

Association of Apolipoprotein A1 Gene Polymorphism and Coronary Heart Diseases in Patients with Type 2 Diabetes Mellitus

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ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) is one of the world's most common diseases with a high prevalence and incidence.

Objective: To investigate the association of APOA1 polymorphism (-75 G/A) with dyslipidemia and susceptibility to CAD among Egyptian patients with T2DM.

Patients and Methods: A total number of 72 subjects with T2DM and 72 age and sex matched healthy controls were enrolled for the study. T2DM patients were subdivided to T2DM without CAD (n=36) and T2DM with CAD (n=36). Genotyping of the APOA1 was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) combined with gel electrophoresis, and then confirmed by direct sequencing.

Results: Our findings revealed that GA and AA genotypes and A allele of APOA1 G-75A were significantly higher in controls than T2DM without CAD cases. Also, GA and AA genotypes and A allele of APOA1 G-75A were significantly higher in controls than T2DM with CAD cases. The risk of CAD were significantly lower among patients carrying GA and AA genotypes than those carrying GG genotype (OR (95%CI): 0.16 (0.06-0.48), $P < 0.001$; OR (95%CI): 0.08 (0.01-0.78), $P < 0.01$, respectively). Our findings revealed significantly higher values of HDL in AA genotype of APOA1 G-75A compared to GA and GG. On the other hand, there were significantly lower values of TC and TG in AA genotype of APOA1 G-75A compared to GA and GG.

Conclusions: The individuals with the APOA1 -75 GA and AA genotypes and A allele were likely to have a lower risk of T2DM and CAD as a result of its effect on HDL-C serum levels.

Keywords: Apolipoprotein A1, Coronary artery disease, Gene polymorphism, T2DM

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is considered one of the most common diseases with a high prevalence and incidence worldwide ⁽¹⁾. Owing to increased life expectancy, obesity, sedentary life, the incidence of type 2 diabetes is growing at an alarming pace ⁽²⁾. In developing countries, the number of subjects with diabetes is expected to increase by 150 % in the next couples of years. In terms of prevalence of diabetes, Egypt is eighth in the world ⁽³⁾.

The leading cause of death and morbidity worldwide is coronary artery disease (CAD) ⁽⁴⁾. Although the exact mechanisms responsible for the onset of CAD are still unclear, it is a multifactorial disease affected by endogenous and exogenous factors that interact ⁽⁵⁾. Age, stress, hypertension, diabetes mellitus, hyperlipidemia, smoking, high alcohol intake, CAD, family history and obesity are the main risk factors for CAD ⁽⁶⁾.

Lipid metabolism disorder and genetic predisposition are important risk factors for CAD ⁽⁷⁾. Genetic risk factors have been estimated to explain approximately 20%-60% of CAD cases ⁽⁸⁾. Some genes have been investigated for their participation in the development of CAD ⁽⁹⁾.

The main apolipoprotein portion of high density lipoprotein (HDL) is apolipoprotein A1 (apoA1) and is involved in the transport of reverse cholesterol ⁽¹⁰⁾. In the APOA1 gene located on the long arm of chromosome 11, several single nucleotide polymorphisms (SNPs) have so far been identified ⁽¹¹⁾. A common-to-A transition of -75 base pairs (bp) located upstream from the APOA1 gene transcription start site has been extensively studied ⁽¹²⁾. In the first intron of the APOA1 gene, which includes the transition of C-to-T at +83 bp, another polymorphism site was found that has also been shown to affect HDL and apoA1 levels ⁽¹³⁾.

CAD is the most common fatal complication of T2DM, accounting for almost 60 percent of deaths among patients with T2DM. Patients with T2DM are subject to a two-fourfold higher risk of developing CAD than the overall population ⁽¹⁴⁾. Thus, identifying the variables that predispose these patients to develop CAD is of fundamental importance in T2DM management. Insulin controls multiple steps in the metabolism of plasma lipoproteins ⁽¹⁵⁾. Dyslipidemia, characterized by hypertriglyceridemia and high density lipoprotein (HDL)



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cholesterol, is frequent in T2DM and can increase the risk of CAD ⁽¹⁶⁾.

This is the first research to investigate, to the best of our knowledge, the association of APOA1 polymorphism (75 G/A) with dyslipidemia and CAD susceptibility among Egyptian patients with T2DM.

SUBJECTS AND METHODS

This study included 72 patients with T2DM recruited from diabetes and endocrinology outpatient clinic of Internal Medicine Department of Zagazig University Hospitals and 36 healthy lean control subjects, who were matched to cases by gender and ethnic origin.

The diagnosis of diabetes was according ADA 2020 ; fasting plasma glucose levels of ≥ 126 mg/dL (7.0 mmol/L) or 2-h postprandial plasma glucose levels of ≥ 200 mg/dL (11.1 mmol/L)⁽¹⁷⁾. The enrolled diabetic patients were classified into two groups: 36 patients without CAD and 36 CAD cases were defined as those having severe angiostenosis (>50 %) in at least one major coronary artery determined by angiography based on ACC/AHA criteria ⁽¹⁸⁾.

CAD cases were selected randomly from patients admitted to outpatient clinic of Internal Medicine Department of Zagazig University Hospitals and who fulfilled the inclusion criteria. All patients were subjected to thorough history taking and full clinical assessment including blood pressure and anthropometric variables. BMI was calculated as weight in kg/ height in meters². Patients that were using statins or other lipid-lowering drugs discontinued this treatment for at least 45 days before they participated in this study. None of the participants had liver, kidney, thyroid, or any active inflammatory diseases, as well as receiving medications that affect glucose metabolism or for weight reduction or participating in a dietary or exercise programs.

Ethical approval:

The Ethical Committee of Faculty of Medicine, Zagazig University approved our study protocol, and all participants assigned written informed consent.

Blood samples:

Blood samples were drawn from all subjects after an overnight fasting and divided into 3 portions: 1 ml of whole blood was collected into evacuated tubes containing EDTA, for genomic DNA extraction and HbA1c; 1 ml of whole blood was collected into evacuated tubes containing fluoride for fasting blood glucose. Serum were separated immediately from remaining part of the sample and stored at -20°C until analysis.

Biochemical analysis:

We determined fasting blood glucose using the glucose oxidase method (Spinreact, Girona, Spain). Total cholesterol and triglycerides were measured by routine enzymatic methods (Spinreact, Girona, Spain). HDL cholesterol was determined after precipitation of the apoB-containing lipoproteins. LDL cholesterol was calculated using the Friedewald formula ⁽¹⁹⁾.

DNA Extraction:

All the work was carried out in the molecular biology laboratory in the Medical Biochemistry Department. All the reagents were highly purified analytical PCR-materials. All the tubes, tips pipettes used for DNA extraction were DNase, RNase free tubes to avoid contamination; purchased from Gentra (Minneapolis. USA).

Genomic DNA was isolated and purified from blood samples using the QIAamp DSP DNA Blood Mini Kit purchased from Qiagen GmbH (Hilden, Germany). DNA was extracted according to the method of **Miller *et al.*** ⁽²⁰⁾ in which standard DNA isolation from 200 μl whole blood in standard 45 minutes protocol, including RNase treatment. Expected yield: 5-15 μg DNA.

Each QIAamp DSP DNA Blood Mini procedure comprises 4 steps:

1. Lysing the cells in the blood sample.
2. Binding the genomic DNA in the cell lysate to the membrane of a QIAamp Mini spin column.
3. Washing the membrane.
4. Eluting the genomic DNA from the membrane.

DNA quantitation:

For DNA concentration determination and DNA purity evaluation, in 1 ml of deionized water in a quartz cuvette, 20 μL of each extracted DNA sample was added and absorption was measured at 260 and 280 nm wavelengths using the Milton Roy Spectronic 3000 Array. DNA has a maximum absorbance at 260 nm because the pyrimidine resonance structures and purine bases are responsible for the absorbance. At 260 nm, an absorption of 1.0 gives a DNA concentration of 50 $\mu\text{g}/\text{mL}$. Because of the presence of tyrosine, phenylalanine and tryptophan, proteins absorb maximally at 280 nm and absorption at this wavelength is used for protein detection in DNA samples. This is done through the A260/A280 ratio determination. This ratio was commonly considered to be between 1.8 and 1.9 for pure double-stranded DNA ⁽²¹⁾.

Apo lipoprotein A1 polymorphism genotyping: The Egyptian subjects were genotyped by the multiplex amplification refractory mutation system PCRR for Apo lipoprotein A1

polymorphism. (Multi-ARMS PCR) as described previously.

The upstream internal primers were P1 (5'-ATGCCGAT GACCTGCAGAATT-3') and P2 (5'-ATGCCGATGACCTGCAGAATC-3'), the upstream external primers were P3 (5'-CGCGGACAT GGAGGACGTTT-3') and P4 (5'-CGCGGACATGGAGGACGTTC-3'), the downstream primer was P5 (5'-GTTCAGTGATTGTCGCTG GGCA-3'). Two tubes were used to duplicate the amplification reactions: tube A containing P5, P1 and P3, tube B containing P5, P2 and P4. The PCR amplification profile was as follows: initial denaturation for 4 min at 95 °C, 35 cycles with denaturation for 45 s at 96 °C, annealing for 45 s at 66 °C, and extension for 45 s at 72 °C, followed by a final extension cycle for 5 min at 72 °C. The fragments were separated on 2 percent agarose gels ⁽²²⁾.

Statistical analysis

Data were verified, coded by the researcher and analyzed using IBM-SPSS Statistics for windows, version 23.0 (Copyright IBM Corp., Armonk, N.Y., USA. 2015). Descriptive statistics: Means, standard deviations, and percentages were calculated. Test of significances: Chi-square test was used to compare the difference in distribution of frequencies among different groups. For continuous variables; independent t-test analysis was carried out to compare the means of

dichotomous data. There was no specific calculation of the sample size. A significant p-value was considered when it is equal or less than 0.05.

RESULTS

Among studied subjects, in the control group, 69.6 % were male and 30.4% were female, their mean age was 45.66±7.56 year. In the diabetic group, 64.3% were male and 35.7% were female, their mean age was 45.58±8.3 year. Control and T2DM groups were matched for age, ethnicity and gender.

Among T2DM groups, T2DM without CAD 67.8 % were male and 32.2% female, their mean age was 47.13±8.08 year. In T2DM with CAD, 71.4% were male and 28.6% were female, their mean age was 44.02±8.33 year. Both diabetic groups were matched for age and gender.

Diabetic patients had significantly higher values of BMI, waist/hip ratio, systolic and diastolic blood pressure as well as lipid profile; TG, TC and LDL compared to control group.

Furthermore, diabetic patients had significantly higher values of FPG, HbA1c and 2hr PP plasma glucose compared to control group. On the other hand, T2DM patients had significantly lower values of HDL compared to control group (Table 1)

Table (1): Clinical, anthropometric and laboratory characteristics of studied groups

Variables	Control group (Mean± SD)	T2DM group (Mean± SD)	P-value
Systolic blood pressure (mmHg)	118.33±6.97	130.56±18.76	<0.001*
Diastolic blood pressure (mmHg)	78.33±6.97	90.83±17.82	<0.001*
Body mass index (kg/m ²)	20.83±1.15	23.71±1.91	<0.001*
Waist circumference	69.50±7.06	83.74±8.74	<0.001*
Fasting plasma glucose (mg/dl)	92.11±10.46	191.68±36.35	<0.001*
2hr PP plasma glucose (mg/dl)	122.97±16.34	311.07±9.26	<0.001*
HbA1c (%)	4.60±0.45	9.77±2.09	<0.001*
HDL cholesterol (mg/dl)	55.89±7.28	34.83±5.91	<0.001*
LDL cholesterol (mg/dl)	85.03±8.23	120.29±9.73	<0.001*
Total cholesterol (mg/dl)	156.33±23.24	251.96±36.15	<0.001*
Triglycerides (mg/dl)	113.89±24.76	208.40±35.21	<0.001*

T2DM, type 2 diabetes mellitus; * P< 0.05.

Anthropometric and biochemical characteristics of the T2DM groups

Among T2DM patients (n=72), 36 patients had CAD confirmed by coronary angiography, they had significantly longer duration of DM and higher values of systolic as well as diastolic blood pressure compared to T2DM patients without CAD. Furthermore, diabetic patients had significantly lower values of waist circumference and HDL compared to control group (Table 2).

Table (2): Clinical, anthropometric and laboratory characteristics of T2DM groups

	Without CAD (Mean ±SD)	With CAD (Mean ±SD)	P-value
Duration of DM (years)	5.18±3.44	10.72±4.48	<0.001*
Systolic blood pressure (mmHg)	119.72±15.58	141.39±15.15	<0.001*
Diastolic blood pressure (mmHg)	80.56±14.13	101.11±15.08	<0.001*
Body mass index (kg/m ²)	23.76±1.92	23.66±1.92	0.826
Waist circumference(cm)	86.19±8.08	81.28±8.79	<0.05*
Fasting plasma glucose (mg/dl)	195.17±38.54	188.19±34.20	0.420
2hr PP plasma glucose (mg/dl)	317.06±8.56	305.08±78.62	0.525
HbA1c (%)	10.17±2.26	9.37±1.85	0.106
HDL cholesterol (mg/dl)	39.83±3.07	29.83±3.16	<0.001*
LDL cholesterol (mg/dl)	123.11±31.40	117.47±24.36	0.425
Total cholesterol (mg/dl)	250.56±35.13	253.36±37.58	0.744
Triglycerides (mg/dl)	208.92±36.22	207.89±34.67	0.902

T2DM, type 2 diabetes mellitus; CAD coronary artery disease.*P< 0.05.

Distribution of genotype and allele frequencies of APOA1 (G-75A) in controls and T2DM without CAD cases

In T2DM cases without CAD and healthy controls, genotype and allelic frequencies of APOA1 (G-75A) gene polymorphisms are shown in table 3. In each studied group, the distributions of genotypes were in Hardy-Weinberg equilibrium. Our findings revealed that GA and AA genotypes and A allele of APOA1 G-75A were significantly higher in controls than T2DM without CAD cases. The risk of T2DM were significantly lower among patients carrying GA and AA genotypes than those carrying GG genotype or at least carrying one A allele than among patients carrying the G allele (Table 3).

Table (3): Distribution of APOA1 (G-75A) genotypes and allele frequencies in healthy controls and T2DM without CAD

		Controls		T2DM without CAD		OR (95%CI)	P
APOA1 G-75A	GG	33	(30.5)	63	(58.3)	-----	
	GA	60	(55.6)	52	(38.9)	0.37 (0.13-1.00)	0.043*
	AA	15	(13.9)	3	(2.8)	0.11 (0.01-1.01)	0.022*
	G allele	126	(58.3)	168	(77.8)		
	A allele	90	(41.7)	48	(22.2)	0.4(0.19-0.83)	0.011*

*P< 0.05.

Distribution of genotype and allele frequencies of APOA1 (G-75A) in controls and T2DM with CAD cases

Genotype and allelic frequencies of APOA1 (G-75A) genes polymorphisms in T2DM cases with CAD and healthy controls. The genotypes distributions were in Hardy–Weinberg equilibrium in each studied group.

Our findings revealed that GA and AA genotypes and A allele of APOA1 G-75A were significantly higher in controls than T2DM with CAD cases. The risk of CAD were significantly lower among patients carrying GA and AA genotypes than those carrying GG genotype or at least carrying one A allele than among patients carrying the G (Table 4).

Table (4): Distribution of APOA1 (G-75A) genotypes and allele frequencies in healthy controls and T2DM with CAD.

		Controls		T2DM with CAD		OR (95%CI)	P
APOA1 G-75A	GG	33	(30.5)	81	(75)		
	GA	60	(55.6)	24	(22.2)	0.16(0.06-0.48)	<0.001*
	AA	15	(13.9)	3	(2.8)	0.08(0.01-0.78)	<0.01*
	G allele	126	(58.3)	186	(86.1)		
	A allele	90	(41.7)	30	(13.9)	0.23(0.10-0.51)	<0.001*

*P< 0.05.

DISCUSSION

T2DM, a leading independent risk factor for cardiovascular disease, is the most frequent cause of morbidity and mortality among diabetic patients (CVD) ⁽²³⁾. Egypt's healthcare services are heavily burdened by the growing prevalence of non-communicable diseases (NCDs), including diabetes and cardiovascular diseases, and nearly 41 percent of all deaths in Egypt are attributed to NCDs ⁽²⁴⁾. Modifiable factors have been reported as risk factors for both T2DM and CVD, such as dyslipidemia, obesity, oxidative stress, smoking, exercise and alcohol consumption, as well as non-modifiable factors: age, sex, positive family history and genetic predisposition of the person ⁽²⁵⁾. Lipoprotein-related mechanisms have been associated with damage to the cardiovascular system in patients with diabetes ⁽²⁶⁾.

Lipid metabolism disorders, such as elevated TC serum levels ⁽²⁷⁾, TGs ⁽²⁸⁾ LDL-c, ⁽²⁹⁾ and (Apo) B ⁽³⁰⁾ Or low levels of HDL-c ⁽³¹⁾ and ApoAI ⁽³²⁾, have been considered to be important risk factors in the development of atherosclerosis CAD ⁽³³⁾.

In reality, for early prediction and identification of at-risk patients and for better clinical management, better understanding of the pathogenesis of CVD and T2DM is of great importance. The goal of the current study was therefore to investigate the association of APOA1 polymorphism (-75 G/A and +83 C/T) with dyslipidemia and CAD susceptibility in Egyptian T2DM patients.

We further sub-group our T2DM patients according to the findings of coronary angiography to T2DM without CAD and T2DM with CAD in order to better elucidate the CAD risk factors. The CAD subgroup of T2DM patients had a substantially longer DM period and higher systolic and diastolic blood pressure relative to T2DM patients without CAD.

This research was the first study examining, to the best of our knowledge, the APOA1G-75A genes mutation in both T2DM with or without CAD for early detection of cardiovascular risks. Accordingly, we can use protective measures of CAD. APOA1 G-75A was the most important finding in our research that GA and AA genotypes and A allele of APOA1 G-75A were significantly higher in controls than T2DD.

Thus, the risk of T2DM was significantly lower among patients carrying GA and AA genotypes than those carrying GG genotype.

In a pilot study in a population in northern India, APOA1 polymorphisms (-75 G/A and +83 C/T) may be susceptible to myocardial infarction ⁽³⁴⁾. In addition, a Brazilian elderly cohort showed

that polymorphisms of APOA1 (-75 G/A and +83 C/T) may be risk factors for hypertension and obesity ⁽³⁵⁾.

The protective effect of HDL-c and ApoAI on the risk of CAD is primarily mediated by the promotion of cholesterol efflux from peripheral cells. Moreover, both HDL-c and ApoAI may have antioxidant, anti-thrombotic and anti-inflammatory properties that may have major anti-atherogenic effects ⁽³⁶⁾. The apo AI gene is located in the 11q23 chromosomal region and is part of the multigene apolipoprotein superfamily, which includes genes encoding exchangeable apolipoproteins such as ApoAI, AII, Cs, and E. The protective effect of GA and AA genotypes and the A allele of APOA1 G-75A against CAD production was an important outcome of our research.

Our results showed that in the control group, the GA and AA genotypes and A allele of APOA1 G-75A were significantly higher than T2DM with CAD cases. Therefore, among patients carrying GA and AA genotypes, the risk of T2DM was significantly lower than those carrying the GG genotype.

Elevated HDL-c levels have been associated with the unusual allele A of the promoter region polymorphism 75G/A, and this elevated level is expected to be due to its effects on the transcription of the apo AI gene and increase in ApoAI levels.

Similarly, **BiHong et al.**'s ⁽³⁷⁾ findings suggested that individuals with the APOA1-75 A allele were likely to have a lower risk of CAD due to its effect on higher serum ApoA1 and HDL-C concentrations. To confirm this finding, further studies are required.

Yang et al. ⁽³⁸⁾ investigated APOA1 polymorphism in the same alleles and genotypes in accordance with our study and found GA and AA genotypes and A allele to be more distributed in the control group than the diabetic or diabetic cardiac group so that they are protective.

Also, similar results were described in **Angotti et al.** ⁽³⁹⁾ study. They track the distribution of different genotypes of APOA1 G 75 A polymorphism in diabetic patients with previous myocardial infarction.

CONCLUSION

Our results revealed that individual carrying GA and AA genotypes and A allele of APOA1 G-75A were significantly higher in controls than both T2DM groups (without or with CAD). Thus, the individuals with the APOA1 -75 GA and AA genotypes and A allele were likely to have a lower risk of T2DM and CAD as a result of its effect on HDL-C. Regarding APOA1 C+83T, TT and CT

genotypes and T allele of APOA1 C+83T were significantly higher in T2DM with CAD cases than control.

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