Original Article

PGHN

Role of Tumor Necrosis Factor-α Promoter Polymorphism and Insulin Resistance in the Development of Non-alcoholic Fatty Liver Disease in Obese Children

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Purpose: Tumor necrosis factor-α (TNF-α) polymorphism has been suggested to play an important role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) in obese adults, and known to be a mediator of insulin resistance. In this study, we evaluated the role of TNF-α promoter polymorphisms and insulin resistance in the development of NAFLD in obese children.

Methods: A total of 111 obese children (M : F=74 : 37; mean age, 11.1±2.0 yrs) were included. The children were divided into 3 groups: controls (group I, n=61), children with simple steatosis (group II, n=17), and children with non-alcoholic steatohepatitis (group III, n=33). Serum TNF-α levels, homeostasis model assessment of insulin resistance (HOMA-IR), and TNF-α -308 and -238 polymorphisms were evaluated.

Results: There were no differences in TNF-α polymorphism at the -308 or the -238 loci between group I and group II + III (p=0.134 and p=0.133). The medians of HOMA-IR were significantly different between group I and group II + III (p=0.001), with significant difference between group II and group III (p=0.007). No difference was observed in the HOMA-IR among the genotypes at the -308 locus (p=0.061) or the -238 locus (p=0.207) in obese children.

Conclusion: TNF-α promoter polymorphisms at the -308 and -238 loci were not significantly associated with the development of NAFLD in children; nevertheless, insulin resistance remains a likely essential factor in the pathogenesis of NAFLD in obese children, especially in the progression to NASH. **(Pediatr Gastroenterol Hepatol Nutr 2012; 15: 44-51)**

Key Words: TNF-α polymorphism, Insulin resistance, Fatty liver, Obesity, Child

INTRODUCTION

The incidence of childhood obesity has been increasing, and with this comes the concern that obesity-related complications, including non-alcoholic fatty liver disease (NAFLD), will also increase. NAFLD is defined as a chronic liver disease characterized by macrovesicular steatosis with histopathologic findings similar to alcoholic fatty liver disease in those who do not drink alcohol [1]. It has a disease spectrum ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis; NASH progressing towards cirrhosis is regarded as a significant cause of liver-related morbidity, even in children [2,3].

The most common risk factor in children for the development of NAFLD is obesity [4], and the pathogenesis of NAFLD in obese patients has been explained by the 2-hit theory; the first hit is the accumulation of fat in the liver (simple steatosis), and the second hit is the induction of inflammation and fibrosis in NASH. These second stimuli include oxidative stress and adipokines or proinflammatory cytokines including tumor necrosis factor-α (TNF-α) [5]. Insulin resistance and hyperinsulinemia have also been suggested to be strongly associated with the pathogenesis and severity of NAFLD [6,7]. NAFLD is now regarded as a hepatic manifestation of the metabolic syndrome, with insulin resistance representing a common mechanism for developing both simple steatosis and NASH in cases of obesity [8].

TNF-α is overexpressed in the adipose tissue of obese patients [9], and is known to be an important mediator of insulin resistance through multiple mechanisms including modulation of the tyrosine kinase activity of the insulin receptor [10]. TNF-α is also overexpressed in the adipose tissue and liver of obese patients diagnosed with NASH [11]. Furthermore, several reports have been published in adults on the relationship between TNF-α and NAFLD in association with insulin resistance [12-14].

The TNF-α gene is located in chromosome 6p21.3 and is polymorphic at several positions including -G308A, -G238A, -T1031C, -C863A, -C851T, -G419C, and -G376A [15]. Among these single nucleotide polymorphisms (SNPs), -G308A and -G238A lie within the promoter, and previous studies have shown that the -308, and possibly the -238, alleles are associated with the increased production of TNF-α in various inflammatory diseases [15,16]. Since TNF-α polymorphism was first suggested to be associated with the pathogenesis of alcoholic liver disease [17], several studies have been published supporting the hypothesis that TNF-α polymorphism might also be associated with the pathogenesis and progression of NAFLD [16,18-21]. However, as yet, there have been no related studies in pediatric populations.

Therefore, the aim of this study was to evaluate the role of TNF-α polymorphism and insulin resistance in the development of NAFLD in obese children, and possibly, the relationship between TNF-α polymorphism and insulin resistance in children with NAFLD.

MATERIALS AND METHODS

A total of 111 children who visited pediatric gastroenterology outpatient clinics were included. The subjects were divided into 3 groups according to their NAFLD status: group I, controls without NAFLD; group II, obese children with simple steatosis; and group III, obese children with NASH.

NAFLD was diagnosed in each patient based on elevated serum aminotransferase levels, abdominal ultrasonographic or computed tomographic findings, and histopathologic findings on liver biopsy [22]. Simple steatosis was defined as parenchymal involvement by steatosis of more than 5% of the liver without fibrosis or inflammation, and NASH as hepatic steatosis with lobular inflammation, ballooning of hepatocytes, and hepatic fibrosis [22].

Children with other causes of chronic liver disease including viral hepatitis, metabolic disease, autoimmune hepatitis, and children with a drug history were excluded from the study. There were no children with a history of alcohol intake.

Parents or legal guardians of the subjects gave their informed consent prior to the study. This study was approved by the institutional review board.

Anthropometric data collection

Clinical data on body weight and height were reviewed in each patient; body weight was measured to the nearest 0.1 kg and height to the nearest 0.1 cm. Body mass index (BMI) was calculated as the body weight (kg) divided by the square of the height (m^2). BMI percentile was determined according to the Korean national growth charts [23]. Obesity was defined as a BMI greater than the 95th percentile for the child's age and sex, and overweight as BMI between the 85th and 95th percentiles.

Biochemical tests and laboratory investigation

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured, and levels exceeding 40 IU/L were regarded as abnormal for both tests. Serum concentrations of fasting glucose, total cholesterol, triglycerides (TGs), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, C-reactive protein (CRP), γ-glutamyl transpeptidase (GGT), and insulin levels were measured after 12 h of fasting at the time of the liver biopsy. Insulin resistance was determined by the homeostatic model assessment of insulin resistance [HOMA-IR, insulin (mmol/L)×glucose (μU/mL)/22.5] [24].

For the differential diagnosis based on other causes of chronic hepatitis, creatine phosphokinase, lactate dehydrogenase, ammonia, lactate, pyruvate, anti-HAV IgM antibody, HBs antigen, and anti-HBs antibody, anti-HCV antibody, EBV VCA IgM antibody, CMV IgM antibody, serum ceruloplasmin, and anti-nuclear antibody levels were measured in all patients with elevated AST and ALT levels.

Serum TNF-α levels were measured by ELISA (human TNF-α ELISA kit; R&D Systems, Minneapolis, USA) using an anti-human TNF-α antibody.

Radiologic evaluation of NAFLD

The presence and the severity of fatty liver were evaluated in all subjects by abdominal sonography or abdominal computed tomography (CT). The degree of fatty liver on abdominal sonography was defined as mild, moderate, or severe [25]. Using non-contrast abdominal CT, the diagnosis of fatty liver was made when CTL (the CT number of the liver) was lower than CTS (the CT number of the spleen) by more than 10.

Histopathologic evaluation in NAFLD

Percutaneous needle liver biopsy was performed on all patients with elevated AST, ALT levels. The diagnosis of NAFLD was evaluated on the basis of the histopathologic grades of steatosis, inflammation, ballooning, and stages of fibrosis, according to the classification by Brunt [26] and Kleiner [22].

Genotyping of TNF-α polymorphisms

At least 3 cc of blood were obtained from the patients and the controls, and DNA was extracted using the QIAamp DNA Mini Kit (Applied Biosystems, Foster City, CA, USA). Extracted DNA was frozen at -20°C before testing.

For amplification of the TNF-α G308A locus, the sense and antisense primers used were 5'-CTGAAGC CCCTCCCAGTTCT-3' and 5'-CGGTTTCTTCTCCATCG CG-3', respectively [27]. For the TNF-α G238A locus, the sense and antisense primers were 5'-GGAG GCAATAGGTTTTGAGGG-3' and 5'-GGTTTCTTCTCC ATCGCGG-3', respectively [27].

For DNA template preparation, amplification of genomic DNA was performed on a PCR (polymerase chain reaction) thermal cycler in a final volume of 50 μL. The reaction mixture contained 1.25 pmol of each primer, 250 μM dNTP, 2.5 mM MgCl$_2$, 10× buffer, 0.15 unit Taq polymerase, and the genomic DNA. The PCR cycling conditions were denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s. PCR products were purified using a PCR purification kit (Roche Molecular Biochemicals, Indianapolis, IN, USA), to give purified DNA templates.

For genotyping of TNF-α promoter polymorphisms, a reaction mixture was prepared containing the PCR products, SNaPshot Multiplex Ready Reac-

tion Mix (ABI PRISM SNaPshot^{TM} Multiplex kit, Applied Biosystems, Foster, CA, USA), and SNaPshot primers (5'-ATATAATCATGATTATAATCAATGATGAT CAATAGGTTTTGAGGGGCATG-3' for the -G308A locus, and 5'-TAATCAATGATGATAAGACCCCCCTCGG AATC-3' for the -G238A locus). Thermal cycling conditions for amplification were 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s. After 25 cycles, the PCR mixture was treated with 1 unit of shrimp alkaline phosphatase at 37°C for 1 h to remove unincorporated ddNTPs and primers. The mixture was then incubated 75°C for 15 min to inactivate the enzyme.

For electrophoresis, 0.5 μL of the SNaPshot product, 0.5 μL Genescan-120 LIZ Size Standard (Applied Biosystems), and 9 μL Hi-Di formamide were mixed and vortexed. After denaturing at 95°C for 5 min, the samples were placed on ice. Electrophoresis was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The data were analyzed with the ABI PRISM GeneScan Analysis Software and Genotyper Software (Applied Biosystems).

Statistical analysis

The results are expressed as medians and ranges. The data were analyzed using the SPSS 18.0 software program (SPSS Inc., Chicago, IL, USA). Frequency data were compared using Fisher's exact test. Wilcoxon-Mann-Whitney rank sum test was used for the comparison of means in 2 groups, and Kruskal-Wallis test was used for 3 or more groups. Pearson's correlation was applied to evaluate the correlation between 2 variables. p-values less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

A total of 111 obese children (74 boys, 37 girls; age, 6.4 to 15.5 yrs; mean±SD=11.1±2.0 yrs) were divided into 3 groups according to their NAFLD status: group I, normal control subjects (n=61); group II, children with simple steatosis (n=17); and group III, children with NASH (n=33).

Clinical features and laboratory findings of the patients are listed in Table 1. Other than the AST and ALT levels, only the serum insulin levels and HOMA-IR showed statistically significant differences among three groups ($p=0.000$ & $p=0.001$) (Table 1).

Table 1. Clinical Manifestations and Laboratory Findings Among the 3 Groups Based on the Spectrum of NAFLD

	Group I	Group II	Group III	p-value*
	Controls (n=61)	Simple steatosis (n=17)	NASH (n=33)	
Sex (M : F)	36 : 25	10 : 7	28 : 5	0.016
Age (yrs)	10.5 (6.4-15.4)†	11.3 (9.0-15.5)	11.5 (8.7-15.0)	0.165
Number of Ob/Ow	27/34	16/1	27/6	0.000
AST (IU/L)	24 (13-43)	25 (16-34)	53 (27-224)	0.000
ALT (IU/L)	17 (6-49)	34 (11-46)	110 (51-357)	0.000
Fasting glucose (mg/dL)	94 (77-111)	93 (72-106)	92 (75-108)	0.703
Insulin (μU/mL)	10.8 (3.1-65.9)	14.4 (5.9-132.9)	23.5 (5.7-72.6)	0.000
Cholesterol (mg/dL)	175 (134-256)	183 (103-237)	192 (106-283)	0.174
CRP (mg/dL)	0.05 (0.01-0.34)	0.01 (0.01-0.44)	0.13 (0.01-0.42)	0.916
TNF-α (pg/mL)	21.0 (16.0-42.0)	19.0 (14.0-32.0)	21.0 (2.0-47.0)	0.664
HOMA-IR	2.8 (0.7-5.4)	3.3 (1.5-32.2)	6.1 (1.2-15.3)	0.001

*p-value less than 0.05 was regarded as statistically significant. †The values are expressed as medians (ranges). Ob: obese children with BMI ≥ 95 percentile, Ow: overweight with BMI between 85 and 95 percentile, AST: aspartate aminotransferase, ALT: alanine aminotransferase, BMI: body mass index, CRP: C-reactive protein, HOMA-IR: insulin resistance determined by homeostasis model assessment, NAFLD: non-alcoholic fatty liver disease, NASH: non-alcoholic steatohepatitis, TNF-α: tumor necrosis factor-α.

Table 2. Frequencies of TNF-α Promoter Polymorphisms at -308 and -238 Loci in Children with NAFLD and Controls

TNF-α polymorphism	Group I controls (n=61)	Groups II+III NAFLD (n=50)	p-value*
-308 locus			
GG genotype	59 (96.7%)	44 (88.0%)	
GA genotype	2 (3.3%)	3 (6.0%)	0.134
AA genotype	0 (0%)	3 (6.0%)	
G allele frequency	98.4%	91%	
A allele frequency	1.6%	9%	
-238 locus			
GG genotype	57 (93.4%)	42 (84.0%)	
GA genotype	4 (6.6%)	8 (16.0%)	0.133
AA genotype	0 (0%)	0 (0%)	
G allele frequency	96.7%	92%	
A allele frequency	3.3%	8%	

*p-value less than 0.05 was statistically significant when Fisher's exact test was applied. NAFLD: non-alcoholic fatty liver disease, TNF-α: tumor necrosis factor-α.

Comparison of TNF-α polymorphism between control subjects and NAFLD patients

No significant differences were observed in the TNF-α polymorphism at the -308 locus between the controls (group I) and children with NAFLD (groups II+III combined) (GG 96.7%, GA 3.3%, and AA 0% in group I; GG 88.0%, GA 6.0%, and AA 6.0% in groups II+III; p=0.134) (Table 2). No corresponding difference was found between the 2 groups at the -238 locus (GG 93.4%, GA 6.6%, AA 0% in group I; GG 84.0%, GA 16.0%, AA 0% in groups II+III; p=0.133) (Table 2). The frequency of the TNF -308 A allele was 1.6% in group I and 9% in groups II+III, and the frequency of the TNF -238 A allele was 3.3% in group I and 8% in groups II+III (Table 2).

Serum TNF-α levels, NAFLD, and TNF-α polymorphism

Serum TNF-α levels were not significantly different among the 3 groups, based on their NAFLD status (p=0.664) (Table 1). Moreover, no significant difference was found in the serum TNF-α levels among the genotypes at the -308 locus [median 20.5 pg/mL (range: 2.0-47.0 pg/mL) in
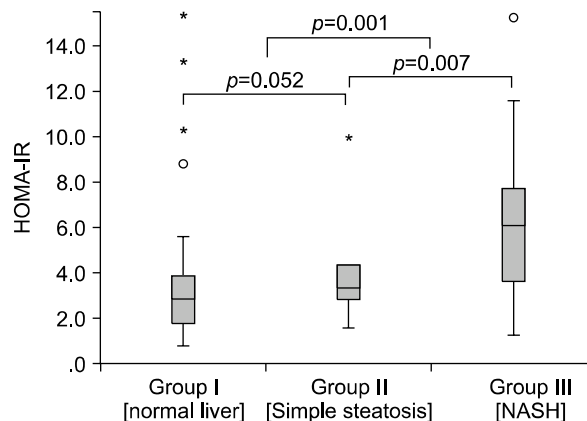

Fig. 1. The medians of HOMA-IR were significantly different among the 3 groups based on the status of NAFLD in obese children (p=0.001) (HOMA-IR: insulin resistance determined by homeostasis model assessment, NAFLD: non-alcoholic fatty liver disease, NASH: non-alcoholic steatohepatitis).

the GG genotype, 21.0 pg/mL (16.0-21.0 pg/mL) in the GA genotype, and 21.0 pg/mL (19.0-22.0 pg/mL) in the AA genotype; p=0.267], or at the -238 locus [median 21.0 pg/mL (range: 2.0-42.0 pg/mL) in the GG genotype and 22.0 pg/mL (16.0-47.0 pg/mL) in the GA genotype; p=0.703].

Relationship between insulin resistance and NAFLD or TNF-α polymorphism

The medians of HOMA-IR values were significantly different between the controls and children with NAFLD [median 2.8 (range: 0.7-15.4) in group I and median 4.6 (range: 1.2-32.2) in group II+III combined; p=0.001] (Fig. 1). No difference in medians of HOMA-IR was observed between group I and group II (p=0.052), and significant difference in medians of HOMA-IR was observed between group II and group III [median 3.3 (range: 1.5-32.2) in group II and median 6.1 (range: 1.2-15.3) in group III; p=0.007] (Fig. 1). The HOMA-IR values correlated significantly with the serum aminotransferase levels (AST, r=0.229, p=0.019; ALT, r=0.284, p=0.003).

No difference was observed in the medians of HOMA-IR values among the genotypes at the -308 locus [median 3.6 (range: 0.7-32.2) in the GG geno-

type, 2.8 (1.4-3.3) in the GA genotype, and 2.6 (2.5-3.6) in the AA genotype; $p=0.061$], or at the -238 locus [median 3.3 (range: 0.7-32.2) in the GG genotype and 5.3 (1.1-15.4) in the GA genotype; $p=0.207$] in children with NAFLD.

DISCUSSION

This study investigated the role of TNF-α promoter polymorphism and insulin resistance in the development of NAFLD in obese children. TNF-α is a well-known proinflammatory cytokine produced in the adipose tissue, and acts as a mediator of insulin resistance [10]. Recently, TNF-α has been suggested to play an important role in the pathogenesis of NAFLD, and may be involved in hepatocyte injury, apoptosis, inflammation, and hepatic stellate cell activation causing fibrosis in the liver, as well as in modulating insulin resistance in NAFLD patients [28].

Until now, only one previous study has been reported on TNF-α and NAFLD in the pediatric population [29], which showed that serum TNF-α levels correlated significantly with the histologic grading of NASH in children with NAFLD, and seemed to be predictive of NASH. However, we found no difference in serum TNF-α levels between the children with NAFLD and the control subjects, and serum TNF-α levels did not reflect the status of NAFLD. Furthermore, serum TNF-α levels did not reflect the severity of NASH in our study when histopathologic grading and staging of fibrosis were considered (steatosis, $p=0.165$; lobular inflammation, $p=0.770$; hepatocyte ballooning, $p=0.064$; fibrosis, $p=0.232$). In earlier studies, increased TNF-α mRNA expression was observed in hepatic tissue and peripheral adipose tissue of NASH patients, and increased expression of TNF-α was correlated with more advanced NASH [11]. Collectively, these findings suggest that future studies on children with NAFLD should look beyond serum TNF-α level, and possibly focus on the expression of TNF-α in hepatic and adipose tissue itself to understand the pathogenesis of NAFLD related to childhood obesity.

Recently, several studies have analyzed TNF-α polymorphism in adults with NAFLD [16,18-21]. As first reported by Valenti et al. [16], the prevalence of the -238 TNF-α polymorphism was higher in adults with NAFLD, and these patients also showed higher insulin resistance. Moreover, Aller et al. [21] reported that patients with the GA genotype at -308 locus had more severe portal inflammation and hepatic fibrosis than patients with the wild-type genotype, with significant elevations in serum TNF-α levels and HOMA-IR. In contrast, other studies have reported no significant differences in the prevalence of several TNF-α promoter polymorphisms, including those at the -308 and -238 loci, between patients with NAFLD and controls [18,19]. Therefore, even in adults, it remains unclear whether TNF-α polymorphism affects the development of NAFLD, and to date, no such studies have been performed in children with NAFLD.

When we analyzed TNF-α promoter polymorphism at -308 and -238 loci, we found no significant differences in polymorphisms at the -308 and the -238 loci between the controls and children with NAFLD. This result suggests that at least the TNF-α promoter polymorphisms at the -308 locus or the -238 locus are not associated with the development of NAFLD in pediatric populations.

Beyond TNF-α promoter polymorphisms, previous studies have suggested that insulin resistance might be a cause of NAFLD in obese patients [30], and to address this, we evaluated HOMA-IR as an indicator of insulin resistance. In our study, HOMA-IR was significantly different between the NAFLD group and the control subjects, and additionally, was significantly correlated with liver enzymes levels. Furthermore, we found a significant difference in HOMA-IR values between children with simple steatosis and children with NASH. Nevertheless, insulin resistance was not directly related to TNF-α promoter polymorphisms at the -308 or the -238 loci. These results suggest that insulin resistance might be an important factor in the pathogenesis of NAFLD in obese children, especially in the progression of steatosis to NASH, regardless of TNF-α promoter polymorphisms.

In conclusion, TNF-α promoter polymorphisms at the -308 and -238 loci were not significantly associated with the development of NAFLD in obese children. Nevertheless, insulin resistance remains a likely essential factor in the pathogenesis of NAFLD in obese children, especially in the progression to NASH. Therefore, insulin resistance should be considered as a possible link between NAFLD and childhood obesity beyond TNF-α promoter polymorphism.

ACKNOWLEDGEMENTS

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