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Influence of TNF gene polymorphisms in Japanese patients with NASH and simple steatosis

Katsutoshi Tokushige*, Mihoko Takakura, Noriko Tsuchiya-Matsushita, Makiko Taniai, Etsuko Hashimoto, Keiko Shiratori

Tokyo Women's Medical University, Department of Medicine and Gastroenterology, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

***Background/Aims*: Tumor necrosis factor (TNF) is considered to play a role in the second hit of non-alcoholic steatohepatitis (NASH). To clarify the effects of TNF in NASH we investigated TNF gene polymorphisms that might influence TNF production were investigated.**

***Methods*: We analyzed 102 patients with non-alcoholic fatty liver disease (NAFLD; 36 with simple steatosis and 66 with NASH) and 100 control subjects. The serum level of soluble TNF receptor (sTNFR)-2 was measured. The TNF-α promoter region positions −1031, −863, −857, −308, and −238 and the TNF-β gene Nco1 polymorphism site were investigated.**

***Results*: The level of sTNFR-2 was significantly higher in NASH patients than in those with simple steatosis or control subjects. In the analysis of TNF gene polymorphisms, there were no significant deviations between the group of all NAFLD patients and the control subjects. The carrier frequencies of polymorphisms at positions −1031C and −863A were significantly higher in patients with NASH than in those with simple steatosis. In the multivariate analysis, TNF-α promoter polymorphisms proved to be significant independent factors distinguishing NASH from simple steatosis.**

***Conclusions*: TNF polymorphisms, which influence TNF production, might be associated with the progression of NAFLD.**

***Keywords*: NASH; Simple steatosis; TNF gene polymorphism**

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has recently been recognized as a leading cause of abnormal liver function. Its spectrum ranges from fatty liver alone, usually a benign and non-progressive condition, to non-alcoholic steatohepatitis (NASH), which may progress to liver cirrhosis [1,2]. Patients with NASH usually have insulin resistance syndrome [3]. The etiology of NASH remains unclear, but most investigators agree that development of the disease requires a baseline of steatosis followed by a "second hit" capable of inducing necroinflammation and fibrosis [2]. The interaction of cytokines with oxidative stress and lipid peroxidation has been postulated to play a key role in NASH [2,4,5]. Tumor necrosis factor (TNF)-α has been noted as an especially important cytokine in the development of many forms of liver injury, including steatohepatitis. Previous studies showed that TNF-α was associated with the pathogenesis of NASH [6–8].

Recently, we reported that there were significant increases in the serum levels of soluble TNF receptor (sTNFR) in NASH patients [9]. Regarding the relationship to hepatic histological features, the serum levels of sTNFR in NASH patients with advanced fibrosis were increased compared with those of patients with low-grade fibrosis. These findings suggested that TNF might be associated with the occurrence and progression of NASH.

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* Corresponding author. Tel.: +81 3 3353 8111; fax: +81 3 5269 7430.

E-mail address: ktoku@pg7.so-net.ne.jp (K. Tokushige).

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There are variations in the production rates of cytokines among individuals [10,11]. Some of these inter-individual differences in cytokine production may be related to polymorphisms in the cytokine genes themselves, or to polymorphisms in genes that regulate cytokine gene transcription. Recent studies have described extensive polymorphisms within the TNF-α promoter region at positions −1031, −863, −857, −308, and −238, and at the Nco1 site in the TNF-β gene, and currently there is considerable interest in the relationships between these TNF gene polymorphisms and susceptibility/resistance of individuals to both autoimmune and infectious diseases [11–13]. Valenti et al. reported that a significant deviation of the TNF promoter region polymorphism at position −238 was observed in NAFLD patients in Italy [6]. However, in Japanese NASH or NAFLD patients, the genomic features remain unknown. In addition, it remains unclear whether genomic variants play a role in the second hit from simple steatosis to NASH. Farrell reported that Asian patients with NAFLD had a significantly lower BMI than all other racial groups [14]. It is thus important to be aware of the variable presentation in different racial and ethnic groups. In this study, we investigated the polymorphisms of the TNF gene in Japanese patients with simple steatosis and NASH.

2. Materials and methods

2.1. Patients and methods

One hundred and two Japanese patients who were histologically diagnosed as having NAFLD at Tokyo Women's Medical University between 1995 and 2005 were evaluated along with 100 healthy subjects who served as controls. All liver biopsy specimens were examined using the following stains: hematoxylin-eosin, Mallory, and silver reticulin. Fibrosis was scored using a 5-grade scale: F0, normal connective tissue; F1, foci of perivenular or pericellular fibrosis in zone 3; F2, perivenular or pericellular fibrosis confined to zones 3 and 2, with or without portal/periportal fibrosis; F3, bridging or septal fibrosis; F4, cirrhosis [2,15,16]. Steatosis was graded on a scale of 1–3: 1, mild (affecting 10–33% of hepatocytes); 2, moderate (33–66% of hepatocytes); 3, severe (>66% of hepatocytes). Inflammation was graded as mild, moderate or severe based on the reviewer's overall impression. The diagnosis of NAFLD was established based on the following criteria. (1) Histologically macrovesicular steatosis affecting at least 10% of hepatocytes and classified as either simple steatosis or steatohepatitis (NASH). In addition to steatosis, the minimum criteria for the diagnosis of NASH included the presence of lobular inflammation, ballooning degeneration and perivenular or/and pericellular fibrosis (fibrosis stage ≧ 1) [2,15–17]. (2) Intake of less than 40 g of ethanol per week, as confirmed by physicians and family members with the patients. (3) Appropriate exclusion of other liver diseases such as alcoholic liver disease, viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, primary sclerosing cholangitis, and metabolic liver diseases. In addition, the NAFLD activity score (NAS) was used to classify NAFLD into simple steatosis (NAS 0–2) or borderline steatosis/steatohepatitis (NAS 3–4) and steatohepatitis (NASH) (NAS ≧ 5) as shown in Table 5B [18].

Body mass index (BMI) was calculated by the standard formula: weight (kg)/(height (m^2)). The diagnosis of type II diabetes mellitus (DM) was based on Japanese criteria (random glucose in excess of 200 mg/dL, or hemoglobin A1c (HbA1c) greater than 6.5% on two occasions) [19]. The criteria for a diagnosis of hyperlipidemia were above-normal fasting levels of total cholesterol and/or triglycerides. Hypertension was considered to be present in patients with a systolic blood pressure over 140 mm Hg or diastolic blood pressure over 90 mm Hg on at least two occasions. Homeostasis model assessment-insulin resistance (HOMA-IR; fasting serum insulin μU/ml × fasting glucose mg/dl/405) was measured in NAFLD patients without DM [20,21]. None of the patients received drug treatment for NAFLD before the liver biopsy. A complete history was obtained and physical examination was performed in all patients. All patients underwent liver tests for measurement of the following laboratory parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), platelet count, hepatitis B serology (hepatitis B surface antigen, antibody to hepatitis B surface antigen, and antibody to hepatitis B core antigen), hepatitis C serology (antibody to hepatitis C virus and hepatitis C RNA polymerase chain reaction), and autoantibodies (antinuclear antibody (ANA), antismooth muscle antibody, and antimitochondrial antibody).

All control subjects were Japanese and matched for age and gender with NAFLD patients. All control subjects were confirmed to have normal liver function and no viral hepatitis infection by blood test. This group was formed by enrolling volunteers from hospital staff, medical students, and acquired relatives of hospital staff and patients. None of the control subjects were alcoholics or had a BMI > 25. Seventy-two of one-hundred control subjects had previously undergone ultrasonographic examination which confirmed the absence of steatosis [22].

Informed consent was obtained from all patients and healthy controls before their entry into the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by our Institution's Research Committee.

When NAFLD patients were referred to us for a liver biopsy or another medical treatment, informed consent and a blood sample were obtained. The serum concentration of sTNFR-2 was measured using commercially available enzyme-linked immunosorbent assay kits (Biosource Europe S.A., Fleurus, Belgium) [9]. All sera from patients were kept at −20 °C until assayed.

Genomic DNAs were obtained from peripheral blood leukocytes by standard phenol–chloroform extraction [23]. PCR was carried out using Taq polymerase (TOYOBO, Tokyo, Japan) in 1.25 μL reaction, dNTP (TOYOBO) at 200 μM each and PCR primers at 1 μM final concentration. Restriction digests were performed on unpurified PCR products, adding the specific restriction buffer to achieve optimal reaction conditions, as described previously [24,25]. The polymorphisms of the TNF-α promoter region at −857, −863, and −1031 were analyzed by direct sequence analysis.

2.2. Statistical analysis

Results were expressed as means ± standard deviation (SD). The statistical analysis in Tables 1–4, 6 and 7 was performed using Mann–Whitney U test or χ^2 test. The multivariate analysis in Table 5 was performed with Dr. SPSS II software (SPSS Institute, Tokyo). To clarify the difference between simple steatosis and NASH, ORs and 95% CIs were estimated by a multivariate unconditional logistic regression model. The influence of profile, interaction, and multicollinearity was examined by regression diagnostics. A p-value of <0.05 was considered to be statistically significant in all analyses.

3. Results

3.1. Soluble TNF receptor

The clinical data and histological features of patients in the NASH, simple steatosis and control groups are presented in Table 1. Serum sTNFR-2 was measured in 83 (30 with simple steatosis and 53 with NASH) of