The expression of thymosin β4 in chronic hepatitis B combined nonalcoholic fatty liver disease

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Abstract
The aim of the study was to detect the expression level of thymosin β4 (Tβ4) in serum and tissues of patients with chronic hepatitis B (CHB) combined nonalcoholic fatty liver disease (NAFLD). The effects of Tβ4 in hepatic steatosis, chronic inflammation, and fibrosis development in CHB combined NAFLD patients were also discussed. The study included 46 patients in the case group with CHB and NAFLD and 42 patients in the control group with CHB. ELISA was applied to detect serum Tβ4 and TNF-α level. Furthermore, the correlation analysis of Tβ4 levels with biochemical index, pathological index, and TNF-α level was performed. The Tβ4 immunohistochemical levels of different inflammatory fibrosis levels were compared, and the correlation analysis with TNF expression was performed. The Tβ4 levels in patients with CHB combined NAFLD showed no statistical difference when compared to the control group. In patients with CHB combined NAFLD group, the Tβ4 level had no correlation with ALT, AST, TG, FGP, hepatitis B virus (HBV)-DNA levels, and fat grading, but had negative correlation with inflammation score and fibrosis score (P <0.01). The immunohistochemical results of hepatic tissues showed that the expression intensity of severe inflammation fibrosis group had statistical significance compared with that of slight group, and the Tβ4 expression both in serum and in liver tissue negatively correlated with TNF-α expression. Tβ4 could be involved in the regulation of chronic inflammation and fibrosis and plays a defense role in the disease progression of CHB combined NAFLD patients.

Abbreviations: ALT = alanine aminotransferase, AST = aspartic transaminase, CHB = chronic hepatitis B, ECM = extracellular matrix, FBG = fasting blood-glucose, HE = hematoxylin-eosin staining, HSCs = hepatic stellate cells, IL8 = interleukin-8, IOD = integral optical density, NAFLD = nonalcoholic fatty liver disease, NF-κB = nuclear factor-kappa B, PPAR = peroxisome proliferator-activated receptor, rGT = r-glutamine transpeptidase, RT-PCR = real-time polymerase chain reaction, SREBP1 = sterol-regulatory element binding proteins 1, Tβ4 = thymosin β4, TBL = total bilirubin, TC = total cholesterol, TG = triglyceride, TGF-β = Transforming growth factor-β, TNF-α = tumor necrosis factor-α.

Keywords: chronic hepatitis B, fibrosis, inflammation, nonalcoholic fatty liver disease, thymosin β4

1. Introduction
Liver is the target organ that can be easily damaged by various pathogenic factors which include infection by viruses, alcohol intake, drugs, toxins, immune factors, and so on. This leads to necrosis and apoptosis of liver cells, and may at times develop into chronic damage and fibrosis.[1] Chronic Hepatitis B (CHB) and nonalcoholic fatty liver disease (NAFLD) are the most common reasons for chronic liver damage, and can also deteriorate into chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Currently, there is an increase in the number of cases with the coexistence of both the diseases in the same individuals.[2] It has been found that nearly a quarter (38%) of CHB patients could be found along with steatosis in the hepatic cells.[3] With the increased obesity and type 2 diabetes disease conditions all over the world, the CHB patients combined with fatty liver diseases also increased gradually.[4,5] Therefore, it is meaningful to explore the mechanism of NAFLD occurrence in hepatitis B patients, as well as the factors involving in the development and evolution of the disease.

Thymosin beta 4 (Tβ4) is a peptide generated by thymus and is composed of 43 amino acids with a molecular weight of 4964 D. It possesses various biological functions[6] and is involved in many cellular reactions such as shaping of blood vessel, promoting cell migration, accelerating collagen deposition, promoting wound healing, inhibiting fibrosis, and so on.[7–9] Recently, Tβ4 over-expression was found in the occurrence of
tumor. Thus, under normal physiological reaction and pathological status, it plays a role in regulating interactions among many cytokines. The current research found that the serum Tβ4 levels were significant lower in patients with chronic hepatitis B and cirrhosis, especially significantly decreased in acute-on-chronic liver failure and chronic liver failure. So it is postulated that Tβ4 protects the liver from injury and fibrosis development. Recently, Lakshman et al. studied the protective actions of Tβ4 on liver damage caused by alcohol and carbon tetrachloride and found out that Tβ4 could effectively protect against alcoholic hepatosteatosis, inflammation, and fibrogenesis. The steatosis, inflammation, and fibrosis are also the pathological manifestations of CHB combined with NAFLD, so these data have attracted more consideration of whether Tβ4 play an important role in the pathogenesis of CHB combined with NAFLD. However, there is still no report on the patients with CHB combined with NAFLD, and explore the effects of Tβ4 in hepatocyte steatosis, chronic inflammation, and fibrosis development.

2. Materials and methods

2.1. Ethical approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of Tianjin Third Central Hospital and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All the patients had signed the informed consent form before entering into the study and liver biopsy.

2.2. Study population

The study enrolled 137 CHB cases who had liver biopsy examinations in Tianjin Third Central Hospital, China, between September 2013 to April 2015. Those who were positive for hepatitis B surface antigen (HBsAg) for at least 6 months and diagnosed with steatosis by abdomen B-ultrasound and liver biopsy histopathology were enrolled in this study. According to this criteria, 46 cases were included in the CHB combined with NAFLD group (35 were male and 11 were female). Exclusion criteria included: (1) alcoholic fatty liver disease (in the past 5 years, men’s alcohol intake was more than 40 g/d and women’s was more than 20 g/d); (2) coinfection of hepatitis A, hepatitis C, hepatitis D, and hepatitis E simultaneously; (3) autoimmune hepatitis or primary biliary cholangitis; (4) drug-induced hepatitis or toxic hepatitis; (5) genetic and metabolic liver diseases, such as Wilson’s disease, hemochromatosis, antiritpyrin deficiency, and so on; (6) liver or biliary tract malignant tumor; (7) immune suppressive treatment within 1 year; (8) application of interferon or antifibrosis drugs within 6 months; (9) patients older than 70 years old. The control group included 42 patients with CHB without steatosis, among which 31 were male and 11 were female cases.

2.3. Study methods

2.3.1. Lab data detection of serum sample. Fasting venous blood (3 mL) was taken 1 day before liver biopsy. Serum was separated and stored under −80°C. The concentrations of serum Tβ4 and tumor necrosis factor-α (TNF-α) was detected by Enzyme-Linked Immunosorbent Assay (ELISA) Kit from AMS Biotechnology (Europe) Limited, UK, and from ebioscience (San Diego, CA), respectively. The automatic biochemistry analyzer was applied to detect the index for alanine aminotransferase (ALT), aspartic transaminase (AST), r-glutamine transpeptidases (rGT), total bilirubin (Tbil), triglyceride (TG), total cholesterol (TC), and fasting blood-glucose (FBG). Serum hepatitis B virus (HBV)-DNA was tested by real-time polymerase chain reaction (RT-PCR).

2.3.2. Liver histology. In the experiment group, CHB patients combined with NAFLD were first diagnosed as fatty liver or steatosis by B-ultrasound. Furthermore, liver tissue was obtained by rapid biopsy using 16 g biopsy needle (BARD) with length ≥1.5 cm and portal area ≥7. The samples were fixed by 10% paraformaldehyde solution, made into conventional paraffin slices and then stained by hematoxylin-eosin staining (HE). The samples were classified as G (Inflammation score) 1–4 and S (Fibrosis score) 0–4 according to the Scheuer system of liver inflammation activity grading standard by 2 pathologists. Liver steatosis was scored based on Kleiner’s et al. NASH histological scoring system: F1 (0–5%) and F2 (6–15%), and F3 (16–66%)..

2.3.3. Immunohistochemical analysis. In the experimental group, Tβ4 immunohistochemical detection (Abcam biotechnology, USA) was performed on the hepatic tissues. In order to understand the relationship between Tb4 expression and other inflammatory factors, we further performed the immunohistochemical detection of TNF-α (Genetex biotechnology, USA). The process is as follows: the paraffin slices were dewaxed, hydrated, and then fixed by EDTA routinely, added 3% H2O2 solution, and blocked by goat serum. Then, the specific primary antibody (1:150) was added and incubated overnight at 4°C. Secondary antibody was added the following day. Afterwards, the slices were developed by DAB, retained by hematoxylin, differentiated by hydrochloric acid alcohol, followed by lithium carbonate back to blue, dehydrated by gradient alcohol, transparentized by dimethylbenzene and neutral gum sealing piece. Analysis of the immunohistochemical staining of Tβ4 and TNF-α was carried out by 2 experienced pathologists blinded to the clinical conditions using a light microscope (BX50 Olympus, Tokyo, Japan). For each slide, acquired images from 5 views (×400) were selected for analysis. The integral optical density (IOD), of the positive-staining density was measured by the Image-Pro Plus v 6.0 image analysis software (Media Cybernetics, USA).

2.3.4. Statistical analysis. Statistical software SPSS 22 (SPSS Inc. Chicago, IL) was used to analyze the data. Data was expressed as mean ± SD, and HBV-DNA was calculated by denary logarithm. Comparison of data measured between groups was analyzed by the t test. Comparison of data variables between groups was analyzed by the chi-square test. The correlation was analyzed by the Pearson method. P<0.05 was considered as statistical significance for all the analysis.

3. Results

3.1. Tβ4 detection of serum samples

3.1.1. Comparison of general information. There was no statistical significance in the baseline and clinical characteristics of age, gender, E antigen positive ratio, ALT, AST, rGT, Tbil, total cholesterol, and HBV-DNA level between the experiment group (CHB combined NAFLD group, n = 46) and the control group (simple CHB group, n = 42). The TG and FBG showed statistical significance between the 2 groups (P<0.05) (Table 1).
Correlation between serum Tj4 and pathological index in CHB combined with NAFLD group.

| Tj4 | R  | P  |
|-----|----|----|
| G1/G2/G3/G4 | 5/05/6/0 | −0.371 | 0.007
| S1/S2/S3/S4 | 29/16/1/0 | −0.308 | 0.012
| F1/F2/F3 | 22/20/4 | −0.061 | 0.612

ALT = chronic hepatitis B, NAFLD = nonalcoholic fatty liver disease, Tj4 = thymosin β4.

In order to investigate the relationship between Tj4 expression and proinflammatory factor TNF-α, we also detected serum TNF-α level and performed TNF-α immunohistochemical experiment in CHB combined with the NAFLD group. The results showed that the level of serum Tj4 was negatively correlated with TNF (r = −0.458, P = 0.000) (Table 3). TNF-α was expressed in all the CHB combined NAFLD tissues (Fig. 4). Besides, the correlation analysis of their expression intensity in liver tissues also demonstrated negative correlation (r = −0.460, P = 0.001) (Fig. 5).

4. Discussion

The infection of hepatitis B virus and NAFLD are the most common reasons for chronic liver disease. However, the occurrence mechanism of fatty liver in CHB patients is still controversial and is not similar with chronic hepatitis C virus and fatty liver, that is, hepatitis C virus can promote fat deposition in liver and induce fatty liver. Furthermore, whether hepatitis B virus can directly lead to fatty liver or whether NAFLD is caused by other metabolic factors in the host is still not clear. Some scholars thought that hepatitis B virus X protein could promote steatosis by up-regulating the transcriptional activity of sterol-regulatory element binding proteins 1 (SREBP1) and peroxisome proliferator-activated receptor (PPAR), but most other studies reported that lifestyle and metabolic disorders were the main pathogenesis of NAFLD. In comparison, it was found that there were no significant differences in the transaminase level, hepatitis B e antigen ratio, and HBV-DNA loading between the 2 groups and this suggests that hepatitis B virus did not cause fatty liver directly. There were differences in TG and blood glucose in the combined fatty liver group which indicates that the metabolic disorders of blood sugar and fat were the main causative factors.

The formation of steatosis in the CHB patients was caused by metabolic disorder in the host cell, but previous studies showed that insulin resistance and NAFLD occurrence and development, oxidative stress was considered as the central link in the fatty acid metabolism. The redundant free fatty acid in the hepatic cells would generate reactive oxygen species (ROS) during lipid peroxidation. The ROS promoted generation of TNF-α and pathological index in CHB combined with NAFLD group.

TNF-α expression intensity in the G+S≥4 group was higher than in the G+S<4 group (P < 0.01) (Table 4, Fig. 2).

3.2. Correlation between Tj4 and TNF-α expression

### Table 1
Comparison of biological index and serum Tj4 and TNF-α expression between 2 groups.

| Biological index | CHB combined with NAFLD | CHB with non-NAFLD | P  |
|------------------|-------------------------|-------------------|----|
| Male/female      | 35/11                   | 31/11             | 0.758 |
| Age              | 39.28 ±10.78            | 37.65 ±11.77      | 0.497 |
| Positive HBsAg/n | 18/46                   | 22/42             | 0.212 |
| ALT (μ/L)        | 75.59 ±46.92            | 58.57 ±49.18      | 0.172 |
| AST (μ/L)        | 30.39 ±28.59            | 27.79 ±22.93      | 0.640 |
| rGT (μ/L)        | 43.11 ±19.37            | 35.00 ±22.25      | 0.071 |
| TBI (μmol/L)     | 15.65 ±7.11             | 13.67 ±6.11       | 0.118 |
| TG (mmol/L)      | 4.81 ±2.10              | 5.21 ±2.28        | 0.405 |
| TC (mmol/L)      | 4.43 ±1.13              | 4.13 ±0.69        | 0.219 |
| FBP (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 |
| Tj4 (μg/ml)      | 0.80 ±0.32              | 0.68 ±0.30        | 0.062 |
| TNF-α (pg/ml)    | 54.87 ±32.38            | 51.68 ±20.60      | 0.550 |

### Table 2
Correlation between Tj4 and the biochemical index in CHB combined with the NAFLD group.

| Tj4 | R  | P  |
|-----|----|----|
| ALT (μ/L) | −0.127 | 0.402 |
| AST (μ/L) | 0.160 | 0.287 |
| rGT (μ/L) | 0.480 | 0.750 |
| TBI (μmol/L) | −0.124 | 0.412 |
| TC (mmol/L) | 0.107 | 0.479 |
| TG (mmol/L) | 0.620 | 0.685 |
| FBP (mmol/L) | 0.550 | 0.819 |
| LG10 (HBV-DNA) | 0.100 | 0.945 |

ALT = aspartate aminotransferase, AST = aspartic transaminase, CHB = chronic hepatitis B, LG10 = log 10, NAFLD = nonalcoholic fatty liver disease, rGT = γ-glutamyl transpeptidases, Tj4 = thymosin β4, TBI = total bilirubin, TC = total cholesterol, TG = triglyceride, TNF-α = tumor necrosis factor-α.
seek a more specific condition. Some studies reported that Tβ4 level probably develops in those with normal ALT. Therefore, the ALT level could not be used to confirm the reason of inflammation, and also could not reflect the conditions of real inflammation in the hepatic cells. Thus, it was urgent to seek a more specific factor which could reflect the inflammatory condition. Some studies reported that Tβ4 could be a potential biomarker in hepatic cells. This study found that in patients with CHB combined NAFLD, Tβ4 level negatively correlated with tissue inflammation grading level. Besides, Tβ4 expression intensity was significantly lower in high inflammation and fibrosis group compared with low inflammation and fibrosis group. Primarily, it was found that Tβ4 up-regulated antioxidant confronted oxidative stress in human corneal cells, and the stress reaction was the central link of occurrence and development of hepatic cell inflammation, especially steatohepatitis. Therefore, Tβ4 expression was possibly involved in the occurrence and development of inflammation in CHB with steatosis patients by inhibiting oxidative stress. Meanwhile, we found that the Tβ4 expression was negatively correlated with TNF-α expression both in serum and liver tissues. TNF-α involved in the inflammatory reaction in activating inflammatory regulatory factor and programmed cell death, which was commonly known as the inflammatory factor of inflammatory response. It has been reported that Tβ4 inhibited the activation of nuclear factor-κappa B (NF-κB) and expression of interleukin-8 (IL8) induced by TNF-α, playing anti-inflammatory action role. Therefore, we proposed that Tβ4 shows a protective effect on the inflammatory response by inhibiting activation of inflammatory factor and took protective effect in the inflammatory injury, although such an effect was not related with hepatitis B replication, blood lipid, blood glucose, and steatosis degree.

Figure 1. Tβ4 immunohistochemical staining: there were brown granules in the cytoplasm of hepatic cells, some brown granules concentrated in the perinuclear. (A) showed strong expression of Tβ4 in liver tissue with extensive steatosis, inflammatory cells, and fibrosis (hematoxylin-eosin stain, A original magnification ×200). (B) showed strong expression of Tβ4 in liver tissue with extensive steatosis and occasional inflammatory cells (hematoxylin-eosin stain, A original magnification ×400). Tβ4 = thymosin β4.

Table 4

| Biological index and IOD in tissue | G+S<4 N=27 | G+S≥4 N=19 | P |
|-----------------------------------|------------|------------|---|
| Age (m)                           | 39.00±10.54| 38.08±11.39| 0.835 |
| ALT (μL)                          | 62.00±41.36| 94.89±85.99| 0.135 |
| AST (μL)                          | 30.15±28.43| 30.74±29.61| 0.946 |
| rGT (μL)                          | 41.67±16.00| 45.16±23.68| 0.553 |
| LG10 (HBV-DNA)                   | 4.36±2.00  | 5.46±2.14  | 0.081 |
| Steatosis degree (F)             | 1.48±0.70  | 1.79±0.54  | 0.114 |
| Tβ4 (IOD)                        | 25.47±7.68 | 19.35±6.55 | 0.015* |
| TNF-α (IOD)                      | 18.81±5.42 | 26.41±6.10 | 0.000** |

ALT = alanine aminotransferase, AST = aspartic transaminase, IOD = integral optical density, rGT = r-glutamine transferase, Tβ4 = thymosin β4, TNF-α = tumor necrosis factor-α.

*P<0.05, **P<0.01.

Figure 2. Tβ4 immunohistochemical expression in liver tissue. Tβ4 = thymosin β4.
CHB and NAFLD can result in liver inflammation and liver fibrosis, but whether such co-existence can influence liver fibrosis development is still controversial. However, hepatitis B virus or fat over-deposition can both lead to chronic inflammation and collagen deposition of hepatic cells. Hepatic stellate cells (HSCs) are one of the representative cells of hepatic cells. Activated HSCs can promote extracellular matrix (ECM) generation and migration. It was reported that normal hepatic cells expressed Tβ4, and kupffer cells as well as activated HSCs and also expressed Tβ4, which could promote cytothesis and fibrosis formation. There are more studies reported that Tβ4 could repair inflammation and inhibit fibrosis formation. However, whether endogenous Tβ4 was expressed by HSCs and whether it possessed the inhibition or promotion effect on HSCs were still controversial. In our study, we did not find any abnormal expressions of Tβ4 in different areas of liver tissues, but the serum Tβ4 level negatively correlated with the tissue fibrosis level. Through histological observation, in the collected samples, patients with severe fatty liver accounted for 52% (24/46), but those with moderate and severe fibrosis F3/F4 were few (only 1 cases in F3 level). The possible reason for this was that the samples we collected were in early stage of liver disease (average age was only 39.3) or the fatty liver could reverse the development of hepatitis B. Even if the number of cases with severe fibrosis was fewer, we could still observe the decreased Tβ4 level in patients with more severe fibrosis. This support the protective effect of Tβ4 which can inhibit fibrosis formation in CHB combined NAFLD.

Currently, liver biopsy is still considered as a gold standard for assessing chronic hepatitis inflammation and fibrosis. Although the noninvasive fibrosis index and instantaneous elastic liver stiffness detection can reflect liver inflammation fibrosis level, they are still influenced by other factors such as obesity. Therefore, many studies aimed to seek and find noninvasive specific index to reflect the inflammatory fibrosis level. Although abnormal expression was not observed during the formation of steatosis, it was found that Tβ4 expression was related with inflammation and fibrosis in patients with CHB combined NAFLD. It might be involved in the regulation of chronic inflammation and fibrosis. The possible mechanism might be as follows: Tβ4 played a defense role in the disease development by inhibiting oxidative stress and proinflammatory factor. The decreased Tβ4 concentration suggested more significant

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**Figure 3.** Scatter plots of expression of Tβ4 and TNF-α in serum. Tβ4 = thymosin β4, TNF-α = tumor necrosis factor-α.

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**Figure 4.** TNF-α immunohistochemical staining (hematoxylin-eosin stain, A original magnification ×400): (A) showed a strong expression of TNF-α in liver tissue with extensive steatosis and inflammatory cells; (B) showed weak expression of TNF-α in liver tissue; liver tissue could be seen scattered in adipose cell and less inflammatory cells. TNF-α = tumor necrosis factor-α.
inflammation and fibrosis development, which could be the target for effectively preventing the development of disease.

There are still some limitations in this study. First, the sample size was small, and the population sample with high fibrosis was even fewer. Thus, it was difficult to compare the Tβ4 level in cases with different fibrosis levels. Besides, the mechanism for abnormal expression of Tβ4 was not explored, and the study did not perform long-term follow-up. Therefore, in the future study, we should increase sample size, deeply explore the relationship between Tβ4 and fatty acid metabolism and oxidative stress specific factor. Then, long-term follow-up was warranted to observe the dynamic change of Tβ4 and indicate the role in patients with CHB and NAFLD.

In conclusion, the serum Tβ4 levels in patients with CHB combined NAFLD had no statistical difference with those with simple CHB, and Tβ4 concentration suggested more significant progression of inflammation and fibrosis.

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Figure 5. Scatter plots of expression of Tβ4 and TNF-α in liver tissue. Tβ4 = thymosin β4, TNF-α = tumor necrosis factor-α.
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