

Basic Study

Genetic polymorphism in *CD14* gene, a co-receptor of TLR4 associated with non-alcoholic fatty liver disease

Shweta Kapil, Ajay Duseja, Bal Krishan Sharma, Bhupesh Singla, Anuradha Chakraborti, Ashim Das, Pallab Ray, Radha K Dhiman, Yogesh Chawla

Shweta Kapil, Ajay Duseja, Bal Krishan Sharma, Bhupesh Singla, Radha K Dhiman, Yogesh Chawla, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Anuradha Chakraborti, Experimental Medicine and Biotechnology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Ashim Das, Histopathology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Pallab Ray, Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Author contributions: Kapil S and Duseja A conceived the study, performed all analyses, interpreted data, and prepared the manuscript; Kapil S performed all molecular biological tests; Sharma BK and Singla B assisted with the collection of samples and sequencing data analysis; Chakraborti A and Ray P helped interpret the data and assisted in preparing the manuscript; Das A performed all histological analyses and reviewed the manuscript; Dhiman RK and Chawla Y participated in the study's design and critically reviewed the manuscript.

Supported by Indian Council of Medical Research (ICMR), New Delhi, No. 5/4/3-7/2009-NCD-II.

Institutional review board statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional (PGIMER, Chandigarh) and/or national research committee, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the ethical committee of PGIMER, Chandigarh (Ethics committee No. 7923-1Trg-08/2281 dated 21/1/10).

Informed consent statement: Informed consent was obtained from all individual participants included in the study.

Conflict-of-interest statement: All authors have no conflict of interest.

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Manuscript source: Invited manuscript

Correspondence to: Dr. Ajay Duseja, MD, DM, MNAMS, FACG, Department of Hepatology, Postgraduate Institute of Medical Education and Research (PGIMER), Sector-12, Chandigarh 160012, India. ajayduseja@yahoo.co.in
Telephone: +91-172-2756336
Fax: +91-172-2744401

Received: March 31, 2016
Peer-review started: April 6, 2016
First decision: May 30, 2016
Revised: August 3, 2016
Accepted: September 14, 2016
Article in press: September 14, 2016
Published online: November 14, 2016

Abstract

AIM

To evaluate the pathogenic role of toll-like receptor (TLR) gene polymorphisms in patients with non-alcoholic fatty liver disease (NAFLD).

METHODS

Two hundred and fifty subjects (NAFLD = 200, healthy volunteers = 50) underwent polymerase chain reaction and restriction fragment length polymorphism to assess one polymorphism in the toll-like receptor 2

(*TLR2*) gene (A753G), two polymorphisms in the *TLR4* gene (TLR4 Asp299Gly and Thr399Ile allele), and two polymorphisms in the cluster of differentiation 14 (*CD14*) (C-159T and C-550T) gene, a co-receptor of TLR4. Association of *TLR* gene polymorphisms with NAFLD and its severity was evaluated by genetic models of association.

RESULTS

On both multiplicative and recessive models of gene polymorphism association, there was significant association of CD14 C (-159) T polymorphism with NAFLD; patients with TT genotype had a 2.6 fold increased risk of developing NAFLD in comparison to CC genotype. There was no association of TLR2 Arg753Gln, TLR4 Asp299Gly, Thr399Ile, and CD14 C (-550) T polymorphisms with NAFLD. None of the TLR gene polymorphisms had an association with histological severity of NAFLD.

CONCLUSION

Patients with CD14 C (-159) T gene polymorphism, a co-receptor of TLR4, have an increased risk of NAFLD development.

Key words: Non-alcoholic steatohepatitis; Non-alcoholic fatty liver disease; Toll-like receptors; Obesity; Cirrhosis; Insulin resistance; Bacterial overgrowth

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Core tip: Our study demonstrated that non-alcoholic fatty liver disease (NAFLD) patients with TT genotype of C (-159) T polymorphism in cluster of differentiation 14 promoter gene have a higher risk of NAFLD development. However, this polymorphism did not affect liver disease severity. We found no association of toll-like receptor (TLR) 2 ARG753, TLR4 (Asp299Gly), TLR4 (Thr399Ile), and CD 14 C/T 550 polymorphisms with the risk of NAFLD development.

Kapil S, Duseja A, Sharma BK, Singla B, Chakraborti A, Das A, Ray P, Dhiman RK, Chawla Y. Genetic polymorphism in *CD14* gene, a co-receptor of TLR4 associated with non-alcoholic fatty liver disease. *World J Gastroenterol* 2016; 22(42): 9346-9355 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i42/9346.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i42.9346>

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a spectrum condition, ranging from simple steatosis to its progressive form of non-alcoholic steatohepatitis (NASH), and has emerged as an important cause of the otherwise unexplained increase in hepatic transaminases, cryptogenic cirrhosis, and cryptogenic

hepatocellular carcinoma (HCC)^[1-3]. Familial studies and inter-ethnic variation in susceptibility to the disease suggest that genetic factors are important in the occurrence and determining the risk of progressive NAFLD^[4]. Studies suggests a strong association of NAFLD with patatin-like phospholipase domain containing 3 gene polymorphism^[5-7], as well as inconclusive association with apolipoprotein C-III^[8,9] and human hemochromatosis gene mutations^[10].

Emerging data suggest the role of small intestinal bacterial overgrowth (SIBO) and gut-derived endotoxins in the pathogenesis of NAFLD *via* inducing the release of proinflammatory cytokines, including tumor necrosis factor- α (TNF- α) from hepatic Kupffer cells.

Toll-like receptors (TLRs) are the most important family of pattern recognition receptors^[11,12]; they are the sensors for recognizing bacterial and viral components, such as lipopolysaccharides, bacterial DNA, and peptidoglycan. Of the various TLRs, toll-like receptor 2 (TLR2), TLR4, the co-receptor cluster of differentiation 14 (CD14), and TLR9 have been well studied in the pathogenesis of NAFLD. In addition to animal studies, two human studies^[13,14] have also suggested a higher risk of developing NASH in patients with NAFLD that possess *CD14* gene polymorphism.

The aim of our study was to evaluate the role of TLR polymorphisms in the causation and severity of NAFLD.

MATERIALS AND METHODS

Patients

This was a case-controlled study where 250 subjects [NAFLD ($n = 200$, males = 122, mean age 38.27 ± 10.3 years) and healthy volunteers (HVs) ($n = 50$, males = 38, mean age 36.56 ± 4.2 years)] were enrolled after informed consent. The study had the approval of the Institute's Ethics Committee. All subjects were enrolled prospectively, with the exception of 28 patients with biopsy-proven NAFLD; their records were retrieved from the existing database and were called again for consent and fresh sampling for TLR polymorphisms. Patients with NAFLD were recruited as per the standard inclusion criteria. Fifty age and gender matched HVs were recruited, as per the guidelines from the Indian Council of Medical Research, and they all had normal liver function tests, normal fasting plasma glucose, normal lipid profile, and no evidence of fatty liver on ultrasound.

Inclusion criteria for NAFLD: (1) age greater than 13 years; (2) non-alcoholic individuals, defined as either total abstainers or individuals who consumed less than 20 g of alcohol per day. History of alcohol consumption was confirmed by two family members of the patient; (3) raised serum transaminases more than one and a half times the upper limit of normal for at least 3 mo; (4) ultrasound showing

features of steatosis; (5) negative viral markers (HBsAg/Anti HCV), negative autoimmune markers [antinuclear antibody (ANA), anti-smooth muscle antibody, anti-liver kidney microsomal antibody, and anti-mitochondrial antibody (AMA)]; (6) normal ceruloplasmin/negative Kayser-Fleischer rings; (7) normal iron work up [serum iron, total iron binding capacity (TIBC), ferritin, and transferrin saturation]; and (8) liver biopsy consistent with NAFLD (60 cases where liver biopsy was performed).

Exclusion criteria for NAFLD: (1) pregnant females; (2) patients with history of drug intake likely to cause NAFLD (e.g., corticosteroids, methotrexate, and tamoxifen); (3) jejunioileal bypass or extensive small bowel resection; (4) total parenteral nutrition at the time of liver biopsy; and (5) clinical, imaging, or liver biopsy features of liver cirrhosis.

Anthropometry

Height was determined with a measuring tape to the nearest cm. Subjects were requested to stand upright without shoes with their heels tight against the wall and eyes directed forward. Weight was measured in kilograms (kg) with a traditional spring balance, which was kept on a firm horizontal surface and the scale checked every day. Body Mass Index (BMI) was calculated using the formula: weight (kg)/height (m^2). Waist circumference was taken as the average of two measurements taken after inspiration and after expiration at the midpoint between the lowest rib and the iliac crest. Hip circumference was taken at the level of greater trochanter and waist-to-hip ratio was defined as the ratio of the waist and hip circumference.

Patients were classified as being lean, overweight, class I obese, class II obese, or centrally obese as per the Asian Pacific criteria (lean: BMI = 18-23 kg/ m^2 ; overweight: $> 23 < BMI < 25$ kg/ m^2 ; class I obesity: BMI ≥ 25 -30 kg/ m^2 ; class II obesity: BMI > 30 kg/ m^2 ; central obesity: waist circumference > 90 cm in males and > 80 cm in females)^[15,16].

Biochemical assessment

All patients with NAFLD underwent detailed baseline investigations, with selective investigations performed with HVs. In patients undergoing liver biopsy (32 = prospective, with 28 retrieved from the existing database), laboratory parameters were measured before the procedure. Serum bilirubin, aspartate aminotransferases, alanine aminotransferases (ALT) (Diagnosticum Rt., Budapest, Hungary), alkaline phosphatase (Reckon Diagnostics, Baroda, India), albumin, globulin (Far Diagnostics, Verona, Italy), total cholesterol, triglycerides (TG), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol (Roche Diagnostics, Indianapolis, United States) were determined as per the standard

methodology. A fasting plasma glucose of > 126 mg/dL on more than one occasion or a random plasma glucose of > 200 mg/dL in a symptomatic patient was defined as diabetes mellitus. Fasting plasma glucose of > 110 and < 126 mg/dL was defined as impaired fasting glucose and 2 h post-prandial plasma glucose between 140 and 200 mg/dL as impaired glucose tolerance. Lipid profile was determined in all patients and serum cholesterol > 200 mg/dL, HDL < 40 mg/dL in males and < 50 mg/dL in females, LDL > 130 mg/dL, and serum TG > 150 mg/dL was taken as abnormal. Serum iron and TIBC were measured by the colorimetric method and serum ferritin was measured using an enzyme immunoassay kit (Orgentec Diagnostika GmbH, Germany). Virological markers, such as HBV (HBsAg, HBeAg), and HCV (anti-HCV), and auto immune markers including ANA anti-smooth muscle antibodies, anti-liver-kidney microsomes, and AMA were determined using enzyme-linked immunosorbent assay (ELISA).

Imaging

All patients with NAFLD and CVH, as well as the HVs, were subjected to an abdominal ultrasound to detect and grade the degree of hepatic steatosis.

Metabolic syndrome

Metabolic syndrome was defined by the presence of ≥ 3 out of 5 modified adult treatment panel III criteria, including modified abnormal waist as per the Asia Pacific criteria, FPG > 110 mg/dL or known diabetes, hypertension (blood pressure $\geq 130/85$ mmHg or on antihypertensive drugs), serum TG > 150 mg/dL, and HDL < 40 mg/dL in males or < 50 mg/dL in females^[17].

Histopathology

Sixty patients with NAFLD (32 recruited prospectively and 28 retrieved from database) were histologically assessed for degree of hepatic steatosis and fibrosis, and then divided into NASH, borderline NASH and no-NASH, as per the NAFLD activity score (NAS) given by the Nonalcoholic Steatohepatitis Clinical Research Network^[18].

Polymorphisms in the genes encoding for receptors (TLR2, TLR4, CD14)

Polymorphisms in the genes encoding for receptors (TLR2, TLR4, and CD14) were detected by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) to assess one polymorphism in the TLR2 gene (Arg753Gln), two in the TLR4 gene (TLR4 Asp299Gly and Thr399Ile allele), and two polymorphisms in the CD14 gene (C-159T and C-550T).

PBMC and genomic DNA isolation: PBMC were isolated from whole blood samples using Ficoll-Hypaque differential density gradient centrifugation procedures.

Table 1 Primer sequences, polymerase chain reaction conditions, and product sizes of different genes

Polymorphism site	Primer sequence (5'-3')	PCR conditions	Product size (bp)
TLR4 (Asp299gly)	F: GATTAGCATACTTAGACTACTACCTCCATG R: GATCAACITCTGAAAAAGCATTCAC	(1) 95 °C for 10 min (2) 35 cycles of : 94 °C for 1 min 61.5 °C for 1 min 72 °C for 1 min (3) 72 °C for 7 min	249
TLR4 (Thr399Ile)	F: GGTTCGTCTCTCAAAGTGATTTGGGAGAA R: CCTGAAGACTGGAGAGTGAGTTAAATGCT	(1) 95 °C for 10 min (2) 35 cycles of : 94 °C for 1 min 64.8 °C for 1 min 72 °C for 1 min (3) 72 °C for 10 min	406
TLR2 (Arg753Gln)	F: 5'-CCTTCAAGTGTGTCTTCATAAG-3' R: 5'-GGCCACTCCAGGTAGGTCTT-3'	(1) 95 °C for 10 min (2) 35 cycles of : 94 °C for 1 min 58.6 °C for 1 min 72 °C for 1 min (3) 72 °C for 10 min	289
CD14 (-550C/T)	F: GGAAGGGGAATTTTCTTTAGGC R: -GGCAGTGTCTGATGACTCA	(1) 95 °C for 10 min (2) 32 cycles of : 94 °C for 1 min 59.8 °C for 1 min 72 °C for 1 min (3) 72 °C for 7 min	368
CD14 (-159C/T)	F: ATCATCCTTTTCCACACC R: AACTCTTCGGCTGCCTCT	(1) 95 °C for 10 min (2) 35 cycles of : 94 °C for 40 s 61 °C for 40 s 72 °C for 40 s (3) 72 °C for 10 min	296

Table 2 Restriction fragment length polymorphism conditions

Polymorphic site	Restriction enzyme (units used)	Incubation temperature and duration	Genotype and restriction fragment pattern (bp)
TLR4 (Asp299gly)	NcoI	37 °C for 16 h	AA: 249 AG: 249, 223, 26 GG: 223, 26
TLR4 (Thr399Ile)	HinfI	37 °C for 8 h	CC: 406 CT: 406, 377, 29 TT: 377, 29
TLR2 (Arg753Gln)	AciI	37 °C for 16 h	GG: 252 and 37 GA: 252, 37, 289 AA: 289
CD14 (-550C/T)	HaeIII	37 °C for 12 h	CC: 23, 236, 109 CT: 23, 236, 109, 259 TT: 109, 259
CD14 (-159C/T)	HaeIII	37 °C for 16 h	CC: 141, 154 CT: 141, 154, 296 TT: 296

Genomic DNA was extracted from cells in the buffy coat using QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was used for detecting polymorphisms in different genes *via* PCR and RFLP.

PCR: Genomic DNA was amplified at specific regions containing the polymorphic sites using specific primer pairs flanking the respective polymorphic sites^[19,20]. A list of the PCR's conducted primer sequences, reaction

conditions, and product sizes are given in Table 1.

RFLP: PCR products were digested with appropriate restriction endonucleases to differentiate different genotypes. A list of restriction enzymes, incubation temperatures and times, and RFLP patterns of different genotypes are given in Table 2.

DNA sequencing

PCR products for all genotypes of all genes were validated commercially by DNA sequencing.

Estimation of adipocytokines and insulin resistance

Serum levels of various cytokines viz., adiponectin, TNF- α , and interleukin-1 β were estimated by (ELISA, Ray Biotech, Norcross GA.). Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) was calculated as the product of fasting insulin (μ U/mL) (Roche Diagnostics GmbH, Mannheim, Germany) and fasting plasma glucose (mmol/L) divided by 22.5. An absolute value of HOMA-IR > 1.64 was taken as abnormal^[21].

Statistical analysis

Data were analyzed for comparison between the two groups using SPSS version 15 for Windows (SPSS Inc. Chicago IL United States). Skewed clinical and biochemical data were expressed as a median (interquartile range), whereas normally-distributed

Table 3 Anthropometric and biochemical characteristics of patients with non-alcoholic fatty liver disease and healthy volunteers *n* (%)

Parameters	NAFLD (<i>n</i> = 200)	HVs (<i>n</i> = 50)	<i>P</i> value
Mean age (yr)	38.27 ± 10.3	36.56 ± 4.2	0.218
Gender	122 M/78 F	38 M/12 F	
Mean BMI (kg/m ²)	27.16 ± 4.7	22.1 ± 1.2	0.0002
Lean	35 (17.5)	46 (92)	0.0001
Overweight	39 (19.5)	3 (6)	0.02
Class I obesity	87 (43.5)	1 (2)	0.0001
Class II obesity	39 (19.5)	0 (0)	0.003
Waist (cm)	91.20 ± 9.5	78.12 ± 4.6	0.0001
Hip (cm)	91.32 (89.90-93.94)	89.18 (86.18-93.98)	0.3
Waist/hip ratio	0.99 (0.98-1.01)	0.93 (0.89-0.97)	0.016
Central obesity	135 (67.5)	0 (0)	0.0001
Mean AST (IU/L)	60.27 (46.0-78.7)	22.9 (18.75-29.50)	0.0001
Mean ALT (IU/L)	88.04 (68.9-118.9)	24 (17-29.5)	0.0001
Mean fasting sugar (mg/dL)	94.95 (87-105.8)	83.50 (75-89.25)	0.0001
Diabetes mellitus	29 (14.5)	0 (0)	0.005
Mean HDL (mg/dL)	43 (38-48.9)	52 (47.5-56)	0.0001
Mean Triglyceride (mg/dL)	156 (126-200.2)	102 (93-127.5)	0.0001
Hypertension	48 (24)	0 (0)	0.0001

NAFLD: Non-alcoholic fatty liver disease; HVs: Healthy volunteers; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HDL: High-density lipoprotein.

variables were expressed as mean ± SD. For continuous data, groups were tested for normal distribution using the Kolmogorov-Smirnov test (K-S test). For skewed data, the Mann-Whitney *U* test was applied for comparison between the two groups. For categorical data, the Chi-square test or Fischer exact test was applied. Genotypic association and the odds ratio with 95%CI were estimated by binary or multinomial logistic regression analysis. Haplotype analysis was carried out for *TLR4* and *CD14* gene using SHEsis software. In all cases, a *P* value less than 0.05 was considered significant.

Models and measures of association of gene polymorphisms: Recessive and dominant models were applied to determine the association of these polymorphisms with the recessive and dominant alleles; a recessive model indicates that two copies of allele A are required for a γ -fold increase in disease risk, while a dominant model indicates that either one or two copies of allele A are required for a γ -fold increase in disease risk^[22,23]. Patients with and without significant polymorphisms were compared to assess the difference between the two groups.

RESULTS

The demographic, anthropometric, and biochemical characteristics of the two groups (NAFLD and HVs) are summarized in Table 3.

Patients with NAFLD had higher BMI, higher overall and central obesity, elevated liver enzymes, and a higher prevalence of diabetes mellitus and dyslipidemia in comparison to HVs (Table 3). In the NAFLD group, 78 patients (39%) displayed the presence of metabolic syndrome.

Polymorphism analysis of *TLR2* gene

TLR2 Arg753Gln polymorphism: A PCR product size of 289 bp was subjected to restriction digestion with ACI 1 restriction enzyme at 37 °C to get the respective band size of 289 bp, 252 bp, and 37 bp, depending upon the deletion of restriction sites by mutant allele. GG was found to be the predominant genotype (89.5%), followed by GA (7.5%) and AA (3%) in NAFLD patients and GG (90%), GA (10%) and AA (0%) in HVs. There was no difference in GG [179/200 (89.5%) vs 45/50 (90%), *P* = 0.91] GA [15 (7.5%) vs 5 (10%), *P* = 0.56] and AA [6/200 (3%) vs 0/50 (0%), *P* = 0.21] genotypes in either study group. No association of TLR2 Arg753Gln polymorphism with NAFLD was found on multiplicative, dominant, or recessive models of analysis.

Polymorphism analysis of *TLR4* gene

TLR4 Asp299Gly polymorphism: TLR4 Asp299Gly polymorphism in exon 4 is an A/G polymorphism that creates a recognition site for restriction enzyme NcoI. Amplification with primer pairs yielded a 249 bp fragment. Respective band sizes of 249 bp, 223 bp, and 26 bp, depending upon the restriction sites created by mutant allele, were obtained. Digestion with enzyme gave a 249 bp fragment in the presence of AA genotype, three bands of 249 bp, 223 bp, and 26 bp in the presence of AG genotype, and two bands of 223 bp and 26 bp fragments in presence of GG genotype. The genotype frequency of AA, AG, and GG were 79%, 17%, and 4%, respectively, in the NAFLD group and 82%, 18%, and 0%, respectively, in the HV group. Distribution was in accordance with Hardy-Weinberg equilibrium. The AA, AG, and GG genotype were not statistically different in NAFLD compared to HVs (*P* = 0.15). No association of TLR4 Asp299Gly

Table 4 Distribution of CD14 -550C/T genotype, allele frequency and genetic models for CD14-550C/T *n* (%)

CD14 C (-550) T genotypes	CC	CT	TT
Cases (<i>n</i> = 200)	120 (60)	65 (32.5)	15 (7.5)
Control (<i>n</i> = 50)	34 (68)	14 (28)	2 (4)
OR	1	1.3 (0.6-2.6) <i>P</i> = 0.43	2.1 (0.4-9.7) <i>P</i> = 0.322
Genetic models			
Multiplicative model	Allele C	Allele T	
Cases	305 (76.25)	95 (23.75)	<i>P</i> = 0.21
Control	82 (82)	18 (18)	OR = 1.41 CI: 0.81-2.48
Dominant model	CC	CT + TT	
Cases	120 (60)	80 (40)	<i>P</i> = 0.29
Control	34 (68)	16 (32)	OR = 0.7 CI: 0.3-1.3
Recessive model	TT	CC + CT	
			<i>P</i> = 0.37 OR = 1.9 CI: 0.4-8.8

polymorphism with NAFLD was found on multiplicative, dominant, or recessive models of analysis.

TLR4 Thr399Ile polymorphism: Our findings demonstrated a PCR product size of 406bp for TLR4 Thr399Ile gene. After restriction digestion of PCR product with HinfI restriction enzyme at 37 °C, respective band sizes of 406 bp, 377 bp, and 29 bp were obtained depending upon the creation of restriction sites by mutant allele. CC was found to be the predominant genotype (83%), followed by CT (12.5%) and TT (4.5%) in NAFLD patients vs CC (84%), and by CT (16%) and TT (0%) in HVs.

Among TLR4 Thr399Ile polymorphisms, the frequency of CC [166/200 (83%) vs 42/50 (84%), *P* = 0.86], CT [25 (12.5%) vs 8 (16%), *P* = 0.51], and TT [(9 (4.5%) vs 0 (0%), *P* = 0.12] genotypes were not different among patients with NAFLD and HVs. There was no difference in T allele frequency between the NAFLD or HV groups (10.75% vs 8%, *P* = 0.41), and no association of TLR4 Thr399Ile polymorphism with NAFLD was found on multiplicative, dominant, or recessive models of analysis.

Haplotype analysis for TLR4 Asp299Gly and TLR4 Thr399Ile polymorphism: A haplotype comprised of a combination of alleles present on the same chromosome. Haplotype analysis revealed that haplotypes AC, AT, GC, and GT were not associated with an increased risk of NAFLD.

Polymorphism analysis of CD14 gene

CD14 C (-550) T polymorphism: CD14 C (-550) T is a C/T polymorphism that creates a recognition site for restriction enzyme Hae111. Amplification with primer pairs yielded a 368 bp fragment. Respective band sizes of 259, 236, 109, and 23 bp were obtained

depending upon the deletion of restriction sites by mutant allele. Digestion with enzyme gave 23, 236, and 109 fragments in the presence of CC genotype, four bands of 23, 236, 109, and 259 in the presence of CT genotype, and two bands of 109 bp and 259 bp fragments in the presence of TT genotype. The genotype frequency of CC, CT, and TT was 60%, 32.5%, and 7.5%, respectively, in the NAFLD group and 68%, 28%, and 4%, respectively, in the HV group (Table 3). Distribution was in accordance with Hardy-Weinberg equilibrium. The TT genotype was not significantly overrepresented in NAFLD (*P* = 0.322) compared to HVs.

Among CD14 C (-550) T polymorphisms, the frequency of CC [120/200 (60%) vs 34/50 (68%)], CT [65 (32.5%) vs 14 (28%), OR = 1.3 (0.6-2.6), *P* = 0.43], and TT [(15 (7.5%) vs 2 (4%), OR = 2.1 (0.4-9.7), *P* = 0.322] genotypes were not different between patients with NAFLD and HVs. There was no difference in T allele frequency between the NAFLD or HV groups (23.75% vs 18%, *P* = 0.21) and no association of CD14 C (-550) T polymorphism with NAFLD was found on multiplicative, dominant, or recessive models of analysis (Table 4).

CD14 C (-159) T polymorphism

CD14 C (-159) T polymorphism is a C/T polymorphism that creates a recognition site for restriction enzyme Hae111. Amplification with primer pairs yielded a 296 bp fragment. Respective band sizes 259, 236, 109, and 23 bp were obtained depending upon the deletion of restriction sites by mutant allele. Digestion with enzyme gave a 296 bp fragment in the presence of CC genotype, three bands of 296, 154, and 141 in the presence of CT genotype, and two bands of 154 bp and 141 bp fragments in the presence of TT genotype (Figure 1A and Table 5).

There was no difference in the CC [36 (18%) vs 10 (20%), *P* = 0.74] genotype between patients with NAFLD and HVs. Even though there was difference in the CT [70/200 (35%) vs 30 (60%), *P* = 0.001] genotypes between patients with NAFLD and HVs, the CT genotype was not associated with an increased risk of NAFLD (Table 2). Distribution was in accordance with Hardy-Weinberg equilibrium. The TT genotype [94/200 (47%) vs 10/50 (20%), *P* = 0.0005] and T allele frequency (64% vs 50%, *P* = 0.007) were significantly higher among patients with NAFLD than HVs, with significant association of C (-159) T polymorphism with NAFLD on multiplicative (*P* = 0.007) and recessive models (*P* = 0.0005). The risk of developing NAFLD with the TT genotype was 2.6 fold higher than in CC genotypes (Table 5).

DNA sequencing

The DNA sequencing data for CD14 C (-159) T polymorphism confirmed our PCR-RFLP findings, wherein

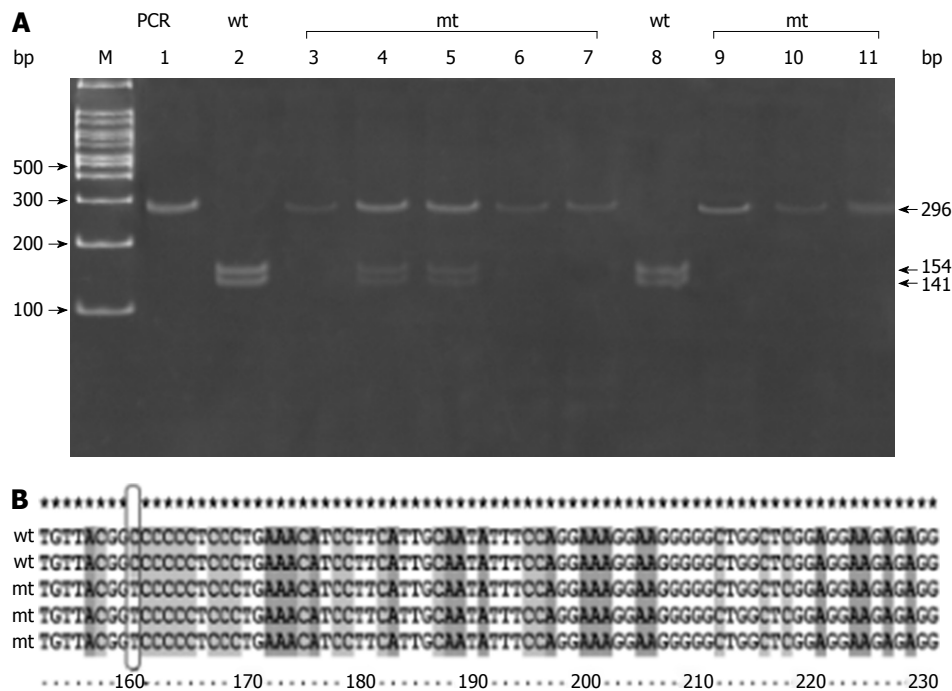


Figure 1 Polymorphism analysis of C14-159C/T. A: Representative PAGE gel picture of PCR-RFLP assay with restriction digested products. Lane M: 100 bp DNA ladder; Lanes 1-8: RE digested product, CC: 141 bp and 154 bp (Lanes 2, 8), CT: 296 bp, 141 bp, and 154 bp (Lanes 4 and 5), TT: 296 bp (Lanes 1, 3, 6, and 7); Lanes 9-11: PCR product; B: DNA sequencing showing different genotypes. Gap indicates the SNP or mutation (C→T). wt: Homozygous wild; mt: Homozygous or heterozygous mutant; PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism.

Table 5 Distribution of CD14 -159C/T genotype, allele frequency and genetic models for CD14-159C/T n (%)			
CD14 C (-159) T Genotypes	CC	CT	TT
Cases (n = 200)	36 (18)	70 (35)	94 (47)
Control (n = 50)	10 (20)	30 (60)	10 (20)
OR	1	0.6 (0.2-1.4)	2.6 (1-6.7)
		P = 0.29	P = 0.043
Genetic models			
Multiplicative model	Allele C	Allele T	
Cases	142 (35.50)	258 (64.50)	P = 0.007
Control	50 (50)	50 (50)	OR = 0.8
			CI: 1.1-2.8
Dominant model	CC	CT + TT	
Cases	36 (18)	164 (82)	P = 0.74
Control	10 (20)	16 (32)	OR = 0.7
			CI: 0.4-1.9
Recessive model	TT	CC + CT	
Cases	94 (47)	106 (53)	P = 0.0005
Control	10 (20)	40 (80)	OR = 3.5
			CI: 1.6-7.4 -8.8

we found that C allele is replaced by T allele (Figure 1B). The results of PCR-RFLP of all other studied polymorphisms were comprised of 5 samples of each genotype in patients with NAFLD, which was also confirmed by DNA sequencing (5 homozygous wild, 5 heterozygous, and 5 homozygous variant) (data not shown).

Haplotype analysis for CD14 C (-159) T and CD14 C (-550) T polymorphisms: Haplotype analysis revealed that haplotype TC had a significantly higher

($P = 0.0002$) frequency in patients with NAFLD in comparison to HVs, with an odds ratio of 2.3; 95%CI: 1.4-3.7. Analysis of individual haplotypes in the CD14 gene as a method of determining the risk of developing NAFLD revealed that the TC haplotype was more frequently seen in NAFLD. None of the other haplotypes showed any association with the risk of developing NAFLD (Table 6).

Role of CD14 C (-159) T polymorphism in determining the severity of NAFLD: Owing to the significant association between CD14 C (-159) T polymorphism and NAFLD, we compared different parameters amongst NAFLD patients with ($n = 94$) and without this polymorphism ($n = 106$). There was a significant difference in serum ALT [95 (72-130) IU/L vs 83 (68-108) IU/L, $P = 0.016$] and TNF α levels [62 (40-112) pg/mL vs 56 (34-80) pg/mL, $P = 0.04$] amongst NAFLD patients with and without CD14 C (-159) T polymorphism (Table 7). However, no difference was observed between the two groups with regard to the degree of hepatic steatosis, hepatic fibrosis, NAS score, and presence of NASH, borderline NASH, and no-NASH amongst the biopsy proven patients with NAFLD in each group.

DISCUSSION

NAFLD is one of the most predominant causes of liver disease in the world, and is considered a hepatic manifestation of metabolic syndrome. Its histology spectrum ranges from steatosis to NASH, and can

Table 6 Haplotypes for CD14 C (-159) T and C (-550) T polymorphism

Haplotypes	NAFLD (frequency)	HVs (frequency)	P value (Fisher's exact test)	OR (95%CI)
CC	23.4%	50%	1.58E-007	0.3 (0.19-0.48)
TC	52.3%	32%	0.0002	2.3 (1.4-3.7)
TT	12.2%	18%	0.12	0.6 (0.3-1.1)
CT	12.1%	0%	0.0002	Undefined

HVs: Healthy volunteers; NAFLD: Non-alcoholic fatty liver disease.

Table 7 Comparison of non-alcoholic fatty liver disease patients with and without CD14 C (-159) T polymorphism

CD14 C (-159) T polymorphism (NAFLD = 200)			
Parameters	Without polymorphism (n = 106)	With polymorphism (n = 94)	P value
TNF- α (pg/mL) (n = 200)	56 (34-80)	62 (40-112)	0.04
Adiponectin (pg/mL) (n = 200)	745 (649-893)	745 (634-928)	0.93
IL-1 β (pg/mL) (n = 200)	43 (32-47)	43 (25-47)	0.53
HOMA-IR (n = 200)	1.9 (1.3-2.7)	1.8 (1.4-3.8)	0.34
MS (n = 200)			
≥ 3 components	43 (40.5%)	35 (37.2%)	0.63
ALT (IU/L)	83 (68-108)	95 (-130)	0.016
NAS score (n = 60)	8	6	0.94
No NASH	11	10	
Borderline NASH	13	12	
NASH			
Severity of steatosis (n = 60)			
1	9	6	0.733
2	14	15	
3	9	7	
Severity of fibrosis (n = 60)			
0	13	11	0.90
1	16	13	
2	1	2	
3	2	2	

TNF- α : Tumor necrosis factor- α ; NASH: Non-alcoholic steatohepatitis; ALT: Alanine aminotransferases; NAFLD: Non-alcoholic fatty liver disease.

progress to cirrhosis and HCC^[1-3]. NAFLD progression is governed by genetic susceptibility, environmental factors, SIBO, lifestyle, and features of metabolic syndrome. Gene expression and genome-wide association studies have identified novel disease pathways and polymorphisms in genes that may be potential biomarkers of NAFLD development and progression. Pathways that include SIBO and toll-like receptor signaling seem to be one of the contributors of NAFLD development. The primary focus of our study was to analyze the polymorphisms of TLR2, TLR4, and CD14 genes in NAFLD patients and to assess their contribution to the causation and severity of the disease.

The overgrowth of bacterial components is recognized by pathogen-associated molecular patterns, including

TLRs. Toll-like receptor 2 (TLR2) are receptors for gram-positive bacterial components. In humans, due to a single nucleotide gene polymorphism at position 753, arginine is replaced by glutamine and the G allele replaced by A allele diminishes the ability of TLR2 to respond to bacterial cell wall components^[24,25]. Although there are animal studies to show the protective role for TLR2-mediated signals in liver injury and occurrence of NASH with TLR2 deficiency^[26,27], ours is the first human study to demonstrate the absence of an association of TLR2 Arg753Gln polymorphism with the risk of developing NAFLD.

In humans, TLR4 is the principal receptor for bacterial endotoxin recognition and functional variants in the gene confer endotoxin hyporesponsiveness^[28]. The missense mutation (Asp299Gly) in the fourth exon of the TLR4 gene alters the extracellular domain of this receptor. An additional missense polymorphism (Thr399Ile) in the extracellular domain of the TLR4 receptor co-segregates with the Asp299Gly substitution in more than 95% of the Caucasian population^[29]. There are conflicting reports on the effects of the Asp299Gly polymorphism on endotoxin responsiveness *in vitro*^[30-34]; however, the authors of several clinical reports associated this polymorphism with the risk of gram-negative infections^[35,36] or severe respiratory syncytial viral infection^[37], as well as such chronic disorders as asthma^[38], arteriosclerosis^[39], and diabetic neuropathy^[40]. We did not observe any association of TLR4 A299G and TLR4 C399T gene polymorphism with the risk of developing NAFLD or NASH. In addition, we did not find any association of haplotypes for TLR4 gene with NAFLD. Our results are in accordance with a study by Day *et al.*^[13], in which biopsy-proven patients with NAFLD were genotyped for Asp299Gly single nucleotide polymorphism (SNP) in the TLR4 gene and no association was found between the susceptibility of NASH and the Asp299Gly TLR4 SNP. A recent study by Kiziltaş *et al.*^[41] demonstrated that subjects with a heterozygous mutation in genotype 299 (Asp299Gly) were significantly lower in the NAFLD group than in the control group. The authors concluded that this mutation may have had a protective role against the genesis of NAFLD.

We found a significant association of C (-159) T polymorphism with NAFLD on multiplicative and recessive models. Patients with NAFLD with C (-159) T polymorphism had a significantly higher prevalence of TT genotype with significantly high levels of TNF- α ($P = 0.04$) and ALT ($P = 0.01$) than those without this polymorphism. Patients with TT genotype had a 2.6 fold higher risk of developing NAFLD in comparison to the CC genotype of CD14 C (-159) T polymorphism. However, this polymorphism did not affect disease severity, as there was no difference in NAS score and the prevalence of NASH or borderline NASH amongst those with and without polymorphism. Brun *et al.*^[14] found that TT genotype distribution was significantly higher in NASH patients than in control subjects,

while subjects carrying TT genotype had higher TNF- α levels than CT and CC genotypes. Another study demonstrated that “high” activity of TT genotype of C-159T SNP in the CD14 promoter gene was associated with NASH, and that patients with CD14 polymorphism had higher levels of serum TNF- α levels in comparison to those without C-159 T SNP^[13].

In contrast to C (-159) T polymorphism, we did not find any association of CD14 C (-550) T polymorphism with NAFLD. Ours is the first study to demonstrate the lack of an association of CD14 C (-550) T polymorphism with the risk of developing NAFLD. However, haplotype analysis of genotypes for CD14 revealed that TC genotype had an increased risk of NAFLD. Haplotype study is now gaining attention because multiple linked SNPs have the potential to provide significantly more power for genetic analysis than individual SNPs^[42]. There was no previously performed haplotype analysis for CD14 gene in patients with liver disease, with ours being the first study to demonstrate its utility in patients with NAFLD.

In conclusion, our study demonstrated that NAFLD patients with TT genotype of C (-159) T polymorphism in CD14 promoter gene have a higher risk of NAFLD development. However, this polymorphism did not affect the severity of liver disease. We did not find any association between TLR 2 ARG753, TLR 4 (Asp299Gly), TLR4 (Thr399Ile), or CD 14 C/T 550 polymorphisms and the risk of NAFLD development. Studies with a larger cohort of patients are required to confirm the results.

COMMENTS

Background

Environmental and genetic factors predispose individuals to the development of non-alcoholic fatty liver disease (NAFLD). In this study, the authors demonstrated that cluster of differentiation 14 (CD14) polymorphism could predict the development of NAFLD.

Research frontiers

NAFLD is the one of the manifestation of the obesity-related complications and incidence of NAFLD-related hepatocellular carcinoma increasing worldwide. It is therefore very important to understand the molecular mechanism underlying the pathogenesis of NAFLD.

Innovations and breakthroughs

Individuals with TT genotype of C (-159) T polymorphism in CD14 promoter gene have a higher risk of NAFLD development.

Applications

Individuals with TT genotype of C (-159) T polymorphism in CD14 promoter gene have a higher risk of NAFLD development. It can be considered a marker for identifying a population at risk of NAFLD progression.

Peer-review

Individuals with CD14 C (-159) T polymorphism have a higher risk of NAFLD development.

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ISSN 1007-9327

