

Serum thymosin beta4 as a noninvasive biomarker in patients with nonalcoholic steatohepatitis

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ABSTRACT

Objective: The aim of the study was to determine whether serum thymosin beta4 (Tβ4) can be a useful noninvasive biomarker to differentiate between nonalcoholic steatohepatitis (NASH) and nonalcoholic fatty liver (NAFL).

Methods: The study included 24 NAFL patients and 21 NASH patients. The levels of Tβ4, 8-hydroxydeoxyguanosine acid (8-OHdG), liver function parameters, blood lipid, and glucose were detected in the venous blood of all patients. The NAFLD histological activity score (NAS) was examined in biopsy specimens from all patients. Statistical analysis was performed in order to find differences between the two abovementioned groups. In addition, receiver operator characteristic (ROC) analyses for alanine aminotransferase (ALT) and Tβ4 levels were performed in NAFL and NASH patients and the cut-off value was determined. Associations between the variables were tested using correlation coefficient calculations. Statistical significance was set at a p value of < 0.05.

Results: Serum Tβ4 content was 5.12 ± 1.87 mg/l in the NAFL group and 2.98 ± 1.35 mg/l in the NASH group ($p < 0.001$). Serum Tβ4 content and NAS, histological features of hepatic steatosis, lobular inflammation and ballooning, ALT, glucose and 8-OHdG levels were negatively correlated ($p < 0.05$ for all) in the NASH group. The correlation coefficient values were -0.530, -0.562, -0.574, -0.438, -0.446, -0.426 and -0.563, respectively. On the basis of ROC analysis, the best predictive Tβ4 cut-off value for detecting NASH was 3.94 mg/l (85.7% sensitivity and 79.2% specificity, which were higher than those of ALT).

Conclusion: Serum Tβ4 level can be used as a biomarker for the diagnosis of NASH and was negatively correlated with the oxidation state of the liver.

Key words: Thymosin Beta4. 8-hydroxy-deoxyguanosine acid. Nonalcoholic steatohepatitis. Nonalcoholic simple fatty liver.

INTRODUCTION

Over the past few decades, non-alcoholic fatty liver disease (NAFLD) has become one of the most common causes of chronic liver disease worldwide due to the increasing prevalence of obesity (1). In contrast, nonalcoholic fatty liver (NAFL) without inflammation is a non-progressive disease with a good prognosis. Attention has shifted from simple steatosis to nonalcoholic steatohepatitis (NASH), which in some cases may progress to end-stage liver disease such as liver cirrhosis or hepatocellular carcinoma (2). Therefore, the early and accurate identification of NASH would be beneficial to these patients. Currently, liver biopsy is the only effective method for differentiating between NAFL and NASH. Accordingly, the search for appropriate noninvasive biomarkers in patients with NASH is urgently needed. Thymosin beta4 (Tβ4), which is a G-actin sequestering peptide, is mainly related to the regulation of actin polymerization in living cells. It acts as an actin-sequestering peptide in mammalian cells and is involved in many critical biological processes, including apoptosis, cell migration, angiogenesis and fibrosis (4). Some studies (5,6) have observed that Tβ4 has anti-inflammatory effects. One study (7) confirmed that serum Tβ4 levels in patients with NAFLD were significantly lower than those of healthy controls. On the basis of the abovementioned findings, we hypothesized that Tβ4 may be a potential biomarker for the diagnosis of NASH.

MATERIALS AND METHODS

Patient selection

Forty-five patients with biopsy-proven NAFLD were enrolled into the study between May 2013 and February 2016 at The

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Second Hospital of Tianjin Medical University. Inclusion criteria included a biopsy sample in accordance with the diagnosis criteria of NAFLD with persistent abnormal aminotransferase levels. Exclusion criteria included hepatic viral infections (including hepatitis A, B, C, D and E), autoimmune liver disease, alcoholic liver disease (males with an alcohol intake of more than 20 g and females with an alcohol intake of more than 10 g per day), use of known hepatotoxic drugs, hereditary metabolic disease (hemochromatosis and Wilson's disease) and insufficient NAS scores to make a diagnosis of NASH even though they are higher than NAFL. Weight and body height were measured on admission and body mass index (BMI) was calculated. The study was approved by the ethics committee of The Second Hospital of Tianjin Medical University. Before participation, all patients provided informed consent for the use of their clinical data for research purposes.

Liver histopathology

All patients were hospitalized for a liver biopsy under standardized conditions with an intensive 24-h follow-up. All liver specimens were soaked in 10% neutral formaldehyde solution and embedded in paraffin blocks. Sections were cut at a thickness of 4- μ m and stained with hematoxylin-eosin and Masson trichrome. Histological results were assessed blindly by at least one experienced pathologist. Histological grading and staging of NAFLD were performed according to the criteria for NAFLD and NAS (8). NAFLD can be divided into four subtypes according to liver histology: a) steatosis only (type 1); b) steatosis accompanied with lobular inflammation (type 2); c) steatosis accompanied with ballooning degeneration (type 3); and d) steatosis accompanied with Mallory-Denk bodies or fibrosis (type 4). The subtypes 3 and 4 were identified as NASH (9) and histological results with stage 2 fibrosis or above were also considered as NASH (10). The NAS score was calculated as follows: steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2). The NAS of numerical pathological scores ranged from 0 to 8 points. Patients with a total NAS \leq 2 were diagnosed as NAFL, while patients with a sum of NAS \geq 5 were diagnosed as NASH.

Clinical assessment

Clinical history, including accompanying illness (such as hypertension, coronary heart disease or diabetes), drug intake and anthropometric, laboratory and clinical data were collected from all patients on the same day of the liver biopsy. BMI was also calculated.

Venous blood samples were collected in the morning on the day of the liver biopsy, after a 12 hour overnight fast. Serum was separated from the cells within one hour of sample collection by centrifugation at 3,000 g for 10 min at room temperature. In order to maintain the serum components intact, the serum samples were quickly sub-packed into microtubes and quickly frozen at -70 °C for further studies. The laboratory measurement in all cases included aspartate (ALT) levels as a liver function parameter, blood lipid levels and glucose levels. These were performed using an automatic biochemical analyzer. Serum 8-hydroxydeoxyguanosine acid (8-OhdG) and T β 4 levels were determined using

an 8-OhdG ELISA (enzyme-linked immunosorbent assay) kit (Nikken Seil, Japan) and a T β 4 ELISA kit (Immunodiagnostik AG, Bensheim, Germany), in strict accordance with the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 (SPSS, Chicago, IL, United States) software. The mean \pm SD was calculated for each data set. Comparisons between the two groups were performed using the Student's t-test. A p-value lower than 0.05 was considered as statistically significant. Correlations between serum T β 4 levels and NAS, histological features of hepatic steatosis, lobular inflammation and ballooning, serum ALT levels, glucose levels and 8-OhdG levels were analyzed within the patient cohort using the Pearson's correlation coefficient test. Serum T β 4 and ALT receiver operator characteristic (ROC) curves were plotted and the areas under the ROC curves (AUROCs) were calculated in order to represent their performance at predicting NASH. Cut-off values of serum T β 4 and ALT levels were evaluated using the ROC curve analysis.

RESULTS

General characteristics of the study population

A total of 24 patients with NAFL and 21 patients with NASH were recruited into the study. The demographic, clinical and biochemical characteristics are provided in table 1. Seven patients (four patients with a NAS sum of 3 points and three patients with a NAS sum of 4 points) were excluded. There were no significant differences between the two groups with regard to data such as sex or age ($p > 0.05$ for both). However, the NASH group had significantly more patients with a past history of diabetes and coronary heart disease (CHD) than the NAFL group ($p < 0.05$ for both). The BMI of patients with NASH was higher than in patients with NAFL (24.8 ± 3.0 vs 26.7 ± 3.2 , $p = 0.042$). ALT, triglyceride (TG) and glucose (GLU) levels in serum were significantly higher in patients with NASH compared to NAFL cases ($p < 0.05$ for all).

Serum concentrations of 8-OhdG and T β 4

Serum concentrations of 8-OhdG in the NAFL and NASH groups were 0.28 ± 0.08 ng/ml and 0.39 ± 0.12 ng/ml, respectively. Serum levels of 8-OhdG were higher in NASH patients compared to NAFL patients and the difference between the two groups was statistically significant ($p = 0.001$). Serum concentrations of T β 4 in the NAFL and NASH groups were 5.12 ± 1.87 mg/l and 2.98 ± 1.35 mg/l, respectively (Fig. 1). Serum levels of T β 4 were lower in NASH patients compared to NAFL patients and the difference between the two groups was statistically significant ($p < 0.001$).

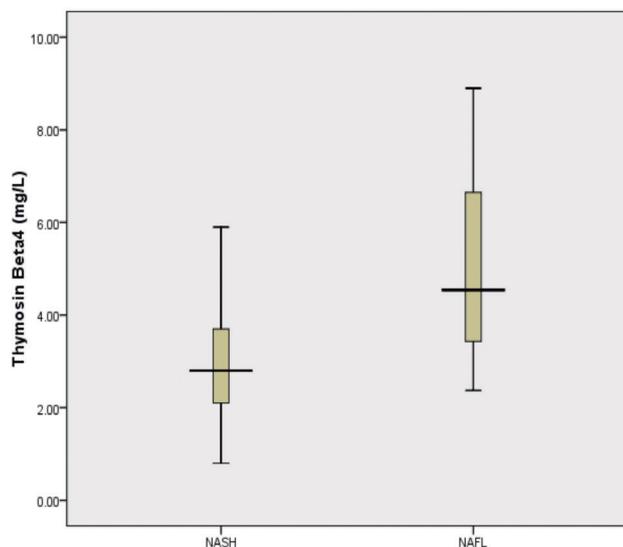
Correlation between T β 4 and histological activity score as well as biochemical indexes and their role in the noninvasive diagnosis of NASH

To verify the value of T β 4 in clinical practice, a linear regression analysis was performed between serum T β 4 levels,

Table 1. Baseline characteristics and factors of all the patients included in the study

Factors	NAFL (n = 24)	NASH (n = 21)	p-value
<i>Demographic data</i>			
Age (years)	57 ± 12	58 ± 12	0.778
Sex (males, n)	17 (70.8%)	14 (66.7%)	0.762
Diabetes history (n)	5 (20.8%)	11 (52.4%)	0.031*
CHD history (n)	3 (12.5%)	9 (42.9%)	0.033*
Hypertension history (n)	4 (16.7%)	6 (28.6)	0.339
BMI (kg/m ²)	24.8 ± 3.0	26.7 ± 3.2	0.042*
<i>Laboratory data</i>			
ALT (U/l)	36 ± 12	54 ± 22	0.003*
AST (U/l)	28 ± 10	32 ± 18	0.291
Albumin (g/l)	43 ± 5	42 ± 6	0.546
GGT (U/l)	97 ± 48	130 ± 71	0.074
ALP (U/l)	139 ± 64	146 ± 67	0.719
TBIL (mg/dl)	0.7 ± 0.2	0.8 ± 0.3	0.188
TG (mmol/l)	2.4 ± 1.5	3.3 ± 1.5	0.041*
CHO (mmol/l)	5.4 ± 1.5	5.7 ± 1.6	0.517
HDL (mmol/l)	1.06 ± 0.21	0.99 ± 0.20	0.195
LDL (mmol/l)	3.19 ± 1.18	3.54 ± 1.26	0.340
GLU (mmol/l)	5.02 ± 0.98	6.01 ± 1.12	0.002*

Data presented as number of patients or mean ± SD; *means p-values less than 0.05. CHD: Coronary heart disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; TBIL: Total bilirubin; GGT: γ -glutamyltransferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TG: Triglyceride; CHO: Cholesterol; GLU: Glucose.

**Fig. 1.** Serum T β 4 in the NASH group and NAFL group ($p < 0.001$).

histological activity score (NAS) and some biochemical parameters (Fig. 2). Serum levels of T β 4 were negatively correlated with NAS and histological features of hepatic steatosis, lobular inflammation and ballooning in the NASH group ($r = -0.530$, $p = 0.013$ [Fig. 2A]; $r = -0.562$, $p = 0.008$

[Fig. 2B]; $r = -0.574$, $p = 0.007$ [Fig. 2C]; and $r = -0.438$, $p = 0.047$ [Fig. 2D], respectively). However, the serum levels of T β 4 were not correlated with NAS in the NAFL group ($r = -0.181$, $p = 0.397$). Biochemical indexes of liver injury in patients with NASH, such as ALT, glucose and 8-OHdG, were also negatively correlated with serum T β 4 levels ($r = -0.446$, $p = 0.043$; $r = -0.426$, $p = 0.004$; and $r = -0.563$, $p = 0.008$, respectively). The serum levels of T β 4 were not correlated with ALT, glucose or 8-OHdG in the NAFL group ($r = -0.050$, $p = 0.816$; $r = -0.277$, $p = 0.065$; and $r = -0.120$, $p = 0.578$).

T β 4 serum level and ALT to create ROC curve

We compared the clinical value of serum T β 4 and ALT for the diagnosis of NASH. The ROC curve analysis included patients from the two groups and was established for different T β 4 and ALT levels in order to evaluate the accuracy of T β 4 for the diagnosis of NASH. The AUROC curve of the T β 4 serum level was highly significant compared to that of ALT (0.916, 95% CI: 0.829-0.967 vs 0.721, 95% CI: 0.570-0.872). The optimal cut-off values of serum T β 4 for NASH was 3.94 mg/l and the sensitivity and specificity values were 85.7% and 79.2%, respectively (Fig. 3A). The optimal cut-off value of serum ALT for NASH was 44 U/l and the sensitivity and specificity values were 63.3% and 57.2%, respectively (Fig. 3B).

DISCUSSION

Westernization of the Chinese lifestyle has led to increased lifestyle-related diseases such as NAFLD in the Chinese population and it is becoming a serious public health problem. NAFL is a relatively benign disease with a good prognosis, while NASH may progress to end-stage liver disease. Hence, early and accurate identification of NASH is crucial. Liver biopsy is the gold standard for the diagnosis of NAFLD and also to confirm the presence of NASH (3). However, liver biopsy is an invasive method with possible serious limitations and complications (11). Therefore, the development of noninvasive diagnostic methods for NAFLD is urgently needed. Some noninvasive methods of NASH diagnosis are under investigation, although these procedures also have limitations. For example, imaging tools such as ultrasound, computed tomography (CT) scans and magnetic resonance imaging (MRI) can reveal lipid accumulation within hepatocytes but they cannot assess the stage of fibrosis (12). As inflammation is considered to be a key component of NASH development, some inflammatory biomarkers may be increased during NASH (13). Many potential inflammatory biomarkers are being considered and their applicability for the diagnosis of NAFLD has been discussed (13-15). However, no definite biomarker has been validated as a predictor of the disease.

In the present study, there were no significant differences between the two groups with regard to patient background data such as sex and age. However, the NASH group had significantly more patients with a history of diabetes and CHD than the NAFL group. The BMI of patients with NASH was also higher than that in patients with NAFL. ALT, TG and glucose levels in serum were significantly higher in patients with NASH than in those with NAFL. The relationship between metabolic syndrome and NAFLD has now been recognized (16). A study also reported that CHD is the

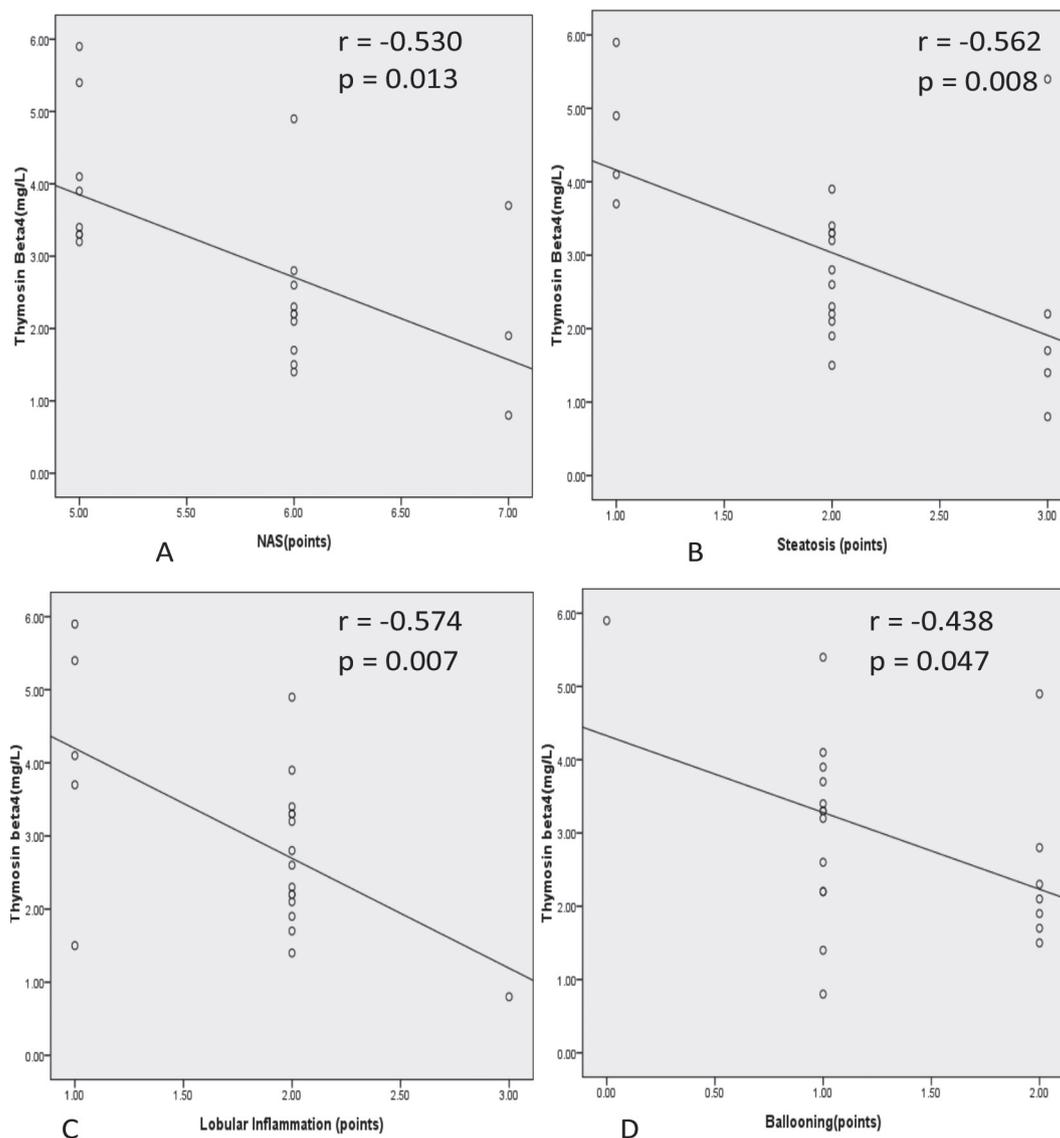


Fig. 2. Correlation between $T\beta 4$ and histological activity score. A. Serum $T\beta 4$ was negatively correlated with NAS in the NASH group. B. Serum $T\beta 4$ was negatively correlated with steatosis in the NASH group. C. Serum $T\beta 4$ was negatively correlated with lobular inflammation in the NASH group. D. Serum $T\beta 4$ was negatively correlated with ballooning in the NASH group.

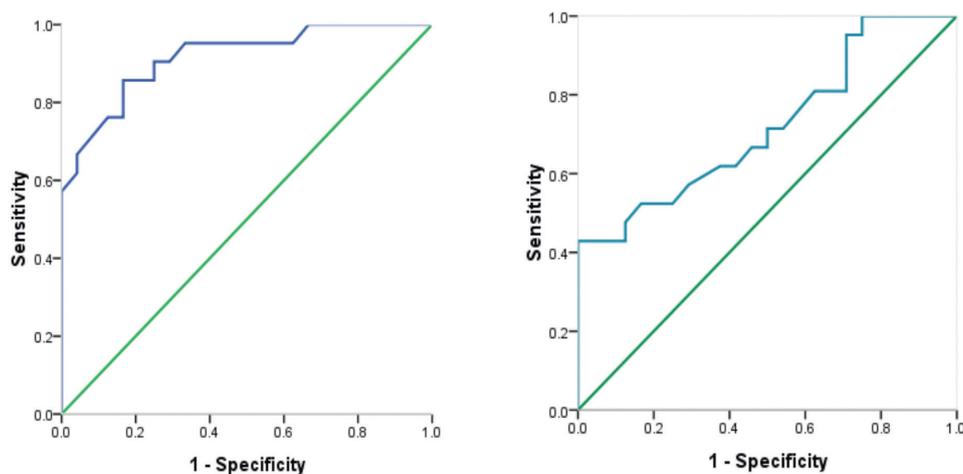


Fig. 3. A. Receiver operator characteristic (ROC) curve of $T\beta 4$ for a NASH diagnosis. B. Receiver operator characteristic (ROC) curve of ALT for a NASH diagnosis.

most common cause of death in patients with NAFLD (17). NASH was initially thought to be more common in females but empirical support for this claim is lacking (18). One study showed that older age is an independent risk factor for significant hepatic steatosis (19), but another research study has found no significant differences in age between individuals with progressive NASH and NAFL (20). NAFLD is more prevalent in patients with co-existing metabolic conditions than in the general population. Type 2 diabetes mellitus and NAFLD have a particularly close relationship (21). Some studies have used abnormal liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as non-invasive biomarkers of NAFLD. However, it is important to emphasize that although elevated ALT is generally associated with histological NASH, a number of patients with normal ALT levels may also have NAFLD and even advanced fibrosis.

Our previous study compared the levels of serum T β 4 in 26 patients and found that serum T β 4 levels in patients with NAFLD were significantly lower than those in healthy controls. However, the concentration of T β 4 in NAFLD increased with the improvement of liver function after treatment (7). Tian et al. (22) studied 83 cases of NAFLD and 80 cases of healthy outpatients and found that T β 4 can effectively reflect the liver function of NAFLD patients. Increased T β 4 concentration indicated the improvement of liver function and decreased T β 4 concentration indicated severe liver cell damage. All studies have indicated that T β 4 is closely related to NAFLD.

In the present study, serum T β 4 levels were negatively correlated with histological activity score (steatosis, inflammation, and ballooning) and with the overall NAS in NASH patients. This finding indicated that T β 4 may be a promising biomarker for the severity of hepatic histological features in NASH. However, the serum levels of T β 4 were not correlated with the histological activity score in the NAFL group. Therefore, T β 4 may be useful for differentiating between NAFL and NASH.

Day and James (23) have suggested that a "second hit", such as oxidative stress, is essential to activate the progression of NASH. Some studies have supported that oxidative stress may also contribute to clinical progression from simple fatty liver to NASH (24). Therefore, oxidative stress plays an important role in the formation of NASH. 8-OhdG is an important marker which can reflect oxidative stress in the body (25). When DNA is exposed to oxidative radicals, it forms the endogenous 8-OhdG, which can serve as a sensitive indicator of physiological damage to DNA (26). We found that serum levels of 8-OhdG were increased in NASH patients compared to NAFL patients and the difference between the two groups was statistically significant. This finding highlighted a higher level of oxidative stress in the NASH group compared to the NAFL group. We also observed a negative correlation between serum levels of T β 4 and serum levels of 8-OhdG in patients with NASH ($p = 0.038$) but not in patients with NAFL. We suggest that in oxidative stress conditions, as occurs in NASH, T β 4 is induced to progress from NAFL to NASH. However, its corresponding molecular mechanisms require further study. Reyes-Gordillo et al. (27) reported that T β 4 has an effective anti-inflammatory and anti-fibrotic effect on carbon tetrachloride-induced acute hepatotoxicity in rats, thus indicating that T β 4 can prevent necrosis, inflammatory infiltration

and upregulation of α 1 (and 2) collagen, alpha-smooth muscle actin (α -SMA), platelet-derived growth factor-beta (PDGF- β) receptor and fibronectin mRNA expression. In addition, the down-regulation of peroxisome proliferator-activated receptor gamma (PPAR γ) and upregulation of MECP2 mRNA levels in acute liver injury may be prevented. Liang et al. (28) detected the expression level of T β 4 in serum and tissue of patients with chronic hepatitis B combined with NAFLD and observed that T β 4 level was negatively correlated with inflammation and fibrosis scores. In addition, T β 4 expression in both serum and liver tissue was negatively correlated with tumor necrosis factor-alpha (TNF- α) expression. Moreover, T β 4 played a defensive role in the development of liver disease by inhibiting oxidative stress and pro-inflammatory factors, and the decreased T β 4 concentration suggested the development of a more significant inflammation. Hence, we can monitor serum T β 4 level in order to differentiate between NAFL and NASH.

We attempted to determine the serum T β 4 cut-off value for a definite diagnosis of NASH compared to NAFL. Our previous study found a cut-off of 4.83 mg/l with an AUC of 0.906 as the best point to distinguish NAFLD from healthy controls (7). The optimal cut-off point was 3.94 mg/l, with an AUC of 0.916 (95% CI: 0.829-0.967) to distinguish NAFLD from NAFL in this study. The corresponding sensitivity and specificity were 85.7% and 79.2%, respectively, which were higher than those of ALT. Some previous studies (29,30) have also reported that ALT levels are strongly correlated with NASH. ALT levels > 45 U/l identified NASH, with every one unit increase in ALT resulting in a 6% increase in the likelihood of NASH (30). These findings mean that the serum level of T β 4 can serve as a better biomarker than ALT for the diagnosis of NASH. This study is the first to focus on the role of T β 4 for the diagnosis of NASH. The major limitation of the study was the sample size. Further studies with a large number of patients are recommended in order to investigate the relevance of serum T β 4 concentrations in relation to the histological grade of steatosis (NAS, hepatic steatosis, lobular inflammation) in NAFLD patients. The causes of significantly lower serum T β 4 in patients with NASH also require further studies.

In conclusion, this study determined that serum T β 4 was significantly lower in patients with NASH compared to NAFL and was negatively correlated with histological activity score and the overall NAS. Furthermore, it was also related with the condition of oxidative stress. Therefore, the serum concentrations of T β 4 may be monitored in order to differentiate between NASH and NAFL.

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