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Research Article
ApolipoproteinC3 Gene Variants in Nonalcoholic Fatty Liver Disease in Egypt

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Abstract

Background and Objective: Apolipoprotein C3 (*APOC3*) is a component of triglyceride-rich lipoproteins and APOC3 gene polymorphisms have been associated with non-alcoholic fatty liver disease (NAFLD), hypertriglyceridaemia and insulin-resistance. This study was undertaken to determine if the APOC3 gene variants were associated with the susceptibility of obese subjects to develop liver damage, hypertriglyceridaemia and insulin-resistance. **Materials and Methods:** The study was carried out on 100 unrelated obese Egyptians. These cases were compared to 83 normal weight healthy controls. All participants were subjected to an estimation of their body mass index (BMI) in addition to liver functions and lipid profile. Polymerase chain reaction Polymerase chain reaction with sequence-specific primers (SSP-PCR) was performed to detect the of APOC3 rs2854116 and rs2854117 polymorphic genotypes. **Results:** Cases showed a significantly higher frequency of the APOC3 T-455C, CC genotype than controls (32 vs. 9.6%, p = 0.0003, Odds ratio = 5.33, 95% CI = 2.2-12.7). In addition, the allelic frequency of the rare APOC3 -455 C allele was significantly higher among cases than controls (51 vs. 30.72%, p = 0.0001, OR = 2.35, 95% CI = 1.5-3.6). On the other hand cases showed a non-significant difference regarding all APOC3 C-482T genotypes (CT vs. CC, TT vs. CC and CT+TT vs. CC) as well as APOC3 -482 T vs. C alleles. All cases showed no significant difference of their hematologic, liver functions and lipid profile related to their genetic polymorphism. **Conclusion:** The polymorphism T-455C but not the C-482T in APOC3 gene was associated with NAFLD in Egyptian obese subjects. However, it did not affect their hematologic, liver and lipid profile parameters.

Key words: ApolipoproteinC3, nonalcoholic fatty liver disease, hypertriglyceridemia, gene polymorphism, insulin-resistance

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

One of the most common chronic liver disorders is the nonalcoholic fatty liver disease (NAFLD)[1]which is characterized by hepatic steatosis in the absence of alcohol consumption or other liver disorders[2]. Both environmental and genetic factors contribute to the process of steatosis, steatohepatitis (NASH) and fibrosis[3]. Risk factors include obesity and type 2 diabetes/insulin resistance[4]. Insulin resistance promotes peripheral adipose lipolysis, thereby increasing FFA flux to the liver, which drives hepatic triglyceride production[5].

Human studies have demonstrated peripheral adipose lipolysis, systemic free fatty acid levels and denovo hepatic lipogenesis to be upregulated in subjects with NAFLD[6]. Fatty acid (palmitate) release from peripheral adipose deposits is increased approximately 35% in NAFLD patients compared to age, gender and fat mass matched controls and it accounts for approximately 60% of hepatic lipid in subjects with NAFLD[6]. De novo lipogenesis accounts for 25% of hepatic fat content in NAFLD subjects compared with 10% in obese hyperinsulinemic subjects and 5% in healthy individuals[7]. In fact, a genetic factor underpinning NAFLD has been suggested by familial aggregation studies[8], heritability studies[9], candidate gene studies[3], genome-wide scans[10] and expression studies[11]. The probing into the genetics of NAFLD will help in the identification of individuals at risk, understanding NAFLD pathogenesis and developing new therapies. Apolipoprotein (*apo*) C3, a protein produced by the liver[12], is an essential constituent of VLDL and HDL[13]. Considering the inhibitory effect of APOC3 on lipoprotein lipase (*LPL*) activity and hepatic uptake of lipoproteins[14], reports of APOC3 gene variants have been proposed as being potentially responsible for the occurrence of lipoproteinlipid profile disturbances. Accordingly, numerous polymorphisms in the APOC3 gene have been identified[15].

The aim of this study was to investigate the relation between NAFLD and diabetes and to determine if the APOC3 variants alter the susceptibility of Egyptian obesesubjects to develop liver damage, hypertriglyceridaemia and/or insulin-resistance. This investigation will reinforce current knowledge about the diseases and how to control it.

MATERIALS AND METHODS

Study group: This study has involved 100 subjects affected with NAFLD, recruited from the Department of Obesity and Diabetes Internal Medicine Specialized Hospital, Mansoura University, Egypt during the period from September, 2013 to May, 2015. All practices were approved by the University of Mansoura Committee of Scientific Research Ethics. Their age Mean±SD was 45.4±15.2 years ranging from 18-70 years. They were in the form of 40 (40%) males and 60 (60%) females. According to the definition of metabolic syndrome given by WHO, ATP and IDF[16], (75%) of patients were classified as having metabolic syndrome while the rest, (25%) were not complicated and were characterized as just having simple obesity.

Control group: For comparison, negative control group was selected including 83 healthy non-obese unrelated subjects.

Biochemical analysis: After 12 h of fasting, a blood sample was collected in a tube for measuring. If the samples were not analyzed immediately, they were frozen and stored at -70°C. Glycosylated hemoglobin A1c (HbA1c) was measured using high performance liquid chromatography[3], the rest of the parameters were measured by enzymatic methods on automatic biochemistry analyzer including total cholesterol, triglyceride (TG), high densitylipoprotein cholesterol (HDL-C), low density lipoprotein (LDL-C), fasting and postprandial blood sugar, serum alanine aminotransferase(ALT), aspartate aminotransferase (AST)[10]. Fatty liver was determined by ultrasound[4].

Capture column kit extraction and purification: The generation DNA purification capture column kit (Gentra system, USA) was based on a proprietary system that used two reagents, a DNA purification solution and a DNA elution solution, along with a specially formulated purification matrix. In this kit, a sample was applied directly to the purification matrix contained a spin column. The cells contained in the sample lyse upon contact with the matrix. Once the cells were lysed, DNA was captured by the matrix material which makes it possible to efficiently wash away contaminants, leaving the DNA bound to the matrix. Contaminants, including protein, heme and RNA are removed from the matrix by washing with DNA purification solution. Following removal of contaminants, the DNA released from the matrix using DNA elution solution and heat. Samples of purified DNA were ready for analysis and not require precipitation.

Primer sequences and PCR conditions of each APOC3 studied: SNPs in APOC3 gene, rs2854116 and rs2854117 were genotyped using Polymerase chain reaction Polymerase chain reaction with sequence-specific primers (SSP-PCR)[17]. Primers used for DNA amplification were: forward:

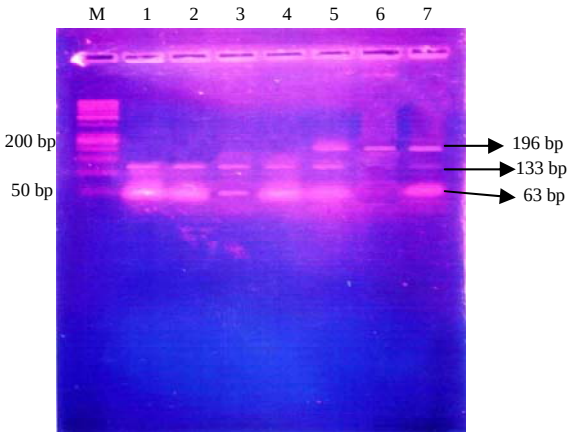

Fig. 1: Genotyping of the Apolipoprotein C3 (-455T>C) polymorphism with Fok I enzyme. Polymerase chain reaction with sequence-specific primers and agarose gel (3%) electrophoresis of the Apolipoprotein C3 (-455T>C) polymorphism illustrated the wild-type homozygote TT (196 bp), heterozygote TC (196, 133 and 63 bp) and variant-type homozygote CC (133 and 63 bp). Lane 1: Digested two bands of 133 and 63 bp for "C" allele, Lane 2: Digested two bands of 133 and 63 bp for "C" allele, Lane 3: Digested two bands of 133 and 63 bp for "C" allele, Lane 4: Digested two bands of 133 and 63 bp for "C" allele, Lane 5: Digested three bands of 196, 133 and 63 bp for heterozygote TC, Lane 6: PCR amplicon (196 bp) of T allele (homozygote TT), Lane 7: Digested three bands of 196, 133 and 63 bp for heterozygote TC

5-GGCTGTGAGAGCTCAGCCCT-3 and reverse: 5 TCACACTGGAATTTCAGGCC-3. 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, Fast Digest) by incubating at 37°C for 5 min followed by separation of fragments on 3%agarose gel. The digestion with Msp I enzyme for "C" allele showed no digestion and PCR amplicon (196 bp) was left undigested, whereas, "T" allele, shows two bands of 143 and 53 bp. Heterozygous -482C/T genotype was detected by three bands of 196, 143 and 53 bp. Restriction digestion with Fok I enzyme showed no digestion for "T" allele and PCR amplicon (196 bp) was left undigested. PCR product was digested into two bands of 133 and 63 bp for "C" allele and for heterozygous -455T/C, it was detected by three bands of 196, 133 and 63 bp (Fig. 1).

Statistical analysis: Statistical analysis of data was done using the software statistical package SPSS program version 17. Student t-test was used to compare the numerical values related to lipid profile, other chemical parameters and body mass index whereas chi square, Fisher exact and odds ratio with 95% confidence interval were used to compare frequencies of different genotypes and alleles among cases and controls. Hardy-Weinberg equilibrium (HWE) law was used to test the concordance of expected genotype frequencies to the observed ones using the chi square test.

RESULTS

Cases and controls showed a non-significant difference regarding their age and gender ratio. However, cases showed a significant lower levels of hemoglobin, red cell count, platelet count, serum albumin and HDL together with a significant higher levels of white cell count, SGOT, serum bilirubin, fasting and postprandial blood sugar, HbA1C level, cholesterol, LDL and TG levels (Table 1).

Regarding gene polymorphism, cases showed a significantly higher frequency of the APOC3T-455C CC genotype than controls (32 vs. 9.6%, p = 0.0003, Odds ratio = 5.33, 95%, CI = 2.2-12.7). Also the allelic frequency of the rare APOC3 -455°C allele was significantly higher among cases than controls (51 vs. 30.72%, p = 0.0001, OR = 2.35, 95%, CI = 1.5 3.6). Hardy Weinberg equilibrium testing showed a non-significant level among controls denoting that the observed genotype frequencies were conforming to the expected ones. On the contrary cases have shown a significant level of HWE meaning that the observed frequencies are not as the expected ones due to increase of the rare genotypes and alleles (Table 2).

On the other hand,cases showed a non-significant difference regarding all APOC3 C-482T genotypes (CT vs. CC, TT vs. CC and CT+TT vs. CC) as well as APOC3 -482 T vs. C alleles from controls. Hardy Weinberg equilibrium showed a significant differences of the observed genotype frequencies from that the expected ones for both cases and controls.

Comparing cases of fatty liver carrying the rare allele of C-482T and T-455C of APOC3 gene vs. others found that the distribution of BMI values, hematologic, liver function and lipid parameters C between showed that all parameters were nearly having a similar distribution denoting that these polymorphismswere not affecting the clinical characteristics of these cases (Table 3 and 4).

Table 1: Demographic, clinical and chemical data of cases of fatty liver compared to controls

	Cases 100 (40/60)	Controls 83 (30/53)	p-value
pM/F	---------- Mean±SD ----------		0.35
AGE	45.45±15.29	45.55±15.53	0.964
HB	11.31±1.74	13.37±1.93	0.000**
RBCs	3.77±0.61	4.77±0.31	0.000**
WBCs	6.28±2.31	5.46±1.34	0.005*
PLATs	167.69±62.75	197.53±49.74	0.001*
SGPT	38.71±43.48	31.52±8.04	0.139
SGOT	43.07±38.97	29.98±6.71	0.003*
BIL	1.29±1.29	0.90±0.14	0.006*
ALB	3.81±0.51	4.27±0.49	0.000**
FBS	131.83±56.25	80.96±9.31	0.000**
PBS	223.23±114.65	127.81±23.39	0.000**
HBA1c	7.00±2.01	4.90±0.38	0.000**
CHOL	230.37±48.58	82.89±16.23	0.000**
LDL	153.05±34.31	83.98±10.99	0.000**
HDL	54.89±11.44	60.96±6.99	0.000**
TG	131.43±72.92	78.49±14.87	0.000**

M: Male, F: Female, SD: Standard deviation, BIL: Bilirubin, ALB: Albumin, SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic pyruvic transaminase, TG: Triglyceride, HDL: High-density lipoprotein, Cholesterol, LDL: Low-density lipoprotein cholesterol, FBS: Fasting blood sugar, PBS: Postprandial blood sugar, PlATs: platelets. *p=<0.05 (significant), **p=<0.001 (extremely significant)

Table 2: Distribution of genotype and allelic frequency of SNP, C-482T and T-455C of APOC3 gene between patients with nonalcoholic fatty liver disease and controls

	Cases N (%)	Controls N (%)	p-value	Odds ratio	95% CI
C-482T					
CC	40 (40.0)	34 (41.0)	Reference		
CT	35 (35.0)	30 (36.1)	0.88	0.99	0.5-1.9
TT	25 (25.0)	19 (22.9)	0.92	1.12	0.5-2.4
CT+TT	60 (60.0)	49 (59.0)	0.98	1.04	0.6-1.9
C allele	115 (57.50)	98 (59.04)	Reference		
T allele	85 (42.50)	68 (40.96)	0.85	1.07	0.7-1.6
HWE	p<0.001**	p<0.001**			
T-455C					
TT	30 (30.0)	40 (48.2)	Reference		
TC	38 (38.0)	35 (42.2)	0.35	1.45	0.7-2.8
CC	32 (32.0)	8 (9.6)	0.0003**	5.33	2.2-12.7
TC+CC	70 (70.0)	43 (51.8)	0.018*	2.17	1.2-4.0
T allele	98 (49.0)	115 (69.28)	Reference		
C allele	102 (51.0)	51 (30.72)	0.0001**	2.35	1.5-3.6
HWE	p<0.05*	p>0.05			

HWE: Hardy Weinberg equilibrium, 95 % CI: 95% confidence interval, N: Number of subjects

Table 3: Distribution of BMI between cases carrying the rare allele of T-455C of APOC3 gene (TC+CC) vs. cases carrying the TT genotype

		APOC3 T-455C		APOC3 C-482T	
BMI_grade		TT N (%)	TC+CC N (%)	CC N (%)	CT+TT N (%)
25.0-29.9	Overweight	0 (0.0)	1 (1.4)	0 (0.0)	1 (1.7)
30.0-34.9	class I obesity	5 (16.7)	19 (27.1)	10 (25.0)	14 (23.3)
35.0-39.9	class II obesity	9 (30.0)	20 (28.6)	12 (30.0)	17 (28.3)
40.0-49.9	class III obesity	16 (53.3)	22 (31.4)	15 (37.5)	23 (38.3)
50.0-59.9	class IV obesity	0 (0.0)	8 (11.4)	3 (7.5)	5 (8.3)
Chi-square		p = 0.112		p = 0.946	

DISCUSSION

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in the world today. This study has shown that all Egyptians patients with obesity associated with NAFLD were found to be diabetic. Insulin resistance is recognized as an essential pathophysiological factor or "first hit" in the development of NAFLD and may play a fundamental role in the pathogenesis of this disorder18. Cases showed also a significant lower levels of hemoglobin, red cell

Table 4: Distribution of hematologic, chemical and lipid parameters between cases carrying the rare allele of C-482T of APOC3 gene vs. others

	APOC3C-482T			APOC3T-455C		
	CC (N = 40)	CT+TT (N = 60)		TT (N = 30)	TC+CC (N = 70)	
	Mean±SD	Mean±SD	p-value	Mean±SD	Mean±SD	p-value
HB	11.17±1.86	11.39±1.67	0.54	11.25±1.85	11.33±1.70	0.84
RBCs	3.74±0.58	3.80±0.63	0.65	3.79±0.57	3.77±0.62	0.83
WBCs	5.84±1.79	6.57±2.58	0.13	6.28±3.05	6.27±1.94	0.98
PLATs	161.3±49.09	171.97±70.48	0.41	176.33±67.51	163.99±60.73	0.37
SGPT	41.03±62.07	37.17±24.89	0.67	33.77±20.50	40.83±50.21	0.46
SGOT	42.30±51.17	43.58±28.57	0.87	44.37±31.81	42.51±41.86	0.83
BIL	1.32±1.58	1.28±1.07	0.87	1.05±0.44	1.40±1.51	0.22
ALB	3.74±0.52	3.86±0.49	0.23	3.84±0.55	3.80±0.49	0.74
FBS	131.1±54.17	132.30±58.04	0.92	130.97±39.78	132.20±62.23	0.92
PBS	228.3±120.17	219.88±111.72	0.72	225.17±91.68	222.40±123.8	0.91
CHOL	232.6±49.06	228.90±48.61	0.71	236.17±41.20	227.89±51.49	0.44
LDL	156.3±33.66	150.92±34.85	0.45	156.20±30.78	151.70±35.84	0.55
HDL	54.70±11.33	55.02±11.61	0.89	56.07±9.01	54.39±12.36	0.50
TG	135.3±74.58	128.87±72.32	0.67	143.33±71.68	126.33±73.36	0.29
HBA1c	7.08±1.91	6.95±2.08	0.75	6.96±1.48	7.02±2.20	0.90
BMI	40.30±6.76	40.03±6.41	0.84	39.68±5.31	40.34±7.00	0.64

count, platelet count, serum albumin and HDL together with a significant higher levels of white cell count, SGOT, serum bilirubin, fasting and postprandial blood sugar, HbA1C level, cholesterol, LDL and TG levels. Cases showed a significantly higher frequency of the APOC3T-455C CC genotype than controls. On the other hand, Cases showed a non-significant difference regarding all APOC3 C-482T genotypes and alleles. Interestingly, this polymorphism showed no relation to patient's BMI grade, hematologic, liver function and lipid parameters.

Li *et al.*[19] study on NAFLD Han Chinese patients found that there was no significant difference between the NAFLD group and the control group with respect to the age and gender distribution. Measurement values of BMI and lipid profile were significantly different between the control group and NAFLD group. Obesity, elevated AST, low HDL, hypercholesterolemia and hypertriglyceridemia were the most common characteristics in the NAFLD group. 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[20]. More recently, these variants have been shown to be associated with the occurrence of NAFLD. Similarly, Li *et al.*[19] study on Chinese patients showed that APOC3 (455T>C) genotypes were associated with NAFLD after adjusting for age, gender and BMI. In partial agreement with our results, Puppala *et al.*[21] have reported that among Southern Indian patients, APOC3 gene polymorphism T-455C (rs2854116) was significantly associated with NAFLD with no 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. Also, Petersen *et al.*[22] studied these polymorphisms among Asian Indian population. They established NAFLD in 38% of the Indian men with variant APOC3 alleles at one or both of these loci. Also, NAFLD was more frequent among those with the variant alleles than those with normal alleles. They proposed that the variant alleles led to increased amounts of APOC3 and inhibition of lipoprotein lipase activity and triglyceride clearance, resulting in hypertriglyceridemia due to increase in chylomicron remnants, which were taken up by the liver resulting in NAFLD[23].

On the contrary to our results, Richartandandhis colleagues 2010, found no relationship between APOC3 mRNA expression and triglycerides content in the livers of morbidly obese women of European descent. Also they did not find any association between gene expression and plasma triglyceride concentrations or insulin-resistance index determined by homeostasis model assessment[24]. Sentinelli *et al.*[25] in their study among Southern European patients found no significant association between APOC3 polymorphisms and fatty liver disease, lipids and insulin-resistance in obese subjects, thus not confirming the suggested role of these APOC3 gene sequence variants. Also the study reported by Niu *et al.*[26] on Chinese Hanpatiens did not find significant associations between these polymorphisms and the risk of NAFLD. Hyysalo *et al.*[27] reported a similar lack of association between the APOC3 gene polymorphisms and NAFLD on their

investigation of Finnish population, but in two SNPs of the APOC3 gene. 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, -482Tor both) had similar amounts of liver fat, plasma APOC3 concentrations, serum triglycerides, HDL and levels of fasting plasma glucose, insulin and transaminases. Furthermore, other subsequent studies in Hispanic, European, American, African. American and European subjects have failed to confirm the association of APOC3 variants with NAFLD28.Interestingly, the study of Yu *et al*.[29] on Chinese Han Population showed the variant APOC3 haplotype was associated with the risk of HTG in individuals without T2DM but not in those with it. The variant APOC3 haplotype was also shown to be associated with a higher plasma TG concentration only in individuals without T2DM and not in those with it. Although present study might suffer some limitations related to its relatively small sample size, including all patients being obese and diabetic and lacking estimation of APOC3 level, it clearly discern a conclusion of positive association of NAFLD in Egyptian obese cases with the T-455C polymorphism and not with C-842T in APOC3 with no apparent impact on their clinical picture.

CONCLUSION

Comparing cases of fatty liver carrying the rare allele of C-482T and T-455C of APOC3 gene against others found that the distribution of BMI values, hematologic, liver function and lipid parameters between showed that all parameters were nearly having a similar distribution denoting that these polymorphisms were not affecting the clinical characteristics of these cases.Moreover, this study will help us in the future, how to control the spread of NAFLD and it will be optimistic to find a new therapies.

SIGNIFICANCE STATEMENT

The significance of current study is to identify a unique T-455C allelic variant in APOC3 of in Egyptian NAFLD. The T-455C allelic position in APOC3 is more abundant than C-482T with people suffering from fatty liver diseases. Hence, the T-455C allelic position in APOC3 can be considered as a good marker to be addressed with people suffering from fatty liver diseases. Likewise, the current study has an advantage, how to regulate the meal of NAFLD and it will be hopeful to discover a novel treatments for NAFLD.

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