation. Multivariate analysis further showed that M65 and HBV DNA independently predicted severe liver inflammation. We built a noninvasive model with logistic regression to predict severe inflammation. $Y=0.001 \times \text{M65 (U/L)} - 0.261 \times \lg \text{HBV DNA} - 0.732$. The AUC was 0.683 (95% CI: 0.583–0.775, $P=.001$) with a sensitivity of 59.5% and a specificity of 73.2% at the cut-off of -1.2 (Fig. 2).

3.5. Correlation of serum M65 and M30 with liver fibrosis

The serum levels of M65 in F1, F2, and cF3 groups were 199.6 U/L (99.4–354.0 U/L), 246.7 U/L (139.5–418.3 U/L), and 226.5 U/L (142.8–674.3 U/L), respectively. The serum M65 in F2 and cF3 groups were higher than that in F1 ($P=.042$, $P=.012$, respectively) and the control groups ($P=.017$, $P=.012$, respectively; Fig. 3A). The serum median levels of M30 in the above groups were 249.9 U/L (173.1–366.0 U/L), 238.4 U/L (171.9–468.3 U/L), and 326.5 U/L (183.2–451.2 U/L), respectively. And there were no significant differences among the 3 groups. And when compared with the control group, there were also no differences (Fig. 3B).

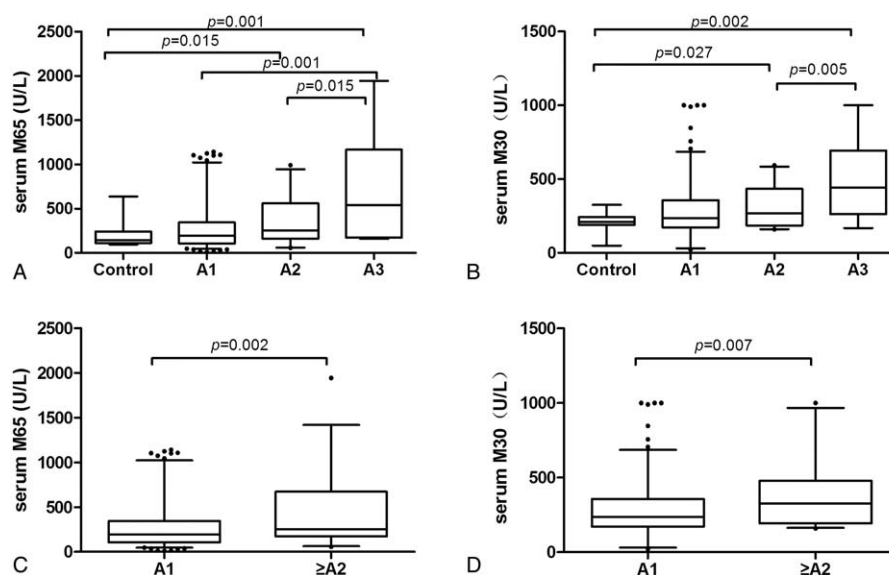


Figure 1. The serum levels of M65 and M30 in different grades of liver inflammation. (A) Serum M65 in A3 group was much higher than that in control group, A1 group and A2 groups. (B) Serum M30 was much higher in A3 group than that in the control and A2 group. Compared with A1 group, serum M65 (C) and M30 (D) were higher in $\geq A2$ group. A1 = patients with liver inflammation of grade 1, A2 = patients with liver inflammation of grade 2, A3 = patients with liver inflammation of grade 3. The top and bottom of each box are the 25th and 75th percentiles. The line through the box is the median and the error bars are the 5th and 95th percentiles.

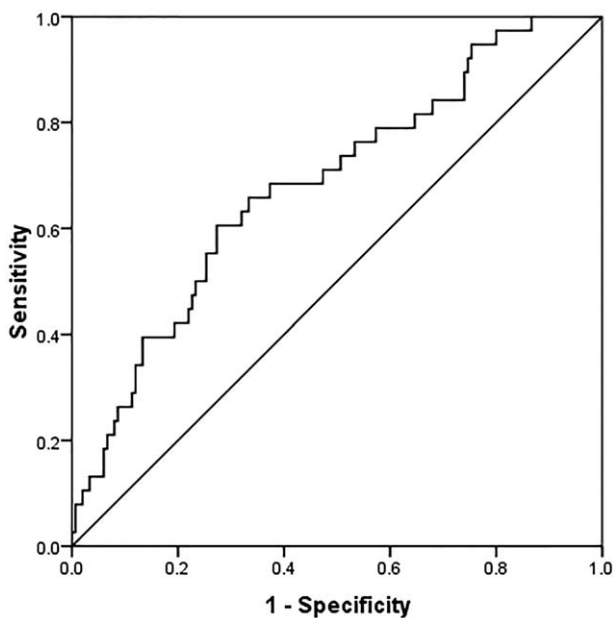


Figure 2. Model of prediction of significant liver inflammation in chronic hepatitis B. The area under the receiver-operating characteristic curve showed the performance of M65 and HBV DNA for predicting severe liver inflammation ($A \geq 2$). $Y = 0.001 \times \text{M65 (U/L)} - 0.261 \times \lg \text{HBV DNA} - 0.732$, area under receiver-operating characteristic curve = 0.683. The cut-off point at -1.2 predicts $A \geq 2$ with a sensitivity of 59.5%, and a specificity of 73.2%, respectively.

The level of serum M65 was significantly higher in advanced fibrosis stages ($\geq F2$, median 245.1 U/L, IQR 141.3–425.3 U/L) than that in the F1 group (median 199.6 U/L, IQR 99.4–354.0 U/L, $P = .006$, Fig. 3C), the M30 had no significant difference between patients with advanced fibrosis ($\geq F2$) and with F1 (F1: median 249.9 U/L, IQR 173.1–366.0 U/L; $\geq F2$: median 245.8 U/L,

IQR 177.3–456.2 U/L, $P = .064$; Fig. 3D). The AUC of serum M65 in identifying patients with $\geq F2$ was 0.592, (95% CI: 0.509–0.674, $P = .033$).

Among 124 patients with normal ALT levels, 72 (58.1%) staged F1, 52 (41.9%) staged $\geq F2$. The serum levels of M65 in F1 and $\geq F2$ groups were 180.7 U/L (98.3–335.5 U/L) and 236.4 U/L (140.6–343.6 U/L), respectively ($P = .082$), whereas the M30 levels were 251.2 U/L (163.8–404.6 U/L) and 209.0 U/L (173.7–424.9 U/L), respectively ($P = .695$). Neither of the difference reached significant level.

3.6. Serum M65 and M30 correlated with inflammation more than with fibrosis

We divided the patients with mild inflammatory damage (A1, $n = 149$) into 2 groups: A1F1 ($n = 105$) and A1F ≥ 2 ($n = 44$). There were no statistical differences of serum M65 and M30 between the 2 groups (Fig. 4A and B). Similarly, we compared the levels of M65 and M30 in A1F1 ($n = 105$) and A ≥ 2 F1 ($n = 8$) groups. Serum M65 and M30 were higher in A ≥ 2 F1 compared with A1F1 (M65: median 564.3 U/L, IQR 266.1–944.3 U/L vs. median 189.4 U/L, IQR 96.4–315.0 U/L, $P = .011$, Fig. 4C; M30: median 430.6 U/L, IQR 277.4–532.6 U/L vs. median 238.5 U/L, IQR 171.5–345.0 U/L, $P = .015$, Fig. 4D). This indicated that serum M65 and M30 may be more closely correlated with liver inflammation than with fibrosis in CHB patients.

4. Discussion

Our study showed that both serum M65 and M30 were useful indicators in the detection of liver inflammation. M65 was superior to M30 in identifying liver inflammation especially in patients with normal ALT. Furthermore, M65 was valuable in the grading of liver fibrosis and M30 had no role in detecting the progress of liver fibrosis.

There are many forms of hepatocyte death, including necrosis, apoptosis, necroptosis, autophagy, and so on. Uncleaved CK-18

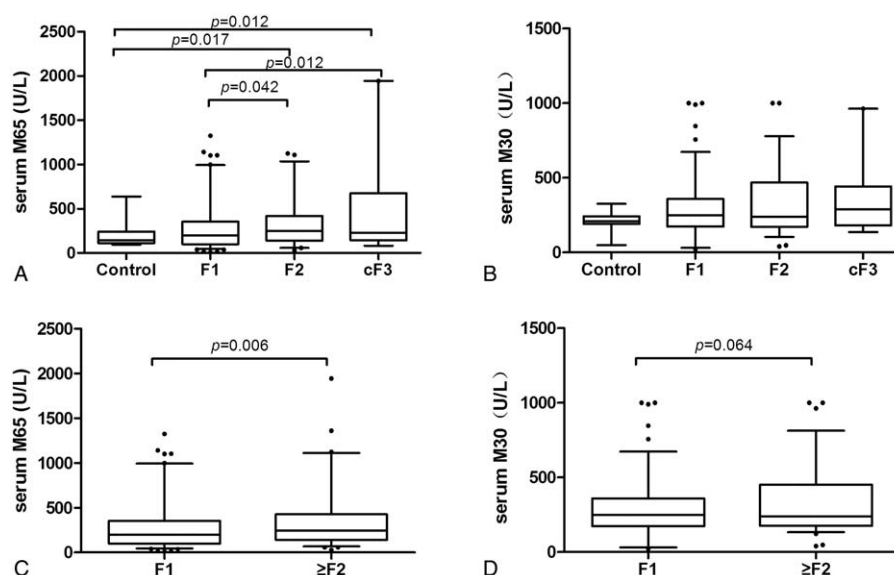


Figure 3. The serum levels of M65 and M30 in different stages of liver fibrosis. (A) Serum M65 in F2 and cF3 groups was higher than that in control and F1 group. (B and D) There was no difference of serum M30 levels between different fibrosis stages. (C) Serum M65 was higher in $\geq F2$ group than that in F1 group. F1 = patients with liver fibrosis stage 1; F2 = patients with liver fibrosis stage 2; cF3 (combined F3) = patients with liver fibrosis stage 3 and 4. The top and bottom of each box are the 25th and 75th percentiles. The line through the box is the median and the error bars are the 5th and 95th percentiles.

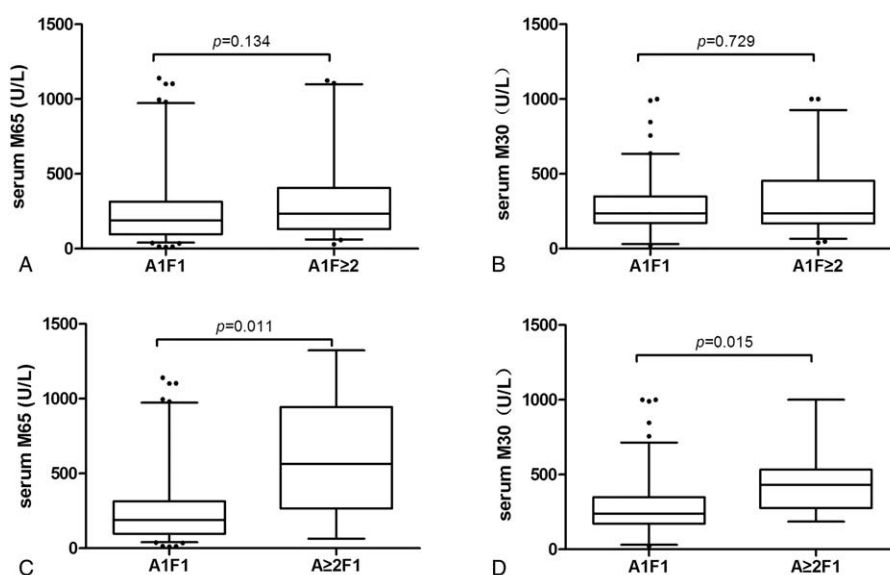


Figure 4. The levels of serum M65 and M30 in A1 or F1. (A and B) No difference of serum M65 and M30 was found between A1F1 and A1F $\geq$ 1 group. (C and D) Serum M65 and M30 were higher in A $\geq$ 2F1. A1F1 = patients with liver inflammation of grade 1 and liver fibrosis of stage1, A $\geq$ 2F1 = patients with liver inflammation of grade 2 and liver fibrosis of stage1, A1F $\geq$ 2 = patients with liver inflammation of grade 1 and liver fibrosis of stage 2. The top and bottom of each box are the 25th and 75th percentiles. The line through the box is the median and the error bars are the 5th and 95th percentiles.

(M65) was assumed to reflect all types of cell death. Apoptosis is definitely an important style of cell death in most liver injuries.^[18] One study discovered that the caspase activity was improved in hepatitis along with the elevated serum cleaved CK-18 (M30).^[19] Another study reported that M65 and M30 were helpful in detecting liver inflammation in viral hepatitis.^[20] Kronenberger et al^[15] and Abdel Haleem et al^[21] found that serum CK-18 neoepitope levels in CHC were strongly correlated with ALT and the histology activity index. The level of CK-18 neoepitope was significantly higher in CHC patients with elevated ALT levels than in those with normal ALT. However, no cutoff line was set up for diagnosis. The other researches with smaller case numbers failed to discover any correlation between serum M65 or M30 and liver inflammation or fibrosis.^[22–24] Papatheodoridis et al^[25] found that in CHB patients, serum CK-18 fragments correlated positively with ALT/AST and grading score. The ROC curve showed high diagnostic accuracy (c-statistic: 0.87) in differentiating inactive carriers from CHB patients. Bae et al^[26] measured serum M30 in 339 cases of CHB with liver biopsy and drew the same conclusion as Papatheodoridis et al. Multivariate analysis showed that AST, albumin, and M30-antigen were the independent predictors of inflammation. The results were confirmed by our present study that the M65 and M30 increased in parallel with the grading score of liver inflammation and also were positively correlated with ALT and AST. Besides, M65 was a risk factor of severe inflammation; the AUC was 0.683 when used together with HBV DNA.

Our data were not totally consistent with the studies of Papatheodoridis et al^[25] and Bea et al.^[26] They demonstrated that M65 could differentiate healthy controls, HBV carriers, and CHB patients. But similar differences were not shown in our study. It may be because of the smaller sample size in our control group. Another phenomenon was that neither M65 nor M30 showed significant difference in control group and A1/F1 group, which indicated that they were not sensitive enough to detect mild lesions.

Apoptosis not only reduces the number of hepatocytes, but also enhances liver fibrosis. After phagocytosis of apoptotic bodies, hepatic stellate cells (HSCs) could be activated to myofibroblasts, which initiate fibrosis.^[27] In addition, apoptotic bodies also activate HSCs indirectly by activating the Kupffer cells.^[28] An animal experiment showed that inhibition of apoptosis of hepatocytes alleviates the progress of fibrosis.^[29] Considering that apoptosis of hepatocytes promotes fibrosis, researchers tried to predict liver fibrosis by apoptotic markers. In NAFLD, Younossi et al^[30] found that M65 and M30 effectively identify patients and healthy people, mild to moderate, as well as advanced fibrosis. Another study^[31] in fatty liver had similar results. In addition, M65 and M30 were also found to be related with fibrosis in CHC^[32] and primary biliary cirrhosis^[33] But other studies failed to prove these notions. Sumer et al^[34] found that M30 was increased in CHB patients compared with controls; the M30 levels were correlated with the fibrotic stages. But Rosso et al^[22] showed that the combination of M30 and fibroscan had no advantage over the diagnostic accuracy of fibroscan alone in patients with CHB. Papatheodoridis et al^[25] and Bea et al^[26] also failed to discover the relationship between M65 and fibrosis. Our data showed that M65, but not M30, increased in parallel with fibrosis grades. Besides, in the CHB group with normal ALT, M65, but not M30, showed significant differences between A1 group and the $\geq$ A2 group. Our results were similar to those of Younossi et al's study^[30] in which M65 was superior to M30 for the diagnosis of fibrosis. It is known that dead hepatocytes provide key fibrosis stimuli. In addition, the dying (but viable) cells may also release factors to promote fibrosis. So it is easy to understand why M65 was more accurate than M30 in the prediction of inflammation and fibrosis. We also found that M65 and M30 levels were correlated with inflammation in patients with the same stage of fibrosis, but not correlated with fibrosis in patients with the same grade of inflammation. We speculated that M65 was a direct indicator of inflammation and an indirect indicator of fibrosis.

There were some limitations in our research. The case numbers in control group and advance fibrotic group were relatively small and this would influence the statistic results. According to our results, we could not suggest that M65 and M30 could be used as diagnostic markers alone in clinical practice. But our results indicated that M65 were helpful in diagnosis of some stages of liver inflammation and fibrosis, and should be studied further.

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