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Original Article

Correlation between TLR4 expression and gene polymorphism in peripheral blood mononuclear cells and condition of nonalcoholic fatty acid disease in Han people of Shaanxi

Mo Yi[1], Jian Zhou[1], Hongzhou Sun[2], Liping Shi[1], Youwei Wu[1], Jian Zhang[1], Qian Chen[1]

[1]*Second Department of Gastroenterology, Shaanxi Provincial People's Hospital, Xi'an, Shaanxi, China;* [2]*Department of Cardiovascular Internal Medicine, Northern Hospital of Xi'an, Xi'an, Shaanxi, China*

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Abstract: Nonalcoholic fatty liver disease (NAFLD) is one metabolic disorder that may develop into liver cirrhosis and liver failure. Toll like receptor 4 (TLR4) signal pathway participate in NAFLD progression. The relationship between single nucleotide polymorphisms (SNPs) of TLR4 and peripheral expression, and NAFLD in Han people has not been fully illustrated. 150 NAFLD patients were recruited in our hospital in parallel with 150 healthy individuals as control. TLR4 mRNA expression was determined by real-time PCR from peripheral blood mononuclear cells (PBMCs). Patient's sex, age, BMI, wrist-hip ratio (WHR), biochemical/metabolic indexes and homeostatic insulin resistant index (HOMA-IR). Correlation between TLR4 expression and disease related index was analyzed. PCR-restriction fragment length polymorphism (RFLP) was used to test TLR4 genotype for gene polymorphism and disease correlation. NAFLD patients had elevated TLR4 expression in PBMCs, whose TLR4 level was positively correlated with clinical indexes but not age, sex or HDL. Genotype frequency at all four loci showed no significant difference or correlation with NAFLD. Locus rs41426344 and rs7873784 were correlated with NAFLD, as mutated allele increased NAFLD risk (OR 1.86, 95% CI, 1.17-2.97; OR 1.92, 95% CI 1.21-2.91, $P<0.05$). In NAFLD patients, TLR4 expression is elevated in PBMCs. Mutation at locus rs41426344 and rs7873784 of TLR4 gene is high risk factor of NAFLD.

Keywords: Nonalcoholic fatty liver disease, toll like receptor 4, genetic polymorphism

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one chronic progressive liver disease and belongs to metabolic disorder. Aggregation of fatty acid in hepatocyte causes denaturation, which may develop into liver cirrhosis or even liver failure. The incidence of NAFLD is widely elevated across all regions of China, including Shanghai, Guangdong and Shaanxi, where NALFD patient numbers are rapidly increasing [1, 2]. NAFLD consists of nonalcoholic fatty hepatitis, nonalcoholic fatty liver, fatty liver fibrosis and fatty liver cirrhosis [3]. Previous study indicated inheritance of NAFLD due to the existence of family history in certain patients, plus the interaction between genetics and environmental factors [4, 5]. In recent years, NAFLD patient number was rapidly increased, with correlation with country, region and ethnic groups [6]. It is estimated that about 20%~30% of adults in Western countries suffered from NAFLD, with relatively lower incidence in underdeveloped countries, which, however, show increasing trends of incidence [7]. NAFLD is related with genetic, nutrient, lipid metabolism, and insulin resistance factors. NAFLD susceptibility is highly correlated with genetic polymorphism [8, 9].

Toll like receptor (TLR4) is one member of Toll like receptor family, and is the major receptor for lipopolysaccharide (LPS) belonging to type I transmembrane signal transduction receptor [10, 11]. TLR4 exerts a critical role in innate immune response, and is expressed in Kupffer cells, hepatocyte, adipocyte, hepatic sinusoidal endothelial cells (HSECs), further participating in occurrence and progression of NAFLD [12,

13]. Entero-endotoxin leakage, overgrowth of bacterium and endotoxaemia are believed to be important for stimulating NAFLD occurrence. The cross-interaction between TLR4 and endotoxin are critical steps for releasing of inflammatory mediator after liver damage and liver fibrosis [14]. Genetic mutation of coding genes lead to various susceptibility of individuals for infection or disease. Single nucleotide polymorphisms (SNPs) of TLR4 gene is correlated with disease susceptibility [15]. Various SNPs of TLR4 gene leads to imbalance between pro-inflammatory and anti-inflammatory factors, further causing disease [16]. NAFLD also has geographic distribution patter, which is correlated with polymorphism of TLR4 gene [6]. This study thus investigated the correlation between polymorphism of TLR4 gene and peripheral TLR4 expression, and NAFLD in Han people of Shaanxi, China.

Materials and methods

Research objects

A total of 150 NAFLD patients who were diagnosed and admitted in Shaanxi Provincial People's Hospital from October 2013 to March 2014 were recruited. The diagnosis of NAFLD followed the guideline stipulated by Liver Disease Sub-committee, Chinese Medial Association [17]. There were 86 males and 64 females in the patient group, aging between 42 and 68 years (average age = 56±6.7 years). Individuals with any of those conditions were excluded: Long-term taking glucocorticoid hormones; Alcohol abuse; abnormal thyroid function; Fatty liver disease caused by virus, drug, auto-immunity, genetic liver disease or bile duct obstruction. Another 150 healthy individuals were recruited in the control group, including 82 males and 68 females, aging between 41 and 69 years (average age = 57±6.1 years). No significant difference of sex ratio or age existed between two groups, which were thus comparable. All research objects were Han people in Shaanxi province, China. This study has obtained informed consents from patients and their families, and has been approved by the ethical committee of Shaanxi Provincial People's Hospital.

Major reagent and equipment

Fasted insulin (FINs) ELISA kit was purchased from R&D (US). RNA extraction kit and reverse transcription kit were purchased from ABI (US). Human lymph tissue separation buffer (Histopaque-1077) was purchased from Sigma (US). DNA extraction kit was purchased from Qiagen (US). Electrophoresis apparatus was purchase from Liuyi Instrument (China). AU680 automatic biochemical analyzer was purchased from Beckman (Germany). DNA amplifier equipment was purchased from PE (US). Labsystem Version 1.3.1 microplate was purchased from Bio-Rad (US). ABI 7500 Real-time PCR was purchased from ABI (US).

General information and sample collection

Height, body mass of research objects were recorded for calculating body-mass index (BMI). Wrist circumference and hip circumference were measured to obtain wrist-hip ratio (WHR). Patients were fasted for 12 hours, after that fasted venous blood were collected from test blood glucose, liver function and lipid levels. PBMCs were isolated from the whole blood, along with DNA extraction. Automatic biochemical analyzer was used to test levels of serum total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), alanine transaminase (ALT), aspartate aminotransferase (AST), fasted blood glucose (FPG). Enzyme linked immunosorbent assay (ELISA) was used to quantify fasted insulin (FINS). Homeostatic insulin resistant index (HOMA-IR) = FPG X FINS/22.5.

Real-time PCR for TLR4 expression in PBMCs

Peripheral blood sample was centrifuged in density gradient for obtaining PBMCs by lymph tissue separation buffer. Trizol reagent was used to extract RNA from PBMCs. Following manual instruction of test kit, DNA reverse transcription was performed. Primers were designed based on Primer Premier 6.0 and were synthesized by Invitrogen (China) as shown in **Table 1**. Real-time PCR assay was performed on targeting genes under the following conditions: 55°C for 1 min, followed by 92°C for 30 s, 58°C for 45 s and 72°C for 35 s in 35 cycles. Data were collected and calculated for CT values of all samples and standards based on relative fluorescent intensity and GAPDH as the internal reference. Using CT values of standard samples as the evidence, a standard curve was plotted for semi-quantitative analysis using $2^{-\Delta Ct}$ method.

Table 1. Primer sequences

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	GAAGCTGAAGGTCGGAGTCA	GGAAGATGGTGATGGGATT
TLR4	GTGGAAGTTGAACGAATG	CCTGGCTTGAGTAGATAACA

Table 2. PCR primers for SNPs of TLR4 gene

SNPs	Primer	Primer sequence 5'-3'
rs10759932	Forward	GAAGTAGACTCTGCTGAGATGG
	Reverse	TATCAGTGTAGGAGGTCTGTGATG3
rs4986790	Forward	GGCCTCTTTCATCACAGACC3
	Reverse	AGATGCAGCAAAGCCAAAGT
rs4986791	Forward	GGTTGCTGTTCTCAAAGTGATTTTGGGAA
	Reverse	ACCTGAAGACTGGAGAGTGAGTTAATCT
rs41426344	Forward	GATTAGCATACTTAGACTACTACCTCCATG
	Reverse	GATCAACTTCTGAAAAAGCATTCCCAC
rs11536889	Forward	CCTGGCAAGTGGATCATTGAC
	Reverse	GGCCACTCCAGGTAGGTCTT
rs7873784	Forward	GCCTACTGGGTGGAGAACCTT
	Reverse	CCAGTTCATACTTGCACCACTC

Table 3. Analysis of clinical information, blood glucose, blood lipid and liver function indexes

Index	Control	NAFLD
Age (year)	57±6.1	56±6.7
M/F	82/68	86/64
BMI (kg/m^2)	22.2±2.1	26.7±3.8*
WHR	0.82±0.07	0.91±0.06*
AST (U/L)	20.6±7.6	24.8±11.9*
ALT (U/L)	24.0±12.5	32.1±15.4*
HDL (mmol/L)	2.02±0.91	2.04±0.98
LDL (mmol/L)	3.24±0.68	3.60±0.88*
TG (mmol/L)	2.02±1.61	3.41±2.72*
TC (mmol/L)	4.98±1.15	5.69±2.59*
FBG (mmol/L)	4.91±0.76	5.46±1.07*
FINS (mUI/L)	6.21±4.28	8.86±4.31*
HOMA-IR	1.31±0.96	2.22±1.39*

*P<0.05 compared to control group.

PCR-RFLP for detecting TLR4 gene polymorphism

Whole blood DNA extraction kit was used to purify DNA, whose quality was detected by spectrometry to measure absorbance (A) values at 260 nm and 280 nm. DNA was identified when 0D260/0D280 = 1.7-1.9. Primers were designed by Primer Premier 6.0 based on target gene sequence, and were synthesized by Invitrogen (China) as shown in **Table 2**.

In a 25 μL total volume of PCR system, we added 10 pmol primer, 5 pmol dNTPs, 3 u TaqDNA polymerase, and 1 μL DNA template (about 300 ng). PCR amplification products were digested in restriction endonuclease Nco I and Hinf I. Digestion products were separated by 3% agarose gel electrophoresis.

Statistics

SPSS 19.0 software was used for data analysis. Measurement data were presented by mean ± standard deviation (SD). Between-group-comparison was performed by t-test. Chi square test was used to compare allele frequency and genotype frequency, and to determine if gene distribution fitted Hardy-Weinberg equilibrium for allele frequency. The correlation between TLR4 and other parameters was analyzed by Spearman method. The correlation between TLR4 SNPs genotype and NAFLD was analyzed. The odd ratio (OR) and 95% confidence interval (CI) were calculated. A statistical significance was defined when P<0.05.

Results

Clinical information, blood glucose, blood lipid and liver function indexes of NAFLD patients

We analyzed blood glucose, blood lipid and liver function indexes in NAFLD patients and control group. Results showed significantly higher BMI, WHR, ALT, AST, LDL, TG, TC, FBG, FINS and HOMA-IR in NAFLD patients than those in control group (P<0.05). HDL had no statistically significant difference between groups (**Table 3**).

TLR4 mRNA expression in PBMCs of NAFLD patients

Real-time PCR was used to test TLR4 mRNA expression in PBMCs of NAFLD and control

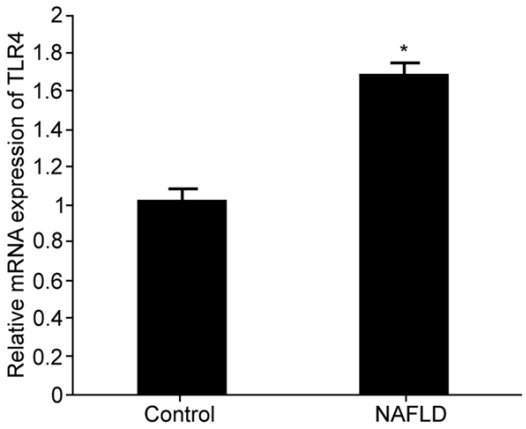

Figure 1. TLR4 mRNA expression in PBMCs of NAFLD and control groups. *P<0.05 compared to control group.

groups. Results showed significantly elevated TLR4 mRNA in PBMCs of NAFLD patients (P<0.05 compared to control group, **Figure 1**).

Correlation analysis between TLR4 and clinical indexes of NALFD patients

We further analyzed the correlation between TLR4 expression and clinical indexes in NAFLD patients. TLR4 was shown to be positively correlated with BMI, WHR, LDL, TG, AST, ALT, FBG, FINS and HOMA-IR (P<0.05), and negatively correlated with TC (P<0.05) but not with age, sex or HDL (**Table 4**).

TLR4 SNPs gene frequency analysis in NAFLD patients

PCR-RFLP was used to detect genotypes of TLR4 gene at various loci (rs10759932, rs4986790, rs4986791, rs41426344, rs11536889, rs7873784). Results showed 99.2% successful rate of SNP genotyping. All six SNPs alleles fitted Hardy-Weinberg equilibrium. Homozygous mutants and heterozygous at rs41426344 locus were CC and TC, respectively. Homozygous mutants and heterozygous at rs11536889 locus were CC and GC, respectively. Homozygous mutants and heterozygous at rs7873784 locus were AA and GA, respectively. Homozygous mutants and heterozygous at rs10759932 locus were CC and GC, respectively. Homozygous mutants and heterozygous at rs4986790 locus were GG and GA, respectively. Homozygous mutants and heterozygous at rs4986791 locus were GC and GG, respectively. After combining homozygous mutant of TLR4 SNPs and heterozygous, we found significant difference of genotype frequency between NAFLD and control group at locus rs41426344 and rs11536889, but not at locus rs10759932, rs4986790, rs4986791 or rs7873784 (**Table 5**).

Correlation analysis between TLR4 SNPs and NAFLD pathogenesis

Allele genotype at rs41426344 and rs11536889 (TC + CC or GC + CC) was correlated with NAFLD onset as they increased NALFD risk (OR 1.86, 95% CI, 1.17-2.97; OR 1.92, 95% CI, 1.21-2.91, P<0.05). The other four SNPs of TLR4 were uncorrelated with NAFLD pathogenesis and had no statistical significance (**Table 6**).

Discussion

During NAFLD progression, TLR4 can bind with its ligand, LPS, to participate in liver injury and repair related functions [18]. This study demonstrated that TLR4 mRNA expression was significantly elevated in PBMCs of NAFLD patients. Previous studies reported higher TLR4 expression under the induction of choline-deficient L-amino acid in nonalcoholic hepatitis (NASH), causing enhanced TNF-α expression, indicating that TLR4 could further induce liver damage [19], as consistent with our results. This study demonstrated significantly higher BMI, WHR, LDL, TG, AST, ALT, FBG, FINS and HOMA-IR levels in NAFLD patients, supporting the common factor of insulin resistance underlying obesity, diabetes, hypertension and NAFLD, indicating that NAFLD was one liver metabolic syndrome [20]. Further study about blood glucose and biochemical indexes related with NAFLD showed positive correlation between TLR4 and BMI, WHR, LDL, TG, AST, ALT, FBG, FINS and HOMA-IR, and negative correlation between TLR4 and TC, suggesting the participation of TLR4 in insulin resistance for further aggravation of liver dysfunction.

Among different people, TLR4 gene was found to have more than 10 loci of SNPs. Such SNPs of TLR4 gene was correlated with Gram-negative bacterial infection and fungal infection, in addition to NAFLD onset [21]. This study reported no statistical significance of genotype frequency at locus rs10759932, rs4986790, rs4986791 and rs7873784, which were uncor-

Table 4. Correlation analysis between TLR4 and clinical indexes in NAFLD patients

	Age	Sex	BMI	WHR	AST	ALT	HDL
R value	0.198	0.147	0.941*	0.782*	0.356*	0.417*	0.131
	LDL	TG	TC	FBG	FINS	HOMA-IR	
R value	0.526*	0.389*	-0.671*	0.289*	0.251*	0.267*	

*P<0.05 compared to control group.

Table 5. Gene frequency of TLR4 SNPs in NAFLD patients

Group	rs10759932			rs4986790			rs4986791		
	GG	GC	CC	AA	GA	GG	CC	GC	GG
Control	13.7	31.1	55.2						
NAFLD	17.4	57.8	22.4						
Group	rs7873784			rs41426344			rs11536889		
	GG	GA	AA	TT	TC	CC	GG	GC	CC
Control	64.9	28.0	7.1	58.8	37.7	3.5	25.2	42.6	32.2
NAFLD	64.1	34.9	1.0	47.6	47.6	8.7	17.1	52.1	31.8

Table 6. Correlation analysis between TLR4 SNPs and NAFLD pathogenesis

SNPs	OR	95% CI
rs41426344	1.86	1.17-2.97
rs11536889	1.92	1.21-2.91
rs10759932	0.82	0.92-1.85
rs4986790	0.61	1.12-2.43
rs4986791	0.45	1.45-2.66
rs7873784	0.56	1.26-2.15

related with NAFLD pathogenesis. Locus rs41426344 and rs11536889 are correlated with NAFLD onset, as elevation of mutant alleles increases NAFLD risk. Previous study reported the inhibition of post-transplant recurrence of hepatitis B in Chinese Han people by C-allele at rs11536889 of TLR4 gene [22], indicating the possible relationship between mutation at rs11536889 and liver disease progression. Locus rs41426344 of TLR4 gene has been shown to be correlated with type II diabetes, as one important SNP of metabolic disorder [23]. Our results further supported this opinion. SNPs at locus rs4986790 and rs4986791 of TLR4 gene were correlated with lower LPS response in airway ductal epithelial cells. Locus rs10759932 and rs7873784 of TLR4 gene were found to be correlated with sepsis or periodontitis or other inflammations [24, 25]. There four SNPs, however, are not involved in NAFLD progression in our study.

Conclusion

Elevation of TLR4 expression, plus mutant allele at rs41426344 and rs11536889 loci of TLR4 gene were found to be high risk factors of NAFLD. This study helps to illustrate the functional role and molecular mechanism of TLR4 in NAFLD pathogenesis, and providing new evidences for drug target of NAFLD treatment.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Mo Yi, Second Department of Gastroenterology, Shaanxi Provincial People's Hospital, 256 West Youyi Road, Xi'an, Shaanxi, China. Tel: +86-29-85251331; Fax: +86-29-85251331; E-mail: moyiqwe@163.com

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