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ORIGINAL ARTICLE

Apolipoprotein C3 gene polymorphisms in Southern Indian patients with nonalcoholic fatty liver disease

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Abstract
Aim Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in the world today. A previous study has suggested an association of apolipoprotein C3 (APOC3) gene variants with the risk of NAFLD in Asian Indian men living in the Western regions. The present study was carried out with an aim to evaluate the association of demographic features, serum lipid profile and APOC3 gene variants (C-482T and T-455C) NAFLD.
Methods One hundred and fifty NAFLD patients and 150 age and gender-matched controls were included in the study. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed to detect the genotypes of APOC3. Serum lipid profile was analyzed.
Results In the present study, body mass index was not a predictive demographic marker for NAFLD. Serum triglycerides were higher in patients (mean 155.95±59.0) with NAFLD compared to the control group (mean 133.75± 44.71) (p=0.016). APOC3 gene polymorphism T-455C (rs2854116) was significantly associated with NAFLD (p= 0.001). However, we did not find a significant association of C-482T polymorphism (rs2854117) of APOC3 gene with NAFLD. Genotype -455C/C of the SNP, rs2854116 associated significantly with the elevated serum triglycerides in patients.
Conclusions The polymorphism T-455C in APOC3 gene and elevated serum triglycerides were associated with NAFLD.

Keywords Nonalcoholic steatohepatitis · Obesity · Type-2 diabetes

Introduction

Nonalcoholic fatty liver disease (NAFLD), including its more severe forms like nonalcoholic steatohepatitis (NASH) and cirrhosis is the most common cause of liver disease worldwide with prevalence varying from 15 % to 40 % in general population depending on the demographic area [1]. NASH is believed to be the liver manifestation of metabolic syndrome [2]. Variants of genes regulating insulin signalling, lipid metabolism, oxidative stress, fibrogenesis and inflammation have been implicated in metabolic syndrome [3]. Previous studies have suggested that two single nucleotide polymorphisms (SNPs), T-455C (Ala455Val) and C-482 T (Met482Thr) in the gene encoding apolipoprotein C3 (APOC3) may be associated with metabolic syndrome [4] as well as NAFLD [5].

Apolipoproteins are involved in the transport of triglycerides, phospholipids and cholesterol in the plasma; binding of apolipoproteins with lipids results in the formation of lipid-protein particles (lipoproteins), the major carriers of lipids in the intra- and extravascular space. The genes coding for apolipoproteins Al (APOAJ), C3 (APOC3) and A4 (APOA4) are closely linked and tandemly organized on the long arm of the human chromosome 11 [6]. The APOC3 gene

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locus on chromosome is 11q23, encodes a protein (APOC3) of 79 amino acids a constituent of triglyceride-rich lipoprotein (TRL), including very low density lipoprotein (VLDL), chylomicron (CM) and high density lipoprotein (HDL). It inhibits the lipoprotein lipase-induced hydrolysis of those particles.

Since there are no reports on the association of genetic variants of APOC3 with NAFLD in an Indian population, the present study was carried out with an aim to evaluate this association in a South Indian population from Andhra Pradesh.

Methods

Subjects

One hundred and fifty patients with NAFLD (92 males; age 44.1±12.1 years) evaluated in the Department of Gastroenterology at Nizam's Institute of Medical Sciences, Hyderabad between July 2013 and December 2013 were included in the study. The study was approved by ethics committee of Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad. All the patients were screened and examined by gastroenterologists for the diagnosis and management of fatty liver. Our diagnostic approach was based on B mode ultrasound imaging which is a well validated tool for the diagnosis of fatty liver. Liver biopsy was not done due to its invasive nature and accompanying mortality of 0.1 %. One hundred and fifty patients, age, and gender-matched healthy individuals (donors from blood bank, staff working in Nizam's Institute of Medical Sciences) (males=92; age 42.6±10.6 years) served as controls. The controls had neither clinical evidence nor family history of any liver disease. Information on demographic features and risk factors was collected by using a structured questionnaire.

Inclusion criteria

Our inclusion criteria is age >18 years (males and non-pregnant females) and BMI >18 kg/m^2, evidence of hepatic steatosis as diagnosed by standard criteria on ultrasound imaging.

Exclusion criteria

Patients taking significant alcohol (>21 drinks/week for males and >14 drinks/week for females), presence of secondary causes of hepatic steatosis like hepatitis B and C, Wilson's disease, hereditary hemochromatosis, lipodystrophy, starvation, parenteral nutrition, drugs known to cause steatosis and inborn errors of metabolism was our exclusion criteria.

DNA isolation and genotyping

Three milliliters of blood was collected in EDTA tubes. Genomic DNA was extracted from blood samples using standard phenol-chloroform method. SNPs in APOC3 gene, rs2854116 and rs2854117 were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method [7]. The primers forward: 5′-GGCTGTGA GAGCTCAGCCCT-3′ and reverse: 5′ TCACACTGGAAT TTCAGGCC-3′ were used for the amplification. The amplified 196 bp PCR product was digested with *MspI* enzyme to genotype polymorphism C-482T, and *Fok I* enzyme for SNP, T-455C (Fermentas, FastDigest) by incubating at 37 °C for 5 min followed by separation of fragments on 10 % polyacrylamide gel. The digestion with *MspI* enzyme for -482C indicated by complete digestion into two bands of 143 and 53 bp whereas, "T" allele, shows two bands of 159 and 37 bp. Heterozygous -482C/T genotype was detected by four bands of 159, 143, 53 and 37 bp. Restriction digestion with *FokI* enzyme showed no digestion for -455 C allele and PCR amplicon (196 bp) was left undigested. PCR product was digested into two bands of 133 and 63 bp for -455T allele and for heterozygous and it results to three bands of 196, 133 and 63 bp.

Plasma lipid measurements

Blood samples for the evaluation of lipid levels were obtained from the subjects between (7 a.m. to 9 a.m.), after at least 12 h of fasting. The plasma total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides were analyzed using commercially available kits (Randox Laboratories, Crumlin, Antrium, UK). Low density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula.

Statistical analysis

The promoter region polymorphisms of APOC3 gene were analyzed using Hardy-Weinberg equilibrium. Association between genotypes and NAFLD was examined by odds ratio with 95 % confidence interval (CI) and chi square analysis using OpenEpi software (OpenEpi Version 2.3.1 from the Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA). Allelic frequencies were calculated according to the number of different alleles observed and the total number of alleles examined. Statistical power was calculated using OpenEpi. We computed the effective sample size and statistical power using a web browser programme, a genetic power calculator developed by Purcell et al., 2003 [8]. In this study, using a 1:1 case control study design, the sample size calculations were estimated based on the assumptions of minor allele frequency (MAF) (0.45). Assumed odds ratio (4.0) would provide 80 %

Table 1 Analysis of demography and serum lipid profile between patients with nonalcoholic fatty liver disease and controls

Parameters	Patients Mean (SD)	Controls Mean(SD)	*p*-value
Age (years)	44.1 (12.1)	42.6 (10.6)	>0.05
Males *n* (%)	92 (61.3)	92 (61.3)	>0.05
Females *n* (%)	58 (38.7)	58 (38.7)	
BMI (kg/m^2)	27.7 (4.7)	23.6 (4.7)	1.580
Total cholesterol (mg/dL)	188.3 (54.5)	174.8 (64.7)	0.086
HDL (mg/dL)	37.4 (6.5)	39.4 (8.8)	0.060
LDL (mg/dL)	120.3 (38.7)	116.4 (41.8)	0.289
Triglycerides (mg/dL)	155.9 (59.0)	133.7 (44.7)	0.016*

BMI body mass index, *HDL* high density lipoprotein, *LDL* low density lipoprotein

Table 3 The distribution of genotype and allelic frequency of SNP, T-455C of APOC3 gene between patients with nonalcoholic fatty liver disease and controls

Study Group	Genotype frequency				Allele Frequency		
	TT	TC	CC	Total	T	C	Total
Patients *n* (%)	44 (29.33)	75 (50)	31 (20.66)	150	163 (0.54)	137 (0.46)	300
Control *n* (%)	60 (40)	81 (54)	9 (6)	150	201 (0.67)	99 (0.33)	300

For CC vs. TT, χ^2 =14.2; p=0.0001*, odds ratio=4.7 (95 % CI; 2.047–11.27). For CC vs. TT+TC, χ^2 =13.92; p=0.0001*, odds ratio=4.08 (95 % CI; 1.8–8.9). For C vs. T χ^2 =10.07; p=0.001*, odds ratio= 1.706 (95 % CI; 1.22–2.37)

power to detect an association. The low density lipoprotein (LDL), HDL and TG levels in patients and controls were compared by Student's *t* test and levels of significance were defined as $p<0.05$. The genotype-phenotype correlation was analyzed using Statistical Package for Social Sciences (SPSS, v. 19) software.

Results

In the present study, 150 NAFLD patients and an equal number of controls were included. All the patients belonged to the Southern Indian population from Andhra Pradesh. The clinical characteristics of the NAFLD patients and controls have been presented in Table 1. In the present study BMI, did not show the significant association with the disease. There was no significant difference between patients and controls in the total cholesterol, HDL and LDL levels. However, triglyceride level was significantly higher in patients (mean 155.9 mg/dL) in comparison with the controls (mean 133.7 mg/dL) (p=0.016).

The genotypic distribution of APOC3 promoter polymorphisms and allelic frequencies for the two polymorphisms studied has been given in Tables 2 and 3. There was no significant difference in the genotypic distribution of SNP, rs2854117 of APOC3 gene between patients with NAFLD and controls. The frequency of -455C/C genotype of SNP, rs2854116 was significantly higher in patients compared to controls. Further, distribution of allelic frequency of both the SNPs was analyzed to understand risk allele for the study phenotype. The allelic distribution of SNP, rs2854117 (C-482T) was found to have no association with the disease. However, allele -455C of SNP, rs2854116 was found to be significantly associated with NAFLD (p=0.001). Further, the genotype-phenotype correlation was analyzed between different genotypes of SNP rs2854116 and serum triglycerides in both patient and control groups. Serum triglycerides were higher in genotype -455C/C NAFLD patients (genotype CC, triglycerides 183.3±19.4 mg/dL, p=0.012) but not in controls (genotype CC, triglycerides 123.9±14.2 mg/dL, p=0.899) (Figs. 1 and 2). The recessive model analysis also showed a significant association of allele -455C with higher concentration of triglycerides in patients (genotype: TC+CC, triglycerides 167.7±48.6 mg/dL, p=0.039) which was not

Table 2 The distribution of genotype and allelic frequency of SNP, C-482T of APOC3 gene between patients with nonalcoholic fatty liver disease and controls

Study group	Genotype frequency				Allele frequency		
	CC	CT	TT	Total	C	T	Total
Patients *n* (%)	55 (36.66)	57 (38)	38 (25.33)	150	167 (0.56)	133 (0.44)	300
Controls *n* (%)	62 (41.3)	46 (30.66)	42 (28)	150	170 (0.57)	130 (0.43)	300

For TT vs. CC, χ^2 =0.005; p=0.9, odds ratio=1.02 (95 % CI; 0.6–1.8). For TT vs. CC+CT, χ^2 =0.27; p=0.6, odds ratio=0.87 (95 % CI; 0.5–1.45). For T vs. C χ^2 =0.06; p=0.8, odds ratio=1.04 (95 % CI; 0.75–1.43)

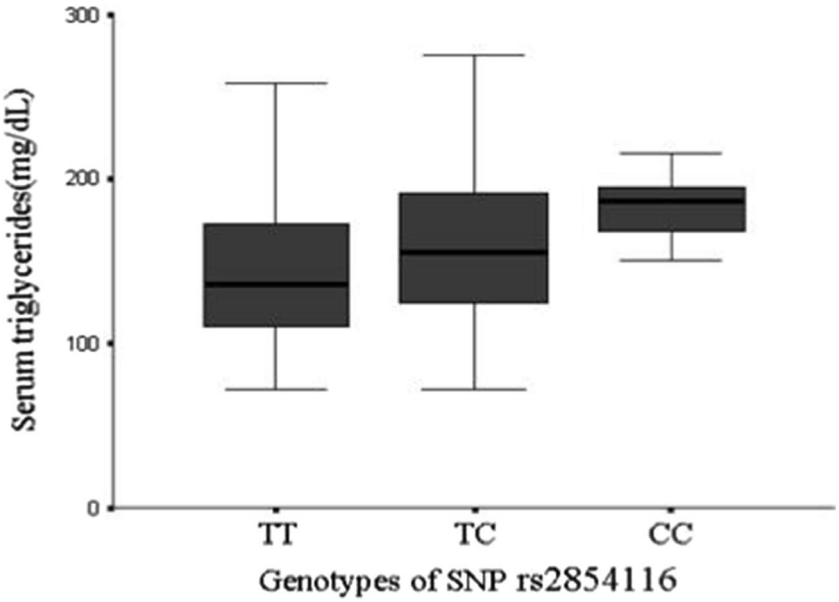

Fig. 1 The distribution of serum triglycerides between genotypes of SNP rs2854116 in patients with nonalcoholic fatty liver disease

found in controls (genotype TC+CC, triglycerides 126.4± 37.4 mg/dL, p=0.688).

Discussion

NAFLD is now becoming an increasingly recognized cause of liver-related morbidity and mortality. In India, the prevalence of NAFLD is estimated to be around 9 % to 32 % in the general population [9]. In the present study, we found a higher prevalence among males in comparison with females. This finding is consistent with a previous study where male patients had significantly higher rates of NAFLD as well as NASH [10]. It has been suggested that sex steroid hormone metabolism may be important in the pathogenesis of NAFLD [11]. This was supported by a recent pediatric study that reported the highest rates of NAFLD among post pubertal boys and the lowest rates among post pubertal girls [12].

Marchesani et al. [13] showed that 80 % of patients with NAFLD were obese. In a study carried out by Deepa et al. [14] only 21 % of the population was found to have normal BMI while the remaining 79 % were either overweight or obese.

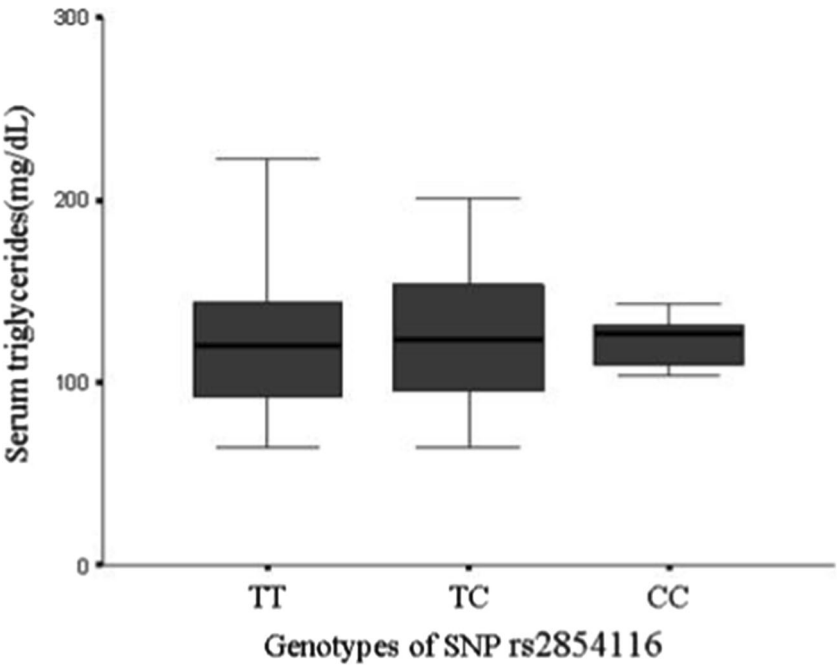

Fig. 2 The distribution of serum triglycerides between genotypes of SNP rs2854116 in control group

Goland et al. [15] reported that patients with NAFLD had a significantly higher BMI. However, in the present study, we did not find an association between BMI and NAFLD.

Lipid metabolism in the liver comprises four major pathways: fatty acid uptake, de novo fatty acid synthesis, oxidation of fatty acids and secretion of VLDL. Changes in any of these pathways can disturb the dynamic balance of the homeostasis of lipids in liver cells, causing abnormal lipid depletion or accumulation. Studies using high-throughput microarray technique verified by qRT-PCR assays were carried out to explain lipid metabolism pathways in the liver [16]. The elevated activity in all lipid metabolism pathways was observed in the NASH livers. Lipid can also be introduced into liver through lipoprotein receptors including LDL receptor-related proteins and LDL receptor. A recent study with non-obese patients carrying apolipoprotein C3 gene variants (C482T, T-455C or both) implicated elevated activity of the LDL receptor in steatosis [5]. These mutations are associated with higher levels of fasting plasma apolipoprotein C3 concentration and NAFLD. In the present study, there was no significant difference in the total cholesterol, HDL and LDL levels between patients and controls. However, triglyceride level was found to be elevated significantly in patients in comparison with the controls, which was in accordance with previous reports, where triglyceride level was found to be significantly high in NAFLD patients [5].

The two SNPs in the promoter region of the APOC3 gene, rs2854117 and rs2854116 have been reported to be associated with hypertriglyceridemia, metabolic syndrome and coronary artery disease [4]. More recently, these variants have been shown to be associated with the occurrence of NAFLD. Petersen studied these polymorphisms in Indian and non-Indian healthy men residing in the USA [5]. They established NAFLD in 38 % of the Indian men with variant APOC3 alleles at one or both of these loci. In the non-Indian men too, NAFLD was more frequent among those with the variant alleles than those with normal alleles. It was proposed that the variant alleles lead to increased amounts of APOC3, and inhibition of lipoprotein lipase activity and triglyceride clearance, resulting in hypertriglyceridemia due to increase in chylomicron remnants, which are taken up by the liver resulting in NAFLD. Their findings are consistent with previous studies where transgenic mice with over expression of human apolipoprotein C3 had hypertriglyceridemia due to decreased lipoprotein lipase activity [17]. However, subsequent studies in Hispanic, European American, African American and European subjects have failed to confirm the association of APOC3 variants with NAFLD [18]. Hyysalo et al. [19] reported a similar lack of association between the two APOC3 gene polymorphisms and NAFLD in a Finnish population. In their study, two SNPs of the APOC3 gene were genotyped and measured the liver fat using magnetic resonance spectroscopy and plasma concentration of APOC3. Individuals with and without the variant alleles (-455C, -482T or both) had similar amounts of liver fat, plasma APOC3 concentrations, serum triglycerides, HDL and levels of fasting plasma glucose, insulin and transaminases.

To the best of our knowledge, this is the first study from India evaluating the association of APOC3 gene variants with NAFLD. We studied the relationship between genetic variants in APOC3 and the risk of NAFLD in a South Indian population from Andhra Pradesh. We found a strong association of SNP (rs2854116) of APOC3 gene with the disease phenotype. Further, the CC genotype was found to be associated with elevated triglyceride levels in the patients.

In conclusion, the present study showed a significant association of genotype -455C/C of SNP, rs2854116 in APOC3 gene with NAFLD. An increased serum triglyceride levels were found to be strongly associated with the study disease. The present study identified a strong genotype -455C/C)-phenotype (elevated serum triglycerides) correlation in patients with NAFLD.

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Conflicts of interest JP, SB, AK, SPS, PDKV, MK, JA and AM all declare that they have no conflict of interest.

Ethical clearance All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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