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Original Article

Serum transforming growth factor beta 3 predicts future development of nonalcoholic fatty liver disease

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Abstract: Transforming growth factor beta 3 (TGFb3) was mainly expressed by liver satellite cells in the normal liver, but it may be expressed by various liver cells during liver diseases, e.g. hepatitis and cirrhosis. However, whether TGFb3 level may be used to predict development of nonalcoholic fatty liver disease (NAFLD) has not been investigated before. Here we evaluated the relationship between TGFb3 and the susceptibility for developing NAFLD by comparing the incidence rates of developing NAFLD and serum TGFb3 levels in 1322 healthy subjects without other risk factors during a 4-year period. These healthy subjects were grouped into tertiles based on their serum TGFb3 levels that were measured in 2009. After 4 years, the odds ratios (ORs) of NAFLD development were analyzed based on the tertiles of TGFb3 levels in 2013. The cumulative incidence of NAFLD was 25.3% (334/1322) after four years. The NAFLD-developing group had higher serum TGFb3 levels in 2009 than those in the group that did not develop NAFLD (554±287 pg/ml vs. 285±173 pg/ml; P=0.002). When the serum TGFb3 levels in 2009 were grouped into tertiles, we found that the incidence of NAFLD in 2013 was significantly higher with increasing tertiles (6.3%, 38.0%, and 55.7%, respectively; P<0.05). Thus, our study demonstrate that higher serum TGFb3 levels in subjects devoid of NAFLD may have a higher chance of its future development, and highlight serum TGFb3 level as a novel predictor for development of NAFLD.

Keywords: Non-alcoholic fatty liver disease, transforming growth factor beta 3

Introduction

Nonalcoholic fatty liver disease (NAFLD) is prevalent disease [1, 2]. Different from alcoholic fatty liver disease (AFLD), NAFLD results from intrahepatic fat accumulation, rather than from excessive alcohol intake. The pathology of NAFLD is complicated, including simple steatosis, nonalcoholic steatohepatitis, fibrosis and cirrhosis [1, 2]. After fat infiltrates the liver, progression to hepatocellular inflammation and fibrosis may occur. Therefore, NAFLD can be predisposed to cirrhosis, which contributes to an increasing mortality related to obesity and diabetes [3, 4]. Moreover, oxidative stress, mitochondrial disorder and cytokine challenge are important in mediating this process.

Transforming growth factor beta (TGFb) is a growth factor that mediates various processes during cell proliferation, differentiation, and other biological events [5-7]. TGFb is secreted by many cell types and exists in at least three isoforms called TGFb1, TGFb2 and TGFb3 [8]. The TGFb family is part of TGFb superfamily [5-9]. TGFb has been shown to play an essential role in the development of liver fibrosis [10-12]. In both normal and fibrotic liver, TGFb1 has been found to be the most abundant among all forms. Kupffer cells are the major source of TGFb1 and stellate cells are the second major source of TGFb1 in the normal liver. During the liver fibrosis, the level of TGFb1 increases essentially and predominantly in stellate cells. TGFb2 is also mainly expressed by Kupffer cells, followed by stellate cells and endothelial cells. On the contrary, TGFb3 is expressed exclusively by stellate cells [13].

Since proliferation, contractility, and chemotaxis of stellate cells is one specific character of liver fibrotic diseases [11], and since there are no previous reports studying the relationship between TGFb3 and development of NAFLD,

here we were prompted to evaluate the role of serum TGFb3 level as a marker for development of NAFLD in a longitudinal analysis.

Methods

Study participants

Participants received medical screening in 2009 and at a follow-up study in 2013. Subjects were excluded from the current study, if found with a fasting blood glucose above 120 mg/dl, or with elevated alanine aminotransferase (ALT) above 30 IU/ml, or infection with viral hepatitis B or hepatitis C, or with a fatty liver by ultrasonography, or with other liver diseases, with various malignancy, or excessive alcohol consumption (>20 g/day). In addition, participants undergoing medications such as peroxisome proliferator-activated receptor-γ agonists, metformin, or antioxidants (vitamin E or C) were excluded. Finally, this study included 1322 subjects who were devoid of diseases that may affect the final conclusion of the current study and this population was evaluated for the development of NAFLD after 4 years in 2013. The study was officially approved by the Institutional Review Board and the Ethics Committee of Linyi Tumor Hospital.

Data obtain

A standardized questionnaire was performed by experienced physicians to collect subject information such as age, sex, alcohol drinking, smoking, and history of diabetes, dyslipidemia, hypertension, stroke and cardiovascular disease. Family history of diabetes or cardiovascular diseases was defined as yes or no based on participants' responses. Patients received complete physical examinations. Anthropometric evaluation including body weight, height and waist circumference. Blood pressure were taken by experienced physicians. Body mass index (BMI) was calculated by dividing the weight in kilograms by the square of height in meters.

Biochemical assays

Blood samples were collected after overnight fasting, and then used to evaluate fasting plasma glucose, fasting plasma insulin, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, direct bilirubin, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP). Homeostatic model assessment (HOMA) of insulin resistance (IR) was determined as: HOMA-IR=[fasting plasma insulin (μIU/ml) × fasting plasma glucose (mmol/l)]/22.5 [14]. Serum samples for TGFb3 quantification were stored at -80°C, and collected for enzyme-linked immunosorbent assay (ELISA, R&D, Minneapolis, MN, USA) analyses.

Ultrasonography

Fatty liver was diagnosed with an abdominal ultrasonography based on hepatorenal echo contrast, deep attenuation, liver brightness and vascular blurring, with a 3.5 MHz probe [14]. The ultrasound examination were blindly and independently performed by two experienced radiologists.

Statistical analyses

Statistical analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Normality was confirmed by the Kolmogorov-Smirnov test. The chi-squared test was applied for comparison of categorical variables between different groups. Comparison of parameters with a normal distribution were done with analysis of variance (ANOVA) and presented as the mean ± standard deviation (SD). Comparison of parameters without a normal distribution were performed with the Mann-Whitney U test and presented as the interquartile range. Serum TGFb3 levels were grouped into tertiles. For calculate odds ratios (ORs) for NAFLD, multiple logistic regression analysis was applied. Subjects with higher serum TGFb3 tertiles were individually compared with the lowest tertile. $P<0.05$ were considered significant.

Results

Baseline characteristics of subjects in 2009 grouped by presence of NAFLD in 2013

The current study included 1322 subjects, from which 708 were males and 614 were females. The subjects had an average age of 41.2 years (range from 36 to 45). All these included subjects were free of liver and other related diseases that may affect the result of our study. The subjects were then divided into 2 groups: 988 without NAFLD and 334 with NAFLD in 2013. We then compared the baseline characteristics between these subjects in 2009,

Table 1. Baseline clinical characteristics of subjects in 2009 grouped by the presence of NAFLD in 2013

	Non-NAFLD (n=988)	NAFLD (n=334)	*P* value
Age, yr	41.3 (36-45)	41.2 (36-45)	0.563
Men, n (%)	495 (50.1)	213 (63.8)	0.002
Smoking, n (%)	142 (14.4)	50 (15.0)	0.135
BMI, kg/m^2	22.9±2.5	23.3±2.8	0.094
Waist circumference (cm)	85.5±9.8	85.8±9.4	0.086
SBP (mm Hg)	131±12.8	133±13.2	0.212
DBP (mm Hg)	81.2±7.2	82.5±7.4	0.105
Cholesterol, mg/dl	195.8±28.5	196.9±26.8	0.658
LDL-C, mg/dl	111.4±24.5	109.7±25.1	0.783
HDL-C, mg/dl	54.5±10.5	55.1±10.7	0.223
FPG, mg/dl	92.0 (87-96)	92.1 (88-96)	0.372
Fasting insulin, μU/ml	5.7±2.3	5.9±2.1	0.078
HOMA-IR	1.58±0.15	1.62±0.22	0.407
AST, IU/l	26.9±4.5	27.3±4.8	0.527
ALT, IU/l	26.3±3.3	27.1±3.8	0.216
ALP, IU/l	48.9±8.9	50.6±9.5	0.089
GGT, IU/l	20.5±4.2	24.2±5.2	0.015
Direct bilirubin, mg/dl	0.34±0.12	0.36±0.13	0.153
Serum creatinine, mg/dl	1.02±0.14	1.04±0.12	0.219
Serum TGFb3, pg/ml	285±173	554±287	0.002

Values are expressed as median (interquartile range) or mean ± SD. NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; FPG, fasting plasma glucose; HOMA-IR, homeostatic model assessment of insulin resistance; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase; TGFb3, transforming growth factor beta 3.

Table 2. Tertile distributions of serum TGFb3 levels in 2009 grouped by the presence of nonalcoholic fatty liver disease in 2013

TGFb3 Tertile	Non-NAFLD (n=988)	NAFLD (n=334)
T1 (0-250 pg/ml)	634 (64.2%)	21 (6.3%)
T2 (250-500 pg/ml)	341 (34.5%)	127 (38.0%)
T3 (500-900 pg/ml)	13 (1.3%)	186 (55.7%)

Values are expressed as number (ratio to total). NAFLD, nonalcoholic fatty liver disease; TGFb3, transforming growth factor beta 3.

based on the presence or absence of NAFLD in 2013, and the data are shown in **Table 1**. Although it appeared that the NAFLD group contained significantly more males (**Table 1**), we failed to detect significant differences in the ages, BMI values and waist circumference between the NAFLD and non-NAFLD groups. Moreover, no difference was detected in diastolic blood pressure (DBP), systolic blood pressure (SBP), cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), fasting plasma insulin, fasting plasma glucose (FPG), HOMA-IR, alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin, alkaline phosphatase (ALP) and creatinine among two groups. However, gamma-glutamyltransferase (GGT) levels were found higher in NAFLD group.

Significant higher serum TGFb3 levels in 2009 was detected in 2013 NAFLD group

Strikingly, serum TGFb3 levels in 2009 were significantly higher in the NAFLD group, compared to those in the non-NAFLD group (554±287 pg/ml vs. 285±173 pg/ml; P=0.002). Since the basic characteristics of these subjects were well distributed, our data highly suggest that serum TGFb3 level may be an early feature predisposed to development of NAFLD.

Tertile distributions of serum TGFb3 levels in 2009 grouped by the presence of NAFLD in 2013

Serum TGFb3 levels in 2009 were used to group the participants into three tertiles (tertile 1: 0-250 pg/ml; tertile 2: 250-500 pg/ml; tertile 3: 500-900 pg/ml), and analyzed for their distribution in the NAFLD and non-NAFLD groups in 2013. The odds ratios (ORs) of the development of NAFLD based on the tertiles of TGFb3 levels in 2013 were then calculated and shown in **Table 2**. We found that the incidence of NAFLD in 2013 appeared to be significantly higher with increasing tertiles (6.3%, 38.0% and 55.7%, respectively; P<0.05) (**Table 2**).

The risk of NAFLD after 4 years based on serum TGFb3 levels grouped by tertiles was then eval-

Table 3. Odds ratio (95% CI) for the development of nonalcoholic fatty liver disease associated with each TGFb3 tertile

	Odds ratio (95% CI)	*P* value
Without correction for GGT		
TGFb3 T1 vs T2	2.35 (1.78-3.86)	0.0012
TGFb3 T1 vs T3	2.89 (2.05-4.43)	0.0001
After correction for GGT		
TGFb3 T1 vs T2	2.28 (1.75-3.81)	0.0019
TGFb3 T1 vs T3	2.86 (2.01-4.29)	0.0001

CI, confidence interval; TGFb3, transforming growth factor beta 3.

uated. A higher tertile OR was at least 2.28 to a lower tertile OR, even after adjustment for the only significantly different parameter GGT (**Table 3**), apart from TGFb3 in the 2009 samples. Thus, our study demonstrate that higher serum TGFb3 levels in NAFLD-devoid subjects may have a higher chance of development of NAFLD in future, and highlight TGFb3 as a novel predictor for development of NAFLD.

Discussion

NAFLD is prevalent disease all over the world and its early predication may substantially improve the current therapeutic approaches. Although the pathology of NAFLD is complicated, progression to fibrosis and cirrhosis seems to be critical outcome [1, 2]. TGFb superfamily members play important roles in various physiological and pathological processes, in which they are primarily secreted as inactive complexes and become biologically active only after proteolysis.

Three isoforms of TGFb (TGFb1, TGFb2 and TGFb3) have been identified in mammalian species. The TGFb binds to the type 2 receptor and initiates the recruitment of type 1 receptor. Subsequent phosphorylation of T type 1 receptor by type 2 receptor enables activation of small and mothers against decapentaplegic (SMAD) 2 and 3, which conduct the downstream signals. TGFb is an important mediator in the development of liver fibrosis [10-12]. In both normal and fibrotic liver, TGFb1 is the most abundant isoform. Moreover, all three isoforms were expressed strongly in adult liver fibrosis. Interestingly, previous studies have demonstrated that neutralization of all three TGFb isoforms is not capable of improving scarring. Moreover, provision of exogenous TGFb3 has been shown to elicit a scar-free or regenerative healing response, whereas TGFb1 and TGFb2 are supposed to lead to a fibrotic scarring response. These data suggest that neutralization of TGFb3 may be detrimental Nevertheless, how TGFb3 may function during liver fibrosis is not completely understood yet.

Hepatic stellate cells are also known as perisinusoidal cells or Ito cells. Hepatic stellate cells are pericytes presenting at the perisinusoidal space of the liver, which is also known as the space of Disse [11, 15-18]. The stellate cells play a key role in liver fibrosis. In normal liver, stellate cells remain quiescent, and they have long protrusions to wrap around the sinusoids. Quiescent stellate cells constitute no more than 8% of the total liver cells, whereas with unclarified biological function [11, 15-18]. Recently, some studies have provided evidence for hepatic stellate cells being liver-resident antigen-presenting cells, in which they present antigens to NKT cells to promote their proliferation [19]. Stellate cells are activated during liver damage, by increasing in cell proliferation, contractility, and chemotaxis. Since proliferation, contractility, and chemotaxis of stellate cells is one specific character of liver fibrosis, and since there are no previous studies addressing the relationship of TGFb3 and development of NAFLD, here we were prompted to evaluate the role of TGFb3 as a marker for development of AFLD in a longitudinal analysis.

In this study, we sought to define whether there is a relationship between TGFb3 and the susceptibility for developing NAFLD. Thus we compared the incidence rates of NAFLD based on serum TGFb3 levels in 1322 healthy subjects without other risk factors after 4 years. The participants were grouped into tertiles according to their serum TGFb3 levels in 2009. We also compared the ORs of the development of NAFLD according to the tertiles of TGFb3 levels in 2013. After 4 years, the cumulative incidence of NAFLD was 25.3% (334/1322). The group that developed NAFLD had higher plasma levels of TGFb3 than those in the group without NAFLD (554±287 pg/ml vs. 285±173 pg/ml; P=0.002). When the 2009 serum TGFb3 levels were categorized into tertiles: incidence of NAFLD observed in 2013 was significantly higher with increasing tertiles (6.3%, 38.0%, and 55.7%, respectively; P<0.05).

To the best of our knowledge, our study should be the first one to demonstrate that higher serum TGFb3 levels in subjects without NAFLD may have a higher change of developing NAFLD in future, which highlights TGFb3 as a novel predictor for development of NAFLD.

However, our study also had several limitations. First, the diagnosis of NAFLD was made by ultrasound, but not confirmed by histology. Ultrasound diagnosis of NAFLD has a good but not the highest sensitivity and specificity [20]. Second, alcohol intake was surveyed by a questionnaire, in which misreporting may occur. Moreover, lifestyle risk factors, which can affect future NAFLD, including exercise and dietary habits, were not considered. Thus, the data may be subject to under- or overestimation at a certain level. Lastly, our cohort was composed of participants who had volunteered for health examinations, which might also introduce a selection bias.

In conclusion, this study provides evidence that higher serum TGFb3 levels in NAFLD-devoid subjects may have a higher chance of developing NAFLD in future, and suggest that TGFb3 may be a novel predictor for development of NAFLD. Future studies are needed to confirm the findings from the current work.

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Disclosure of conflict of interest

None.

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