Apolipoprotein E Gene Variants As A Risk Factor For Coronary Artery Disease In Type 2 Diabetic Egyptian Patients

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Patients with diabetes mellitus (DM) have increased mortality and morbidity of cardiovascular diseases compared with non-diabetic patients. The role of apolipoprotein E in lipid metabolism and cholesterol transport is well established. Apolipoprotein E gene (APO E) polymorphism that confers susceptibility to or protection from CAD in patients with type 2 DM may be quite different in different ethnic populations. We aimed to determine the frequencies of allelic variants of APO E in Egyptian population and to examine the relationship between APO E polymorphism and risk of coronary artery disease (CAD) in Egyptian type 2 diabetic patients. The study included 35 diabetic patients with CAD (group I), 35 diabetic patients without CAD (group II) and 30 control subjects. All were subjected to history taking, clinical examination, and laboratory investigations for lipid profile and APO E genotyping by PCR–RFLP. Results revealed that ε3 allele was the commonest among the studied subjects (84%). The frequencies of ε2 and ε4 alleles were higher in group I (24.3% and 8.6% respectively) than group II and controls. The frequency of E2/E2, E2/E3, and E4/E3 genotypes was significantly higher in group I than group II and controls. Comparing group I vs. controls and group I vs. group II, multivariate analysis demonstrated significantly increased risk for CAD with ε4 and ε2 alleles vs. ε3 (OR=7.02 and 4.97 respectively). In Conclusion, ε4 and ε2 alleles are associated with higher risk of CAD in type2 DM than ε3 allele. Larger scale studies are still needed to either confirm or modify these results.

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, 2010).

Cardiovascular diseases are significantly increased in patients with metabolic syndrome and type 2 diabetes (Zhang *et al.,* 2009).

Apolipoprotein E gene (APO E) consists of three common alleles, designated as ε2, ε3 and ε4 which code for E2, E3 and E4 proteins respectively resulting in three homozygous (E2/E2, E3/E3, E4/E4) and three heterozygous (E3/E2, E4/E2 and E4/E3) genotypes (Anoop *et al.,* 2010). Recently, some reports suggest that APO E polymorphisms may play a role in diabetes and its outcome (Ma *et al.,* 2010). Genetic polymorphism of APO E has been reported as an important dyslipidemia genetic marker associated with coronary artery disease (CAD) and it is possible that the complications of type 2 DM can be also associated with the polymorphism of the APO E (Leiva *et al.,* 2005). APO E polymorphism that confers susceptibility to or protection from CAD in patients with type 2 DM may be quite different in different ethnic populations. This study aimed to determine the frequencies of allelic variants of APO E, examine the relationship between APO E polymorphism and risk of coronary artery disease (CAD) and analyze association of polymorphism with classical lipid risk factors [Serum total cholesterol (TC), triglycerides (TG), high density lipoprotein–cholesterol (HDL-c) and low density lipoprotein-cholesterol (LDL-c)] in Egyptian population.

Subjects and Methods

This study was carried out at Menofiya University Hospital, where samples were collected from the Catheterization Unit inpatient clinic there. The study was approved by the ethical committee of the Faculty of Medicine, Menofiya University Hospital. A signed informed consent was obtained from all the study subjects. The study involved 70 diabetic patients (47 males, 23 females), their ages ranged between 38 - 62 years. Patients were divided into 2 groups: group I included 35 diabetic patients with CAD (24 males, 11 females), their ages ranged between 42-62 years and group II included 35 diabetic patients without CAD (23 males, 12 females), their ages ranged between 38-62 years. In addition 30 apparently healthy, age and gender matched subjects were involved in this study as a control group (21 males, 9 females).

All individuals were subjected to history taking, full clinical examination, and laboratory investigations, including lipid profile (TC, TG, HDL-c and LDL-c) and genotyping of the APO E by PCR–RFLP.

Procedure of PCR -RFLP for Apo-E genotypes

DNA was extracted from EDTA peripheral blood samples using commercially available spin-column technique kit for DNA extraction from human whole blood (QIAamp®DNA Blood Mini Kit, Qiagen, 28159 Avenue Stanford, Valencia, USA). DNA concentration and purity was determined Spectrophotometrically by measuring the optical density at 260 nm and 280 nm of the prepared diluted DNA. PCR reactions were performed in a 50 µL reaction containing 25 µL of a ready for use master mix (Taq PCR master Mix Kit 250 unit, Qiagen), 0.5 µM of each APO E specific primer, 1µg DNA and 2.5 µL DEMSO.

5'ATAAATATAAAATATAAATAACAGAATTC GCCCCGGCCTGGTACAC-3' was used as sense and 5'TAAGCTTGGCACGGCTGTCCAAGGA-3' was used as antisence (Paul et al., 1995). PCR was performed on Perkin Elmer thermal cycler (PCR system 2400 PERKIN ELMER, version 2.11, USA) as followed, 10 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 1 min at 60°C and 1 min at 72°C, followed by a final elongation of 5 min at 72°C. PCR products were separated by standard electrophoresis on 2% agarose gel containing ethidium bromide and UV photographed. The amplified APO E gene was 267 bp (Fig.1).

In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis.

After amplification, DNA was digested with restriction enzyme HhaI (10 units/ µL) (Fermentas International Inc., headquartered in Canada). The digestion mixture contained: PCR product (20 µL), restriction enzyme buffer (2 µL) HhaI (2 µL) and nuclease-free water (10 µL), and incubated for 3 hours at 37°C. Then, it was electrophoresed on 5% agarose gel containing ethidium bromide and UV photographed (Fig.2).

Restriction enzyme digestion of the fragment and electrophoresis allowed the products of 4 out of 6 different APO E genotypes to be visualized. The ε2 allele is characterized by the presence of the 104-bp and 91-bp bands and the absence of the 56-bp and 48-bp bands. The absence of both the 104-bp and 72-bp bands is typical for the presence of the ε3 allele. The ε4 allele is characterized by the absence of both the104-bp and 91-bp bands and the presence of a 72-bp band.

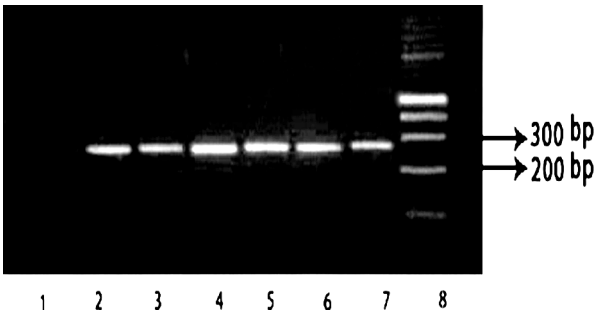

Figure 1. Agarose gel electrophoresis of PCR products after amplification. Lane 8 shows the DNA marker, lane 1 shows non-template control, lanes from 2 to 7 show APO E gene (The size of the amplified fragment is 267 bp).

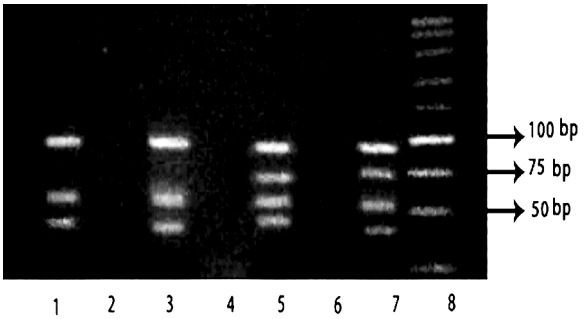

Figure 2. Agarose gel electrophoresis of PCR products after digestion by restriction enzyme (HhaI). Lane 8 shows the DNA marker. Lane 1 and lane 3 shows APO E genotype, E3/E3. Lane 5 and lane 7 shows APO E genotype, E4/E3.

Statistical Analysis

Results were collected, tabulated, statistically analyzed by IBM personal computer and statistical package SPSS version 11 (Chiccago, IL, USA). Data were expressed in the form of percentage (%), mean (x) and standard deviation (SD). Analytic statistics used were Chi-square test (χ^2), Student t-test, Odds ratio (OR), Mann-Whitney test and Kruskal-Wallis test. Differences were considered statistically significant (S) with $P<0.05$.

Results

The study included 100 subjects categorized into 3 groups: group (I) 35 diabetic patients with CAD, group (II) included 35 diabetic patients without CAD and group III included 30 control subjects.

Lipid profile of patients and control groups is shown in table (1). There was a significant increase in the level of TC, LDL-c and TG and a significant decrease in the level of HDL-c in both group I and group II than controls. There was no significant difference in TC, LDL-c, TG and HDL-c between both patient groups (group I and group II).

Table1. Lipid profile of patients and control groups.

Parameter	Studied groups (mean ± SD)			*P* value
	Group I	Group II	Group III	
	N =35	N =35	N =30	
TG (mg/dl)	213.68±21.80	209.65± 19.55	93.66±16.78	*<0.001 **<0.001 ***NS
TC (mg/dl)	199.17±32.64	193.17±25.38	179.1±16.96	*0.003 **0.012 ***NS
HDL-c (mg/dl)	34.05± 5.19	35.91± 3.41	42.53± 2.16	*<0.001 **<0.001 ***NS
LDL-c (mg/dl)	146.74±23.29	154.94±20.27	111.23± 11.42	*<0.001 **<0.001 ***NS

GroupI= diabetic patients with CAD, groupII= diabetic patients without CAD, groupIII=controls, TC=serum total cholesterol, TG=triglycerides, HDL-c=high density lipoprotein–cholesterol and LDL-c=low density lipoprotein-cholesterol, $P<0.05$ is significant. * group I vs III, ** group II vs III, *** group I vs II

Comparing APO E allele frequencies between the studied groups revealed significant difference between group I and control group and between both patient groups, so that, ε3 allele was represented more in group II (94.2%) and group III (91.7%) than in group I (67.1). However, both the ε2 allele and the ε4 allele were significantly higher in group I (24.31.6% and 8.6% respectively) than group II (2.9% and 2.9 % respectively, *P*<0.001) and group III (6.7% and 1.6% respectively, P= 0.003). There was no significant difference between group II and controls (table 2).

Comparing APO E genotype distribution between the studied groups, there were significant difference between group I and control group. Genotypes E2/ E2, E2/ E3, E3/ E4 were represented more in group I, while genotype E3/ E3 was represented more in control group (*P*<0.05). There were significant difference between group I and group II. Genotypes E2/ E2, E2/ E3, E3/ E4 were represented more in group I, while genotype E3/ E3 was represented more in group II (*P*<0.05). There were no significant difference between group II and control group (table 2).

Table2. APO E allele frequencies and genotypes in patients and control groups.

APO E allele	Studied groups			*P* value
	Group I	Group II	Group III	(X^2=chi square)
ε3	47 (67.1%)	66 (94.2%)	55 (91.7%)	*0.003
ε2	17 (24.3%)	2 (2.9%)	4 (6.7%)	**NS
ε4	6 (8.6%)	2 (2.9%)	1 (1.6%)	***<0.001
ApoE genotype	Studied groups			*P* value
	Group I	Group II	Group III	
E3/E3	18 (51.5%)	31 (88.6%)	26 (86.7%)	*0.023
E2/E2	6 (17.1%)	0 (0.0%)	1 (3.3%)	**NS
E2/E3	5 (14.3%)	2 (5.7%)	2 (6.7%)	***0.005
E4/E3	6 (17.1%)	2 (5.7%)	1 (3.3%)	

GroupI= diabetic patients with CAD, groupII= diabetic patients without CAD, groupIII=controls, , *P* <0.05 is significant. NS= not significant * group I vs III, ** group II vs III, *** group I vs II

Multiple logistic regression analysis for APO E allele frequencies between group I and control group and between group I and II showed significant odds ratio of 4.97 for ε2 (*P*=0.007) and significant odds ratio of 7.02 for ε4 (*P*=0.04) versus ε3 (referent). While, between group II and control group, it showed non significant odds ratio for ε2 or ε4 versus ε3 (referent). This indicates that ε4 and ε2 alleles are significant independent risk factors for CAD. Multiple logistic regression analysis for APO E genotypes between group I and control group showed significant odds ratio of 8.76 (for both E2/E2 and E3/E4 genotypes) versus E3/E3 (referent) (*P*=0.05). APO E genotypes between group I and group II showed significant odds ratio of 5.17 (for E3/E4 genotypes) versus E3/E3 (referent)

(*P*=0.05). Non significant difference in odds ratios was observed between group II and control group regarding APO E genotypes (table 3).

Knowing that E3/ E3 genotype corresponds to E3 phenotype, E2/ E2 and E2/ E3 genotypes correspond to E2 phenotype and E4/ E3 genotype corresponds to E4 phenotype; No E4/ E4 and E2/ E4 genotypes had been encountered in our study subjects. In group I, APO E phenotypes were distributed as follows: APO E3 was the commonest (N=18/35), then APO E2 (N=11/35) and finally, APO E4 (N=6/35). In group II, APO E phenotypes were distributed as follows: APO E3 was the commonest (N=31/35), then APO E2 (N=2/35) and APO E4 (N=2/35). In group III, APO E phenotypes were distributed as follows: APO E3 was the commonest (N=26/30), then APO E2 (N=3/30) and finally, APO E4 (N=1/30) (Table 4).

Table 3. Odds ratio of APO E genotype and alleles.

APO E allele	Studied groups			*OR (R)	**P* value	**OR (R)	***P* value	***OR (R)	****P* value
	Group I	Group II	Group III						
ε3	47(67.1%)	66(94.2%)	55(91.7%)						
ε2	17(24.3%)	2(2.9%)	4(6.7%)	4.97	0.007	0.42	NS	4.97	0.007
ε4	6(8.6%)	2(2.9%)	1(1.6%)	7.02	0.040	1.67	NS	7.02	0.040
APO E genotype	Studied groups			*OR (R)	**P* value	**OR (R)	***P* value	***OR (R)	****P* value
	Group I	Group II	Group III						
E3/E3	18(51.5%)	31(88.6%)	26(86.7%)						
E2/E2	6 (17.1%)	0(0.0%)	1(3.3%)	8.76	0.042				0.003
E2/E3	5(14.3%)	2(5.7%)	2(6.7%)	3.61	NS	0.84	NS	4.31	0.08
E4/E3	6(17.1%)	2(5.7%)	1(3.3%)	8.76	0.042	1.68	NS	5.17	0.042

GroupI= diabetic patients with CAD, groupII= diabetic patients without CAD, groupIII=controls, OR=odds ratio, *P*<0.05 is significant, R=referent. * group I vs III, ** group II vs III, *** group I vs II

Studying lipid laboratory parameters among the three APO E phenotypes within both group I and group II revealed that TG, TC, LDL-c were significantly higher and HDL-c was significantly lower in E4 phenotype than in E3 phenotype. There was non significant difference between E2 and E3 phenotypes regarding the studied parameters. Within the control group, apart from significantly higher TC in E2 than E3 phenotype (*P*=0.013), the other laboratory parameters did not differ significantly both between E4 and E3 phenotype and between E2 and E3 phenotype (Table 4).

Table 4. Comparison of the lipid laboratory parameters regarding different APO E phenotypes in group I, group II and group III.

Group I	Phenotypes (mean ± SD)			*P* value
	E2	E3	E4	
	N =11	N =18	N = 6	
TG (mg/dl)	12.09±20.59	207.11±21.20	236.33±8.80	*NS **0.005
TC (mg/dl)	198.45±36.90	188.77±28.72	231.66±7.76	*NS **0.001
HDL-c (mg/dl)	34.18 ± 5.28	36.0 ± 4.33	28.66 ± 3.61	*NS **0.003
LDL-c (mg/dl)	145.36±18.37	136.44±18.51	180.16±10.99	*NS **0.001
Group II	E2	E3	E4	*P* value
	N =2	N = 31	N = 2	
TG (mg/dl)	215.0±24.04	207.03±17.92	245.0 ±2.82	*NS **0.019
TC (mg/dl)	232.0±2.82	186.90±19.01	251.5± 2.12	*NS **0.019
HDL-c (mg/dl)	32.5 ± 0.70	36.51 ± 3.11	30.0 ± 0.0	*NS **0.023
LDL-c (mg/dl)	173.0±4.24	151.35±18.29	192.50±6.36	*NS **0.029
Group III	E2	E3	E4	*P* value
	N =3	N =26	N = 1	
TG (mg/dl)	104.33±8.32	91.15±16.02	127	*NS **NS
TC (mg/dl)	199.0± 7.54	175.57±15.11	211	*0.013 **NS
HDL-c (mg/dl)	42.0 ± 1.73	42.80 ± 1.95	37	*NS **NS
LDL-c (mg/dl)	117.33±15.53	109.69±10.31	133	*NS **NS

GroupI= diabetic patients with CAD, groupII= diabetic patients without CAD, groupIII=controls, TC=serum total cholesterol, TG=triglycerides, HDL-c=high density lipoprotein–cholesterol and LDL-c=low density lipoprotein-cholesterol, *P* value<0.05 is significant, NS= not significant .* E2 vs E3, ** E3 vs E4

Discussion

In this study, we investigated the occurrence of certain APO E alleles and genotypes as a risk for CAD in type 2 diabetes mellitus.

Considering all our studied Egyptian subjects, ε3 allele represented the commonest allele (84%). Thus the distribution of the ε3 allele in Egyptian population is in concordance with its distribution in other ethnic groups as reported by Burman *et al.* (2009) that ε3 allele was the most common (87.6% in South Asians, 86.0% in Chinese, and 78.3% in Europeans). In our studied subjects, the occurrence of ε2 (11.5%) then ε4 (4.5%) came after ε3 allele. Regarding ε4 and ε2 alleles' frequency, Svobodova *et al.* (2007) reported that the distribution of APO E alleles varied across populations so that; the Asian populations traditionally had lower ε4 frequency than Europeans with heterogeneity of ε4 distribution in the European as well as in the Asian populations. Burman *et al.* (2009) reported that South Asians had a lower frequency of ε2 allele (1.9%) compared to Chinese and Europeans (8.4% and 7.2% respectively). Chinese subjects had a lower frequency of ε4 allele (5.6%) compared to South Asians and Europeans (10.4% and 14.5% respectively). This justifies the need for larger scale studies to define the distribution of APO E alleles among Egyptian populations.

The study showed that, both ε2 and ε4 alleles were higher in group I than in group II and group III. This is in agreement with several other studies, Kharrazi *et al.,* (2006), Akanjia *et al.,* (2007), Bennet *et al*. (2007), Vaisi-Raygani *et al.,* (2007) and Winkler *et al.,* (2010). They all reported that the frequencies of ε2 and ε4 allele were more prominent among diabetic patients with CAD than controls. However, Kolovou *et al.* (2002) and Young *et al.* (2004) showed that there was no significant difference between ε2 allele frequencies in diabetic patients with CAD than controls. Ferreira *et al.* (2010) reported that similar frequencies of ε2 and ε4 alleles were observed between controls with normo-lipidima and CAD diabetic patients with dys-lipidemia. In diabetic patients with CAD we showed that, APO E3 phenotype was the commonest, then APO E2 followed by APO E4. These findings are in agreement with Vaisi-Raygani *et al.* (2007).

The present study showed no significant difference between APO E allele frequencies or genotypes between diabetic patients without CAD and controls. These findings are matched with other studies, Leiva *et al.,* (2005), Singh *et al.,* (2006) who reported that APO E polymorphism was not associated with type2 DM. Moreover, Onat *et al*. (2010) proved that APO E gene variants were not independently related to type 2 DM or metabolic syndrome. On the other hand, Errera *et al.* (2006) reported that carriers of ε2 allele in a Brazilian case control study were more significantly frequent in patients with type 2 diabetes than in controls. As well as, Vaisi-Raygani *et al.* (2007) reported that APO E polymorphisms may play a role in diabetes and its outcome.

Both diabetic patients with CAD and those without CAD showed that TG, TC, LDL-c were significantly higher and HDL-c was significantly lower in E4 phenotype than in E3 phenotype ($P<0.005$). Several other studies reported an association between ε4 allele and higher TC level (Guang-daa *et al.* 2005, Leiva *et al.*, 2005, Tziakas *et al.*, 2006, Zee *et al.*, 2006; Vaisi-Raygani *et al.,* 2007), between ε4 allele and higher LDL-c level (Guang-daa *et al.,* 2005, Leiva *et al.,* 2005, Tziakas *et al.,* 2006, Zee *et al.,* 2006; Vaisi-Raygani *et al.,* 2007; Winkler *et al.,* 2010) and between ε4 allele and both higher TG level & lower HDL-c level (Zee *et al.,* 2006;

Vaisi-Raygani *et al.,* 2007). However some studies revealed absence of significant association between ε4 allele and both higher TC & LDL-c levels (Winkler *et al.,* 2010) and between ε4 allele and both higher TG level & lower HDL-c level (Guang-daa *et al.,* 2005; Winkler *et al.,* 2010). Several mechanisms have been proposed to explain the effect of APO E polymorphism on plasma lipid levels. In ε4 carrier subjects, compared with ε2 individuals, there is a more efficient catabolism of chylomicrons and VLDL-remnants, which eventually leads to increased hepatic cholesterol pool, reduced LDL-receptor activity and higher plasma LDL (Takei *et al.,* 2009). Besides this, ε4 carriers have increased intestinal cholesterol absorption, resulting in down-regulated hepatic cholesterol synthesis and LDL-receptor activity. Decreased conversion of VLDL into LDL is observed in ε2 carriers. These effects might give some explanation why individuals with ε4 allele have higher plasma TC and LDL-c levels when compared to those seen with ε2 and ε3 alleles, as demonstrated in different populations (Ferreira *et al.,* 2010).

In this study, ε2 allele significantly increased the risk of CAD in type2 diabetic patients by 4.97 times and ε4 allele significantly increased the risk of CAD in type2 diabetic patients by 7.02 times more than ε3 allele. This ε4 and ε2 alleles' associated higher risk of CAD in type2 diabetic patients is in concordance with what was reported in other studies. Kharrazi *et al.* (2006) reported that type2 DM patients carrying ε2 and ε4 alleles had a higher risk of developing CAD than non diabetic patients in the western population of Iran, with ε4 allele being more closely associated with CAD than ε2 allele. A meta-analysis by Ranjith *et al.* (2004) identified a significant 42 % increased risk for coronary artery diseases among the carriers of ε4 allele. Winkler *et al.* (2010) found that ε4 allele significantly increased the risk of CAD in diabetic patients (odds ratio was 1.29). While, ε2 did not significantly increase the risk of CAD in diabetic patients (odds ratio was 0.96). However, Kolovou *et al.* (2002) and Young *et al.* (2004) found a negative association between ε2 allele and development of CAD in diabetic patients. The mechanism whereby the APO E alleles contribute and increase the risk of CAD in type 2 diabetic patients is unknown. One possibility is that the cholesterol efflux from the macrophage is APO E genotype-dependent. Moreover, the role of ε4 allele as a risk factor for CAD is not only due to its association with a high level of LDL-c, but also by its association with a low level of the anti-atherogenic HDL-c (Kharrazi *et al.,* 2006). In our study, ε4 and ε2 alleles were not associated with increased risk for type2 DM. This is in concordance with Leiva *et al.,* (2007) and Onat *et al.,* (2010) studies.

It is concluded that ε3 allele represents the commonest allele, and that ε4 and ε2 alleles are more associated with increased risk of CAD in type2 DM than ε3 allele. TG, TC, LDL-c levels are significantly higher and HDL-c is significantly lower in E4 phenotype than in E3 phenotype. Larger scale studies are needed to define the distribution of APO E alleles among Egyptian populations and evaluate APO E alleles risk for CAD.

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