

MOLECULAR EVALUATION OF -75G/A AND +83C/T APOLIPOPROTEIN A-1 GENE POLYMORPHISMS IN NON ALCOHOLIC FATTY LIVER DISEASE

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ABSTRACT

Apolipoprotein A-1 (APOA1) is the major protein component of High Density Lipoprotein in plasma, promotes cholesterol efflux from tissues to the liver for excretion, it is a cofactor for Lecithin Cholesterolacyl Transferase (LCAT) which is responsible for the formation of most plasma cholesteryl esters. Therefore, the main objective of the present study was carried out to investigate the association of APOA1 gene polymorphisms among patients with Non Alcoholic Fatty Liver Disease (NAFLD) by using PCR-RFLP analysis. Samples were collected from 100 cases affected with NAFLD as well as 100 healthy samples as a negative control. APOA1 +83C/T gene polymorphism was showed that CC genotype was significantly higher frequency ($p=0.0001$) in healthy control than cases (70% vs. 10%), CT genotype was significantly higher frequency ($p=0.001$) in cases than healthy control (65% vs. 25%) and TT genotype was significantly higher frequency ($p=0.001$) in cases than healthy control (25% vs. 5%). Regarding all types of APOA1 -75G/A gene polymorphism, they were showed that all cases were non-significantly ($p>0.05$) when compared to healthy control. So, NAFLD among Egyptian was strongly associated with mutations related to APOA1 gene polymorphism especially APOA1 +83C/T gene polymorphism.

Key words: Polymorphisms; Nonalcoholic fatty liver disease; APOA1 gene.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is common and may Progress to cirrhosis and its complications. The pathogene of steatosis and cellular injury is thought to be related mostly to insulin resistance and oxidative stress. Therefore, management entails identification and treatment of metabolic risk factor, improving insulin sensitivity, and increasing antioxidant defences in the liver. Weight loss and exercise improve insulin sensitivity. Bariatric surgery may improve liver histology in patients with morbid obesity. Insulin sensitising drugs showed promise in pilot trials as have a number of hepatoprotective agents. Further randomised, well controlled trials are required to determine the efficacy of these drugs (1).

The optimal diet to treat NAFLD is not known. Patients with NAFLD seem more likely to have a diet high in saturated fats and cholesterol and low in fibre and antioxidants (2) Mono and poly unsaturated fats may potentially improve insulin resistance and may be beneficial in improving hepatic steatosis. . Most trials have used a diet similar to that recommended by the American Heart Association with energy restriction and energy intake composed of 40%–50% carbohydrates, 15%–20% protein, and 25%–40% predominately unsaturated fats (3).

Several studies have examined weight loss in obese children with NAFLD. Diet and exercise leading to roughly 500 g/week weight loss in nine children, led to improvement in aminotransaminases and hepatic steatosis as determined by ultrasound. Similarly, weight loss

from diet (1200–1400 calories/day) and exercise (at least six hours/week) was evaluated in 33 obese children aged between 4 and 16 years. Weight loss was associated with normalisation of liver tests and improvement or normalisation of hepatic steatosis on ultrasound. Improvement in aminotransaminases has also been reported in a series of six children with NAFLD, with fluctuating liver enzymes reported in those unable to lose weight **(4)**.

Apolipoproteins are proteins that bind lipids (oil-soluble substances such as fat and cholesterol) to form lipoproteins. They transport the lipids through the lymphatic and circulatory systems **(5)**. Apolipoprotein A1 is a major protein that is a component of high-density lipoprotein, or HDL cholesterol. Also known as Apo A1, it helps clear cholesterol from the blood by removing cholesterol from organs and tissues to be destroyed by the liver, human apoA-1 circulates in plasma primarily as a component of HDL. It is also present on chylomicrons but is rarely found in significant amounts on VLDL or LDL. Apolipoprotein A-1 has two major sites of synthesis: the intestine and the liver **(6)**.

Restriction site polymorphisms have been identified at -75 bp in the promoter region and +83 bp in intron 1 of the apo A1 gene. Association of the +83 bp polymorphism with elevated levels of HDL cholesterol has been stronger than that with the -75 bp polymorphism **(7)**. The genotype effect on circulating apo A1 and HDL cholesterol levels is modulated by sex and environmental factors such as smoking **(8)&(9)**. The differences in HDL cholesterol and apo A1 levels among various genotypes of the apo A1 gene are not modified by different diets, suggesting that the possible benefit is independent of fat and cholesterol intake **(10)**.

SUBJECTS AND METHODS

Subjects

In the present study, Samples were collected from 100 cases affected with NAFLD, as well as, 100 healthy samples as a negative control, using PCR-RFLP analysis. NAFLD patients group had 21 males and 79 females. NAFLD patients age was ranged from 18 to 72 years with mean age = (44.94 ± 15.01) years. All cases were recruited from outpatient clinic, department of obesity and diabetes in Internal Medicine Specialized Hospital (IMSH), Mansoura University, Egypt. These cases were compared to other Negative control was selected including 100 healthy unrelated subjects randomly taken from blood donors, The healthy control group had 26 males and 74 females, their age was ranged from 19 to 65 years with mean age = 45.43 ± 15.54 years.

Blood samples

Peripheral blood was collected from the subjects in 2-ml EDTA tubes, and genomic DNA was isolated using a puregene DNA isolation kit.

Sequence Specific Primers (SSP-PCR)

In the present study the Sequence Specific Primers (SSP-PCR) method was used to detect and determine the polymorphisms -75G/A, and +83C/T genomic DNA was amplified. PCR amplification was performed in single SSP-PCR reaction employing a forward and a reverse primer for polymorphisms of (APOA1) gene. The regions containing RFLPs within the APOA1 gene were amplified with Taq DNA polymerase with gene specific primers

(Forward 5'-AGGGACAGAGCTGATCCTTGAAGCTTAAG-3')

and

(Reverse 5'-TTAGGGGACACCTACCCGTCAGGAAGAGCA-3').

Briefly, The 29 μ l reaction volume contained 10 μ l of 2X PCR Master Mix, 2 μ l of Forward primers, 2 μ l of Reverse primers, 3 μ l of DNA Sample and 12 μ l of distilled water. The amplification was carried out with the following cycling parameters: 240 seconds heat start at

94°C, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds for cycles and a final extension at 72 °C for 7 mint. PCR products were visualized on 2% agarose gel. Afterwards, Eight microliters of PCR product was digested with 2.5 U of the restriction enzyme Msp1 at 37°C for 4 hour, and the digested products were visualized on a 3% Ethidium bromide agarose gel, gel appeared the 433-bp amplicon into 254, 209, 179, and 113.

Statistical analysis of the data

The collected data in the present work were organized and statistically analyzed using SPSS statistical computer package software (version17) and post comparison was carried out with Fisher exact test corrected chi-square test, Odds ratio (OR)& 95% confidence interval (95% CI) and Independent Sample T test. The result were expressed as means \pm standard deviation (SD) for variables of age (years) of cases. The values of $P < 0, 05$ were considered statistically significant.

RESULTS AND DISCUSSION

+83C/T genotype values

Data represented in **Table (1)** shows comparisons among genotypes values between healthy controls and cases regarding APOA1 +83C/T gene polymorphism it was showed that CC genotype was significantly higher frequency in healthy control than cases (70% vs. 10% , OR =0.05 95%CI = (0.02-0.10)) ($p=0.0001$, for each). While CT genotype was significantly higher frequency in cases than healthy control (65% vs. 25%, OR=5.57 95%CI= (3.02 – 10.27) ($p=0.001$, for each). Also TT genotype was significantly higher frequency in cases than healthy control (25% vs. 5% OR = 6.33 95%CI= (2.31- 17.33) ($p=0.001$, for each).

-75G/A genotype values

Data represented in **Table (2)** shows comparisons among polymorphic variants of Apo A1 -75 G/A gene polymorphism among all cases and controls. From this table, cases did not differ significantly from controls ($p>0.05$) regarding all type of apo A1 -75 G/A polymorphism, although the frequency of GG genotype was higher among cases than healthy control (92% vs. 86% OR = 1.87, 95%CI= (0.75- 4.68). Also the frequency of AA genotype was higher among cases than healthy control (4% vs. 0.0% OR=0.49, 95%CI= (0.42 – 0.56)). While the frequency of the GA genotype was higher among healthy control than cases, (14% vs. 4%, OR= 3.83, 95%CI= (1.21 – 12.07)). Also A and G alleles showed no significant among all cases ($p>0.05$).

Comparisons among+83 C/T and -75 G/A genotype of APOA1 gene

Data represented in **Table (3)** shows comparisons among cases to controls as regard +83C/T & -75G/A polymorphisms it was noted that GA/CT genotype of healthy control showed significantly higher frequency than cases (14% vs. 4%, OR=0.256, 95%CI= (0.081 – 0.807) ($p=0.013$). Also GG/CC genotype of healthy control showed significantly higher frequency than cases (70% vs. 10%, OR= 0.048, 95%CI= (0.22 – 0.104) ($p=0.001$), While GG/CT genotype of cases showed significantly higher frequency than control (57% vs. 11%, OR=10.725, 95%CI=5.112 – 22.502) ($p=0.001$ for each). Also GG/TT genotype of cases showed significantly higher frequency than control (25% vs. 5% OR=6.33, 95%CI= (0.231 – 17.33) ($p=0.001$ for each). Also AA/CT genotype of cases showed significantly higher frequency than control (4% vs. 0%, OR=2.042, 95%CI= (1.77 – 2.355) ($p=0.043$).

Table (1): Comparison of +83C/T genotype values among control and cases regarding APOA1 gene polymorphism

APOA1 +83	N	Cases (n = 100)	Control (n = 100)	P	OR (95% CI)
		N (%)	N (%)		
Genotypes	(CC)	10 (10%)	70 (70%)	<0.001*	0.05(0.02 – 0.10)
	(CT)	65 (65%)	25 (25%)	<0.001*	5.57(3.02 – 10.27)
	(TT)	25 (25%)	5 (5%)	<0.001*	6.33(2.31 – 17.33)
Alleles	(C)	85 (42.5%)	165 (82.5%)	<0.001*	0.16(0.10 – 0.25)
	(T)	115 (57.5%)	35 (17.5%)	<0.001*	2.83(2.09 – 3.83)

N= number of subjects, significance using t-test or Chi-square test:

* Significant P < 0.05

P= cases vs. Controls

Table (2): Comparison of -75G/A genotype values among healthy control and cases regarding APOA1 gene polymorphism

-75G/A	APOA1	Cases (n = 100)	Control (n = 100)	P	OR (95% CI)
		N (%)	N (%)		
Genotypes	(GG)	92 (92%)	86 (86%)	0.175	1.87(0.75 – 4.68)
	(GA)	4 (4%)	14 (14%)	0.015*	3.83(1.21 – 12.07)
	(AA)	4 (4%)	0 (0%)	0.041*	0.49(0.42 – 0.56)
Alleles	(G)	188	186	> 0.05	1.18(0.53 – 2.62)
	(A)	12	14	> 0.05	0.92(0.64 – 1.34)

N= number of subjects, significance using t-test or Chi-square test:

* Significant P < 0.05

P= cases vs. Controls

Table (3): Comparison of the combined genotype frequency among healthy control and cases regarding +83 C/T and -75 G/A genotype of APOA1 gene

	Cases	Control	P value	OR (95% CI)
GG/CC	10 (10%)	70 (70%)	< 0.001*	0.048 (0.22 – 0.104)
GG/TT	25 (25%)	5 (5%)	< 0.001*	6.33(2.31 – 17.33)
GG/CT	57 (57%)	11 (11%)	< 0.001*	10.725 (5.112 – 22.502)
AA/CT	4 (4%)	0 (0%)	0.043*	2.042 (1.77 – 2.355)
GA/CT	4 (4%)	14 (14%)	0.013*	0.256 (0.081 – 0.807)

N= number of subjects, significance using t-test or Chi-square test:

* Significant P < 0.05

P= cases vs. Controls

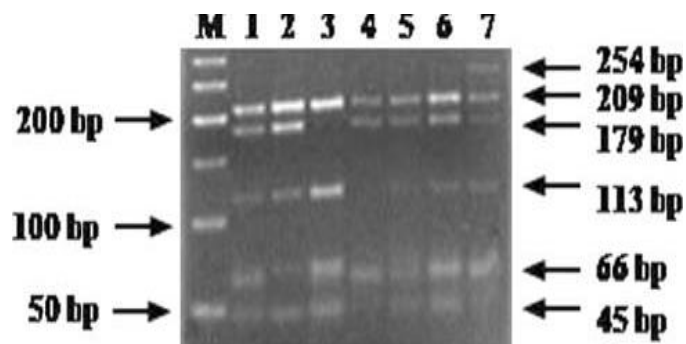


Fig (1): showed cases of -75 G/A and +83C/T polymorphisms of APOA1 gene

Restriction fragment length polymorphism (RFLP) on 3% agarose gel. At lane 1, 2, 5 and 6 there were triple bands 209,179,113bp which refer to GG/CT genotype. At lane 3 there is double bands 209, 113 bp which refer to GG/CC genotype, At lane 4 there is double bands 209 &179 bp which refer to GG/TT genotype, At lane 7 there is 4 bands 254, 209, 179, 113 bp which refer to GA/CT genotype. M DNA marker 50 bp.

Nonalcoholic fatty liver Diseases (NAFLD) has become recognized as one of the most common abnormalities observed in obese individuals (11). It referred to a wide spectrum of liver damage, ranging from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis. Overall, NAFLD has been reported to affect 10% to 24% of populations of various countries and up to 74% of obese individuals (12). NAFLD is

associated with alteration in lipoprotein metabolism resulting in an atherogenic lipid profile with increase in total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL) and reduced level of high density cholesterol (HDL-C) (13) (14). NAFLD might be complicated by disorders like diabetes mellitus,Diabetes mellitus associated with

NAFLD is a complex metabolic disorder characterized by persistent hyperglycemia resulting from defects in insulin secretion, insulin action or both **(15)**. There are also changes to apolipoprotein composition in diabetic patients with increased susceptibility of apolipoproteins A1 and B to glycation which results in decreased affinity for HDL receptors and increased susceptibility to oxidative modification **(16)**.

In the present, study from the cumulative data concluded that there was no relationship between -75(G/A) polymorphism and serum lipid HDL-cholesterol levels when patient values were compared to those of the controls ($p < 0.05$) **(17)**. Also another study suggested that Non difference in apoA1G-75A genotype or allele frequency distributions between the controls and patients were identified. However, there was a significantly lower frequency of the CT genotype ($p = 0.012$) and T allele ($p = 0.011$) in the affected subjects with hypercholesterolaemia or hypertension. Triglyceride levels were significantly lower in the subjects with the CT genotype compared to those with the CC genotype ($p < 0.05$). The G-75A genotypes did not appear to influence the lipid or blood pressure levels. The +83 T allele may be associated with a better lipid profile and blood pressure levels in this group of Chinese subjects **(18)**.

The result of the current work demonstrated that the levels of serum TC, TG, HDL-C and LDL-C were significantly higher than those in healthy control individuals. In addition, the study has compared the liver function tests, CBC, creat, HBA1c and lipid levels of -75G/A & +83C/T combined genotype of apoA1 gene and noted that all values levels between all combined genotype groups showed no significant, while serum TG level was significantly higher in GG/TT genotype than GG/CC genotype. Also, this study observed that all values levels of +83C/T genotype of apo A1 gene showed no significant difference among the three groups (CC/CT/TT).

While the serum TG level in CC genotype was significantly lower than those in TT genotype. Also the study compared the liver function tests, CBC, HBA1c, creat and lipid levels of -75G/A genotype of apoA1 gene and showed no significant, Also the frequency of AA genotype was higher than GG genotype in TG, TC, LDL-C and HDL-C serum levels. While GG genotype was higher than frequency AA genotype in ALB and HBA1c serum levels, Also, this study observed that the mutated homozygous TT & heterozygous CT genotype & mutant T allele of +83C/T polymorphism had a significantly higher frequency among NAFLD cases compared to healthy control. So CT, TT genotype & T allele may be considered as genetic risk factors for NAFLD cases. Also the result found that wild CC genotype and C alleles of +83 C/T polymorphism had significantly higher frequency among healthy controls compared to NAFLD cases. So, CC genotype and C allele may be considered as protective factors against the development of NAFLD.

Comparisons among the results of the current work with populations obesity, the -75G/A polymorphism, A allele frequency was (6.5%) in the the present study. The frequency was close to Brazilian (10.2%) **(19)**, Nigerians (10.1%) **(21)**, other European populations (11.2-15.5%) **(20)**, **(22)**, **(23)**, on other hand A allele was lower than Taiwanese (32%) **(24)**, Spanish (20.3-24%) **(25)**, and omani (22%) **(26)**, Also, The frequency of T allele was (57.5%) in the present study, the results of the current work was higher than the frequency of Brazilian (21%) **(35)**, Nigerian (40.2%) **(21)**, Chinese (4%), Malays (3%) and Indians (2%) from Singapore **(27)**, Omani (6.7%) **(26)** and Australian (4.1%) **(28)**.

In conclusion, the data of the present study concluded that +83C/T and -75 G/A polymorphisms of apo A1 gene were found to be associated with development of NAFLD Egyptian patients. Mutant T allele, CT and TT genotypes of +83 C/T polymorphism may be

considered genetic risk factors for development of NAFLD, there was no relationship between mutant A allele of -75 G/A polymorphism and NAFLD. Maximum risk combined genotypes included (GG and CC), (GG and TT) and (GG and CT) may be consider genetic risk factor. So, This study recommend that studying +83 C/T and -75% G/A polymorphisms of APOA1 gene using simple technique as SSP-PCR can be used as a marker for NAFLD and analysis of these polymorphisms among familial cases.

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