Journal of Advanced Scientific Research
Available online through https://sciensage.info
ISSN 0976-9595
Research Article

ASSOCIATION OF GENETIC VARIANTS IN THE PROMOTER REGION OF APOC3 WITH THE RISK OF NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) IN NORTH INDIAN POPULATION

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Received: 18-12-2021; Accepted: 09-05-2022; Published: 31-08-2022
 https://doi.org/10.55218/JASR.202213714

ABSTRACT

Non-Alcoholic Fatty Liver Disease (NAFLD) is a clinical condition defined by excessive fat accumulation in the liver in the form of triglycerides involving more than 5% of hepatocytes. The present study was carried out with an aim to evaluate the association of demographic features, liver enzymes, serum lipid profile and APOC3 gene variants (C-482T and T-455C). A case-control study was designed with 150 patients with NAFLD, and 180 healthy controls. The mutations were analyzed using PCR-RFLP approach, Case-control studies assessing the relationship between APOC3 rs2854116 C/C and rs 2854117 C/T gene polymorphism with non-alcoholic fatty liver disease. Statistically significant increased frequencies of CC+CT genotype [OR, 2.00; 95 percent C. I (1.18-3.40); P.0006] [OR, 2.00; 9.5 percent C.I (1.38-2.91); P value < 0.0002] and the C allele is seen in NAFLD patients compared to healthy controls. In conclusion, we found that the APOC3 gene and elevated triglyceride levels in -455C/C SNP is significantly associated with the risk of NAFLD, whereas the Apoc3-482C/T showed no association with disease.

Keywords: NAFLD; APOC3; AST; ALT; TG.

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is estimated to affect almost one-fourth of certain populations and contributes to a noticeable proportion of the burden of liver diseases globally [1]. As a result, NAFLD is widely recognized as a major public health issue around the world [1]. The spectrum of liver disorders ranges from simple nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), permanent Cirrhosis and progression to HCC finally [2-3].

The APOC3 gene codes for a 79-amino-acid glycoprotein developed primarily in the liver. It is a part of triglyceride (TG)-rich lipoproteins and high-density lipoprotein (HDL) that is synthesized primarily in the liver and to a lesser extent in the intestine [4-5]. The APOC3 gene is found on chromosome 11q23 and is closely linked to the APOA1 and APOA4 genes. It is involved in the transfer and removal of chylomicron remnants, VLDL and HDL from the bloodstream. The fact that NAFLD prevalence varies by demographical location, clinical profile and histological severity across ethnic groups suggests a genetic component [5]. Since the pathogenesis of NAFLD is unclear, recent studies have engrossed on identifying NAFLD-related genes, especially candidate genes involved in obesity, lipid metabolism, and insulin regulation, which has led to investigations into the polymorphism of these genes [4].

A significant amount of research has been done on the genetic effect of apolipoprotein C3 polymorphisms on the risk of NAFLD [5, 6]. Polymorphisms in the insulin responsive element on the promoter region cause the protein to be overexpressed [7, 8]. The APOC3 promoter polymorphisms C482T (rs2854117) and T455C (rs2854116) have a lower affinity for nuclear transcription factors that mediate insulin responses in insulin resistance. The transition from cysteine to

thymine at position C-482-T is linked to dyslipidemia and insulin resistance [8-10]. A transition from thymine to cysteine at position T- 455-C, which is a risk factor for cardiovascular disease, is related to higher triglyceride levels. rs2854116, rs2854117, rs4520 and rs5128 are four common SNPs in the APOC3 gene that have been identified as putative functional SNPs that influence serum APOC3 concentrations, causing dyslipidemia by influencing triglyceride (TG) and very-low-density lipoprotein (VLDL) levels [11-12] The SNPs in the APOC3 gene have been linked to hypertriglyceridemia in a number of earlier studies [13].

A number of studies have linked polymorphisms in the APOC3 gene to NAFLD resistance in non-Asian Indian males [14]. However, until now no such research has been conducted in our community, we focused to look into the relationship between polymorphic variants of APOC3 gene variants and NAFLD susceptibility.

2. EXPERIMENTAL

2.1. Subjects

This analysis included 150 patients with NAFLD (86 men and 64 women) ranging in age from 25 to 75 years old (average age = 40 years). The controls were a group of 180 safe volunteers (129 men and 51 women) of similar age and sex who had no personal or family history of NAFLD. Each person who took part in the analysis gave their informed consent.

2.2. DNA Isolation and Genotype analysis

In EDTA vials, three milliliters of peripheral blood from patients with NAFLD and healthy controls was obtained. The DNA was isolated using the standard phenol/ chloroform procedure. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods were used to find genetic variants rs2854116 (T-455C) and rs2854117 (C-482T) in the promotor region of the APOC3 gene. The final PCR rxn was 25 µl in which 50 ng genomic DNA base, 1 PCR buffer (Biotools, B & M Labs, S.A. Madrid-Spain) with 2mM MgCl2, 0.20mM dNTPs (Biotools, B & M Labs, S.A. Madrid-Spain), 0.4 mol of each primer (Sigma-Aldrich Co. LLCUSA) and 0.4 mol of each primer (Sigma-Aldrich Co. LLC(USA). 1 unit of DNA polymerase (Biotools, B &M Labs, S.A. Madrid-Spain) was added. The PCR conditions used for PCR amplification were: an initial denaturation stage at 95°C for 4 minutes, followed by 35 cycles of denaturation (95°C for 30s), annealing (58°C for 30s), and extension (72°C for 30s), followed by a final elongation at 72°C for 7 minutes. Table 1 lists the primers used to amplify the APOC3 (T-455C) and APOC3 (C-482T) SNPS of this gene. 10 µl of amplified products of APOC3 (T-455C) and APOC3 (C-482T) SNPS were digested with the enzymes Msp1 and FokI (Fermentas Thermo Fisher Scientific Inc. Massachusetts, USA) (1 U at 37°C for 16 h, respectively) for RFLP analysis. The digested products were visualized on a 3% agarose gel (Table 1).

2.3. Statistical analysis

For statistical analysis, the genotype and allelic frequency distributions of polymorphisms in the control and NAFLD' disease patient groups were matched using the Chi-square test. The disease sensitivity of particular genotypes and alleles was calculated using odds ratios (O.R) with 95 percent confidence intervals (CIs). When the probability of findings happening by chance was less than 5% (P 0.05), the results were statistically found to have significance. SPSS v 20 software and other online tools from http://vassarstats.net were used for the statistical analysis.

3. RESULTS

Our analysis involved a total of 330 people (150 NAFLD patients and 180 healthy controls). Among the control subjects, 51 were females and 129 were males (Female/Male ratio 1:3), while in the patient group, 64 were females and 86 were males (Female/Male ratio 2:3). The average age of the inpatient category was 46.8 years. There were no major demographic or age gaps within the groups and the p-value was greater than 0.05 ($P > 0.05$) (Table 1). A study of Hardy-Weinberg equilibrium (HWE) was carried out. In the control population, the genotypic distributions of APOC3 C-455T and APOC3 T-482C were p value was 0.84 and 0.86 respectively and this was in accordance with Hardy-Weinberg equilibrium.

Genotypes were represented by different RFLP patterns of bands. The wild allele genotype (C/C) has 143 and 53bp, the T mutant allele has two bands at 159 and 37 bp (T/T) whereas the heterozygous (T/C) genotype has four bands of 159, 143, 53, and 37 bp. After performing the statistical analysis, the genotypic and allelic frequencies of the APOC3 C-482 T polymorphism in cases and controls were not significantly different [OR, 0.70; 95 percent CI (0.42-1.17); P=0.2] and [OR, 0.70; 95 percent CI (0.49-1.01; P=0.05], respectively (Table 3). We did not find any association between the APOC3 C-482T (rs28554117) polymorphism and clinicopatho-

logical parameters, and thus the findings were not statistically significant (Table 2).

Similarly, T455 C (rs2854116) genotypes were characterized by a band pattern of 196bp for wild type (T/T), 133 and 63 bp for mutant allele type, and for heterozygous it showed a band pattern of three bands of 196, 133, and 63 bp (T/C) after digestion with Fok1enzyme. Table 3 shows the genotypic and allelic frequency distributions of cases and controls. The prevalence of wild (TT) genotypes in NAFLD cases was 90(60%) and TC (Hetero) plus CC (Mutant) genotypes was 60(40%) whereas in healthy controls wild (TT) genotypes were 103 (57.22%) and TC (Hetero) plus CC (Mutant) were 77(42.77%).

Table 1: Frequency distribution analysis of selected demographic and risk factors of NAFLD cases and controls

Variables	Cases N=150(%)	Controls N=180(%)	p-value chi sq
Age ≥50=90 <50=60	90 (60) 60 (40)	103(57.22) 77(42.77)	0.34
Dwelling Rural=84 Urban=66	84(56) 66(44)	92(61.34) 58(38.66)	0.20

Table 2: Genotypic frequencies of Apoc3 Gene Polymorphism in the NAFLD cases and Controls

Genotype	Apoc3 455c (cases)(150)	Controls(180)	0.D-ratio	p-value
455c **TT** **TC/CC**	**107** **43**	**150** **30**	**2.00(1.18-3.40)**	**0.0006**
Allele 2N **T** **C**	**214** **86**	**300** **60**	**2.00(1.38-2.91)**	**0.0002**
482 **CC** **CT/TT**	**118** **32**	**130** **50**	**0.70(0.42-1.17)**	**0.2**
Allele 2N **C** **T**	**236** **64**	**260** **100**	**0.70(0.49-1.01)**	**0.05**

Table 3: Association of APOC3 Gene polymorphism (snp's 455c and 482T) and various clinical and lab features of NAFLD Patients

Parameter	Cases=150				Controls=180			
	455c				482T			
	TT=107	TC+CC=43	P-VALUE	O-RATIO	CC=118	CT+TT=32	P-VALUE	O.RATIO
SEX Male=86 Female=64	67(77.90) 40(62.5)	19(22.10) 24(37.5	0.04	0.47(0.23-0.96)	68(79.07) 50(78.13)	18(20.93) 14(21.87)	1	0.94(0.42-2.07)
DWELLING Rural=84 Urban=66	62(73.81) 45(68.19)	22(26.19) 21(31.81)	0.28	0.76(0.37-1.54)	72(85.72) 46(69.69)	12(14.28) 20(30.31)	0.01	0.38(0.17-0.85)
LDL High/low=30 Normal=120	19(63.34) 88(73.34)	11(36.66) 32(26.66)	0.36	1.59(0.68-3.70)	25(83.33) 93(77.5)	05(16.67) 27(22.5)	0.33	0.68(0.24-1.97)
TRIGLYCERIDE High/low=104 Normal=46	87(83.65) 20(43.47)	17(16.35) 26(56.53)	**0.001**	0.15(0.06-0.32)	84(80.76) 34(73.91)	20(19.24) 12(26.09)	0.23	0.67(0.29-1.53)
AST HIGH=50 NORMAL=100	34(36.66) 73(45)	16(63.34) 27(55)	0.56	1.27(0.60-2.66)	30 77	20 23	0.03	2.23(1.07-4.64)
ALT HIGH=96 NORMAL=54	63(44.79) 44(38.88)	33(55.21) 10(61.12)	0.05	2.30(1.02-5.15)	74(77.09) 44(81.48)	22(22.91) 10(18.52)	0.54	1.30(0.56-3.01)

The genotypic and allelic frequencies of the APOC3 T455C polymorphism is shown to be slightly different in cases and controls [OR, 2.00; 95 percent C.I (1.18-3.40); P.0006] [OR, 2.00; 9.5 percent confidence interval (1.38-2.91); P value < 0.0002] (Table 3), indicating that carriers of the C allele had a higher chance of developing NAFLD. Furthermore, the relationship between the APOC3 T455C polymorphism and clinicopathological parameters was extensively examined (Table 4). This polymorphism was shown to be significantly linked to a rise in serum triglyceride levels with a P value < 0.001.

Table 4: Primer sequence, Annealing temperatures, Restriction Enzymes & lengths of digested fragments of APOC3- gene for polymorphic variants

Polymorphism	rs number	Primer Sequence	Restriction Enzyme	AT*	Amplicon size (bp)	RFLP pattern
APOC3- 455c	rs2854116	F- 5′-GGCTGTGA GAGCTCAGCCCT-3′	Fok1	58°C	196	TC 133,63 CC 196, 133,64
APOC3-482T	rs2854117	R5′TCACACTGGAAT TTCAGGCC-3′	Msp1	58°C	196	CC 143,53 CT 143,159,53,37 TT 159,37

AT: Annealing, £-The same primer set was used to amplify for both polymorphisms variants.*

4. DISCUSSION

NAFLD is an escalating health concern, and differences in NAFLD prevalence and clinical profile among ethnic groups indicate a genetic component [5]. This has prompted researchers to look at polymorphisms in a wide range of genes, including those involved in lipid metabolism, insulin signaling, oxidative stress, and hepatic fibrosis. We analyzed two SNPs in the APOC3 gene's promoter region, rs2854117 and rs2854116 (T-455-C and C-482-T), and this is the first report from our population on the association of these APOC3 gene variants and the risk of NAFLD [14].

NAFLD is a form of liver disease that affects a large number of people [15-16] despite the fact that the pathogenesis of NAFLD is poorly understood. It is known that insulin resistance, oxidative stress and inflammation all play key roles in the disease's progression and development of NAFLD [17-18] Several candidate genes, including TNF-, PNPLA3, APOC3, PPAR- and adiponectin, have been linked to the risk of NAFLD [18-19]. Two SNPS (T-455C and C-482T) of the APOC3 gene were analyzed in this study, both of which are linked to lipid metabolism. We found a strong association of T-455C (rs 2854116) with NAFLD whereas no association with NAFLD was found for the other SNP C-482T (rs 2854117)] studied. In addition, there was an increase in triglyceride levels in NAFLD patients.

These two SNPs associated with the APOC3 gene [T-455C and C-482T], are in the 5'promoter region and have high linkage instability with each other. According to a study by Petersen et al., Nonalcoholic fatty liver disease and insulin resistance have been linked to the polymorphisms C-482T and T-455C in APOC3 [14]. Many conditions have been associated to APOC3 polymorphisms, including metabolic syndrome [20], type 2 diabetes [21], coronary heart disease [22] and plasma APOC3 and lipid levels [22, 23]. This polymorphism causes a 30 percent rise in APOC3 plasma concentration and postprandial hypertriglyceridemia, causing NAFLD and hepatic insulin resistance in individuals which are carriers of this polymorphism T-455C(rs2854116) [24].

In the case of the APOC3 T-455C polymorphism, we find statistically significant variations between cases and controls in allelic and genotypic frequencies, [OR, 2.00; 95 percent CI (1.18-3.40); P =0.0006] [OR, 2.00; 9.5 percent confidence interval (1.38-2.91); P= 0.0002] (Table 2), showing that having the TC genotype increases the chance of developing NAFLD. Besides, our findings are consistent with those of a study conducted [25, 26], Patients with NAFLD had higher serum triglycerides than the control group (p= 0.016). The T-455C polymorphism in the APOC3 gene (rs2854116) was shown to be associated with NAFLD (p=0.001). Our findings are also similar with the studies of Southern Chinese Han population where triglyceride levels were shown to be substantially higher in patients than in controls groups [14]. Results of the analysis of the association between the APOC3 (T-455C) polymorphism and NAFLD sensitivity in the Southern Chinese Han population were close to those seen in the Asian Indian population, where the CC+CT genotype was associated with higher triglyceride levels and

patients had an increased risk of NAFLD.

In case of (rs2854117) T-482C polymorphism, a negative correlation was observed between polymorphism in cases and controls. The statistical analysis indicated the genotypic and allelic frequencies of various clinicopathological parameters associated with APOC3 T-482C polymorphism in cases and controls are not significant with the SNP(rs2854117) [OR, 0.70; 95% CI (0.42-1.17); P=0.2] and [OR, 0.70; 95% CI (0.49-1.01); P=0.05]. The results of our study are consistent with the findings of Hyssalo et al., 2012 in Finnish population [24-26] where they reported a similar lack of association between the two APOC3 gene polymorphisms and NAFLD [24]. These differences could be attributed to the varied ethnicity in different populations.

5. CONCLUSION

In conclusion, increased serum triglyceride levels were found closely correlated with NAFLD.

Genotype T -455C of SNP, rs2854116 in the APOC3 gene showed significant association with the disease. Further studies with other functional polymorphisms will be helpful to evaluate the gene-gene interactions and the association between APOC3 gene and development of NAFLD.

6. ACKNOWLEDGEMENTS

We acknowledge the Department of Gastroenterology, of Sher-I- Kashmir Institute of Medical Sciences for their Support. We also acknowledge our patients for their participation in the study.

Conflict of Interest:

The manuscript has been seen and approved by all the listed authors, all of whom have contributed significantly to the work and there is no conflict of interest between authors.

Source of funding

None declared

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