Original Article

Angiotensin- Converting Enzyme Insertion/Deletion Polymorphism and Its Association with Coronary Artery Disease in an Iranian Population

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Abstract

Background: *The study of the association between genotype and phenotype is of great importance for the prediction of many diseases and pathophysiological conditions. The relationship between angiotensin-converting enzyme (ACE) gene insertion/ deletion (I/D) polymorphism and pathological processes such as coronary artery disease (CAD) has been investigated previously with discordant results.*

This study was designed to determine the association between ACE gene I/D polymorphism and CAD in an Iranian population.

Methods: *A total of 1050 individuals who were referred to Tehran Heart Center for coronary angiography were recruited. Six hundred seventy-six CAD-positive patients (documented by coronary angiography and Gensini scores higher than 6) and 374 CAD-negative patients were evaluated for ACE gene I/D polymorphism via the Polymerase Chain Reaction Amplification method. The patients' age, sex, smoking status and its duration as well as familial history of CAD, hypertension, and diabetes mellitus were recorded.*

Results: *Five hundred four (74.6%) of the CAD-positive patients were male, and the mean age of this group was 60 (60 ± 10). In the CAD-negative individuals, the mean age was 56 (56 ± 10) and 196 of them were male (52.4%). After the analysis of all the groups and gender subgroups, neither genotype nor allele frequency was significantly different between the CAD-positive and CAD-negative groups (p values for genotypes and allele frequencies were 0.494 and 0.397, respectively).*

Conclusion: *ACE gene I/D polymorphism was not associated with an increased risk of CAD in an Iranian population.*

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Introduction

Individuals suffering from coronary artery disease (CAD) show different types of signs and symptoms and mostly they show no evidence for decades. The symptoms and signs of CAD are noted in the advanced state of disease. As the disease progresses before the first onset of symptoms, often a "sudden" heart attack arises.[1] Identification of predictive factors can considerably help to determine the possibility of such events. Some of these predictive factors are already known and others, especially genetic ones, are currently under investigation.[2]

The study of the association between genotype and phenotype is of great importance for the prediction of many diseases and pathophysiological conditions.[2] Angiotensin-converting enzyme (ACE) is a member of the renin-angiotensin-aldosterone system (RAAS), which converts angiotensin I to angiotensin II: the latter affects vascular tone and alters renal function and consequently controls blood pressure. RAAS is, therefore, an important regulatory system of cardiovascular function and blood pressure.[3, 4] ACE is the most important factor in the production of angiotensin II and is a zinc-dependent enzyme which hydrolyses the dipeptide band of the carboxylic end of angiotensinogen and bradykinin.[5] After the cloning of ACE gene, a deletion-insertion (I/D) polymorphism, involving 287 base pairs (bp) in intron 16 of the ACE gene, was identified which affected the level of ACE serum activity.[6] Additionally, it was shown that deletion/deletion (D/D) genotype had the highest ACE serum level and was associated with the development of myocardial hypertrophy.[7, 8] I/D polymorphism is responsible for 28%-43% of serum-level variance of ACE [9, 10] and it has been shown that this polymorphism affects the transcription of the enzyme and does not affect enzyme secretion.[11]

The relationship between ACE gene I/D polymorphism and pathological processes such as CAD has been investigated previously with discordant results[12, 13] even in the same country, indicating that further studies are required to examine the multi-factorial association between ACE gene I/D polymorphism and the risk of CAD in different population groups and different environmental conditions. There are a limited number of conflicting studies conducted in Iran with incompatible results.[14, 15] However, the available data remain ambiguous as reports of associations have not been consistently confirmed, and even reports on inverse associations have been published.

The aim of this study was to determine whether ACE gene I/D polymorphism can influence CAD in an Iranian population.

Methods

In this cross-sectional study, patients referred to Tehran Heart Center for coronary angiography between January 2007 and March 2008 were included. The study population consisted of 1050 patients, mainly the residents of the Iranian capital, Tehran. The patients' age, sex, smoking situation and its duration, familial history of CAD, blood pressure, and diabetes mellitus were recorded. Smoking any kind of tobacco or cessation of smoking for less than one month was the criterion to be categorized as a smoker in this study. Similarly, consumption of any kind of plasma-glucose-lowering drugs, including insulin and oral tablets, or previous consumption of such drugs was the criterion to be classified as having diabetes. Arterial blood pressure higher than 140/90 mmHg or consumption of antihypertensive drugs was the criterion for having hypertension. Familial history of CAD was considered positive if the patients' parents or siblings under the age of 55 for men and 65 for women had any proven CAD.

The blood samples of all the selected patients were collected after 12 hours' overnight fasting. Biochemical parameters, including cholesterol, triglyceride, and high-density lipoprotein (HDL) levels, were measured in the clinical laboratory via an enzymatic method with an Auto-Analyzer (Beckman CX4, USA). The Friedewald formula was used for the calculation of LDL-cholesterol levels. If the triglyceride level was higher than 400 mg/dl, the LDL-cholesterol level was not measurable. Plasma triglyceride levels higher than 150 mg/dl or total cholesterol levels higher than 200 mg/dl were defined as hypertriglyceridemia and hypercholesterolemia, respectively. These parameters in conjunction with high plasma low-density lipoprotein (LDL) levels (higher than 130 mg/dl), low plasma HDL levels (lower than 40 mg/dl), and consumption of any kind of lipid-lowering drugs at the time of study were considered dyslipidemia.

Two experienced cardiologists, blinded to the genotypes of I/D ACE gene (and other biological data), performed angiography on all the participants with the Philips Integris H5000. Catheters (Judkins, left and right) from Cordis Corporation [US] and guide wires (0.014 inch Hi Torque floppy) from Guidant Corp. [US] were utilized. Twenty per cent of the angiograms, previously interpreted by each cardiologist, were selected randomly and interpreted again by another cardiologist, blinded to the previous data. The inter-observer reliability of the cardiologists' review reports was calculated using the intraclass correlation (ICC).

The angiography results were reported as Gensini scores, which are computed by assigning a severity score to each coronary stenosis according to the degree of luminal narrowing and its importance based on location. Reduction in the lumen diameter and the angiographic appearance of concentric lesions and eccentric plaques were quantitatively evaluated.[16] Gensini scores higher than 6 were considered a criterion for having CAD in the patients.[17]

After the extraction of genomic DNA from all the pa-

tients' blood samples using a salting out method,[18] a single polymerase chain reaction (PCR) method using oligonucleotide primers 5'-CTGGAGACCACTCCCATCCTTTCT-3' (forward) and 5'-GATGTGGCCATCACATTCGTCAGAT-3' (reverse) specific to a 490bp DNA fragment in intron 16 was performed for genotyping.[19] The amplification was carried out in a total volume of 25 μL containing 50 ng template DNA, 10 μM of each primer, 2.5 μL 10X PCR buffer (Fermentas, Lithuania), 3 mM $MgCl_2$, 200 μM each dNTP, and 1.5 units of Taq DNA polymerase (Fermentas, Lithuania). The PCR was performed in a Biorad MJ Mini thermocycler (Biorad, Singapore), and amplification conditions were as follows: an initial denaturation of 5 minutes at 95 °C and then 30 cycles consisting of 30 seconds of denaturation at 95 °C; 105 seconds of annealing at 60 °C; and 1.5 minutes of extension at 72 °C. The PCR products were separated on 2% agarose gel, stained with ethidium bromide and visualized on a trans-illuminator (Vilber-Lourmat, France). Because D allele is deemed a 287 bp deletion in 490 bp DNA fragment, the PCR products for D/D, I/D, and insertion/insertion (I/I) genotypes were respectively: one 190-bp DNA band; two DNA bands of 190 bp and 490 bp; and finally one 490 bp DNA band.

For further assurance about genotyping accuracy, about 20% of the total specimens were selected randomly using SPSS software, and their genotyping procedure was repeated by a technician, blinded to the previous results. Thereafter, the newly obtained results were compared with the previous ones.

Statistical Analysis

The data are presented as mean ± standard deviation (SD) for the numerical variables and are summarized by absolute frequencies and percentages for the categorical variables. The continuous variables were compared using the Student t-test or nonparametric Mann-Whitney U test when the presumption of normality was not met, while the categorical variables were compared using the chi-square test.

Although in the univariate analysis, no statistically significant association was obtained between ACE gene I/D polymorphism and the risk of CAD, multivariable analysis was performed to adjust for potential confounders.

For the statistical analyses, the statistical software SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL) was used. All the p values were two-tailed, with statistical significance defined by a P value ≤ 0.05.

To determine whether the genotypes were in the Hardy-Weinberg equilibrium, the statistical package GenePop version 4.0.10 was used.

The study protocol was approved by the institutional Ethics Committee for Human Studies, and informed consent was obtained from all the participants, who approved the collection of their blood samples for scientific research.

Results

The demographic and the biological data of all the patients participating in this study are presented in Table 1. There were significant differences in the HDL cholesterol and LDL-cholesterol levels between the CAD-positive and CAD-negative groups (P value < 0.05), but the differences between the total cholesterol and triglyceride levels were not statistically significant between the CAD-positive and CAD-negative patients. Age, male sex, diabetes mellitus, and cigarette smoking were significantly different between the CAD-positive and CAD-negative groups, whereas familial history did not differ significantly between these two groups (Table 1).

The calculated ICC for the two reviewing cardiologists was 97.6% to 99.1%, which demonstrated that those two reviewers calculated a similar Gensini score.

Table 1. Baseline characteristics of the study population based on the presence of coronary artery disease (CAD) and genotypes*

Variables	CAD		P value	Genotypes			P value
	CAD-positive (n = 676)	CAD-negative (n = 374)		I/I (n = 479)	I/D (n = 420)	D/D (n = 151)	
Age (y)	60.65±10.15	56.78±10.46	< 0.001	59.5±10.30	59.02±10.43	59.25±10.82	0.816
Male sex	504 (74.6)	196 (52.4)	< 0.001	311 (64.9)	280 (66.7)	109 (72.2)	0.256
Diabetes mellitus	192 (28.5)	70 (18.8)	< 0.001	120 (25.1)	100 (23.8)	42 (27.8)	0.628
Cigarette smoking			< 0.001				0.497
Current smoker	157 (23.3)	57 (15.3)		97 (20.3)	80 (19)	37 (24.5)	
Ex-smoker	144 (21.4)	53 (14.2)		88 (18.5)	77 (18.4)	32 (21.2)	
Non-smoker	372 (55.3)	263 (70.5)		291 (61.2)	262 (62.6)	82 (54.3)	
Age ≤ 45 years	41 (6.1)	46 (12.3)	< 0.001				
Hypertension	277 (41)	146 (39)	0.537	190 (39.7)	167 (39.8)	66 (43.7)	0.670
HDL cholesterol (mg/dl)	41.98±10.89	46.18±10.93	< 0.001	42.72±10.79	44.58±11.40	42.81±10.39	0.039
LDL cholesterol (mg/dl)	110.66±41.60	103.57±37.79	0.006	108.68±41.13	108.72±39.18	104.77±41.55	0.458
Total cholesterol (mg/dl)	187.05±47.65	183.20±42.31	0.178	186±45.97	186.07±45.08	183.55±47.74	0.706
Triglyceride (mg/dl)	154 (107-213)	143 (97-215)	0.834	151 (106-218)	146 (97-210)	155 (111-220)	0.212
Family history of CAD	227 (33.7)	111 (29.8)	0.204	161 (33.6)	126 (30)	51 (33.8)	0.473

*Data are presented as mean±SD or n (%)

Except for triglyceride levels which are presented as median (inter quartile range)

I/I, Insertion/insertion; I/D, Insertion/deletion; D/D, Deletion/deletion; HDL, High-density lipoprotein; LDL, Low-density lipoprotein

The results of the genotyping of ACE gene (Figure 1) and its relationship with CAD are depicted in Table 2. The genotypes of the CAD-negative patients were in the Hardy-Weinberg equilibrium, but the genotypes of CAD-positive patients were not. When all the study groups and subgroups were separated by gender, neither genotype nor allele frequency was significantly different between the CAD-positive and CAD-negative groups and/or male and female subgroups.

The association between ACE I/D polymorphism and CAD was assessed between the male patients and female patients separately, but again there was no significant association (P value = 0.281 for the female group and P value = 0.869 for the male group, Table 3).

After adjustment for the conventional risk factors of CAD, multivariate logistic regression analysis was performed, which showed no statistically significant relationship (P value = 0.949).

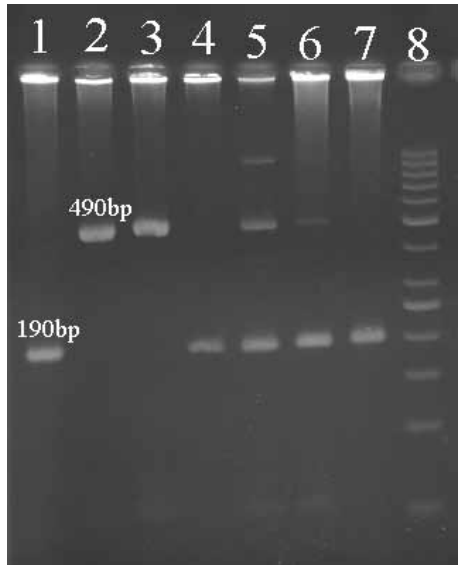

Figure 1. Gel electrophoresis image showing results of Angiotensin-converting enzyme Insertion/deletion polymorphism. Lines 1, 2, 10 and 12 represent normal homozygote patients, lines 5, 6 and 9 represent mutant homozygote patients and lines 3, 4, 7 and 8 represent heterozygote patients. Ladder 50bp (CinnaGen, Iran)

Table 2. Genotypes and allele frequencies of ACE I/D polymorphism and its relationship with CAD in the whole study group and subgroups separated by gender

	CAD-Positive				CAD-Negative				P value
	All (n=676)	Male (n=504)	Female (n=172)	OR in CAD^+ (95%CI)	All (n=374)	Male (n=196)	Female (n=178)	OR in CAD^- (95%CI)	CAD^+ vs. CAD^-
Genotype									0.494
D/D	317 (46.9)	227 (45.0)	90 (52.3)	0.614 (0.352-1.073)	162 (43.3)	84 (42.9)	78 (43.8)	0.799 (0.429-1.487)	
I/D	262 (38.8)	199 (39.5)	63 (36.6)	0.769 (0.433-1.363)	158 (42.2)	81 (41.3)	77 (43.3)	0.780 (0.419-1.455)	
I/I	97 (14.3)	78 (15.5)	19 (11.0)	Ref.	54 (14.4)	31 (15.8)	23 (12.9)	Ref.	
Alleles									0.397
I	456 (33.7)	355 (35.2)	101 (29.4)	Ref.	266 (35.6)	143 (36.5)	123 (34.6)	Ref.	
D	896 (66.3)	653 (64.8)	243 (70.6)	0.765 (0.586-0.997)	482 (64.4)	249 (63.5)	233 (65.4)	0.919 (0.681-1.241)	

Data are presented as n (%)
ACE, Angiotensin-converting enzyme; CAD, Coronary artery disease; OR, Odds ratio; CI, Confidence interval

Table 3. Genotypes of ACE I/D polymorphism and its relationship with CAD in the male and female subgroups

Genotypes	CAD^- Patients	CAD^+ Patients	OR (95%CI)	Total	P value
Female	178 (100)	172 (100)		350 (100)	0.281
D/D	78 (43.8)	90 (52.3)	0.716 (0.363-1.412)	168 (48.0)	
I/D	77 (43.3)	63 (36.6)	1.010 (0.505-2.019)	140 (40.0)	
I/I	23 (12.9)	19 (11.0)	Ref.	42 (12.0)	
Male	374 (100)	676 (100)		1050 (100)	0.869
D/D	84 (42.9)	227 (45.0)	0.931 (0.573-1.513)	311 (44.4)	
I/D	81 (41.3)	199 (39.5)	1.024 (0.628-1.671)	280 (40.0)	
I/I	31 (15.8)	78 (15.5)	Ref.	109 (15.6)	
	196 (100)	504 (100)		700 (100)	

Data are presented as n (%)
ACE, Angiotensin-converting enzyme; CAD, Coronary artery disease; OR, Odds ratio; CI, Confidence interval

After random repeating of genotyping on 20% of the 1050 total specimens by a technician blinded to the previous results, only 6 specimens (< 3%) had different results, showing the good precision of our techniques.

Discussion

Various risk factors are related with CAD, including hypertension,[20] dyslipidemia,[21] and diabetes mellitus.[22] It has been shown that ACE has an important role in blood pressure and remodeling changes of heart and vessels. ACE encoding gene has several alleles (I/I, I/D and D/D) due to an insertion/deletion in intron 16. In contrast to some studies,[13, 23-29] and in line with some other studies[12, 30-32] performed in different geographical areas and different years, we observed no association between different ACE genotypes (D/D, I/D and I/I) and CAD instances. Some of the examples of such studies are as follows:

Fujimura et al.[12] investigated the relationship between ACE gene I/D polymorphism and CAD in 1840 Japanese subjects, including 947 CAD patients and 893 normal individuals, and reported no relationship between the D/D genotype of ACE gene and CAD in the male and female subjects. Another study on 100 healthy individuals and 178 CAD patients in Japan performed by Nakai et al.[13] showed discordant results, including clear association between D/D genotype and CAD. Similar results were achieved by more recent researches (Table 4).

Zintzaras et al.,[33] having assessed 118 studies involving 43,733 cases with CAD and 82,606 healthy controls in their meta-analysis performed in 2008, reported that there was a modest positive association between ACD I/D polymorphism and CAD. Some similar studies have been conducted in Iran with different consequences. Vaisi-Raygani et al. (2010)[14] assessed ACE I/D polymorphism among 323 Iranian individuals undergoing their first coronary angiography and showed that the ACE D allele was a risk factor for the early onset of CAD even after correcting for the conventional risk factors. Shafiee et al.[15] assessed the relationship between ACE gene I/D polymorphism and CAD in 487 age-matched individuals and found out that D/D genotype was not associated with an increased risk of CAD in that group. It is deserving of note, however, that they omitted patients and controls with any history of diabetes, hypertension, and other general illnesses and their control groups were comprised of individuals with no history of heart disease and chest pain proven by angiography. Because of such results and limitations, we recruited more cases (676 CAD-positive and 374 CAD-negative) in our study to obtain more reliable results and our research included patients from throughout the country. Furthermore, in contrast to previous studies conducted hitherto in Iran and many other countries, all of our individuals (CAD-positive and CAD-negative patients) were documented angiographically.

Conclusion

The existence of such differences in such a large number of studies may be due to geographical discrepancies, ethnic diversities, or even the defectiveness of the techniques employed in different researches. It, therefore, seems that an international study with identical techniques, similar criteria for the evaluation of the association between ACE gene I/D polymorphism and CAD, and a greater number of subjects under examination is necessary so as to conclusively determine a positive or negative relationship between these two factors.

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Table 4. Discordant results of different studies on the association between CAD and ACE gene I/D polymorphism

Country	Number of cases	Association between CAD and ACE gene I/D polymorphism	Year	Researcher	Reference number
Japan	1840	No	1997	Fujimura et al.	12
China	130	No	2005	Qiu et al.	30
Greece	309	No	2010	Ragia et al.	31
India	112	No	2010	Ramakrishnan et al.	32
Japan	278	Yes	1994	Nakai et al.	13
Turkey	307	Yes	2005	Acarturk et al.	24
Mexico	247	Yes	2006	Vargas-Alarcon et al.	25
Turkey	178	Yes	2006	Sekin et al.	26
Poland	341	Yes	2007	Niemiec et al.	27
India	200	Yes	2009	Jamil et al.	28
Lebanon	300	Yes	2010	Abchee et al.	29

CAD, Coronary artery disease; ACE, Angiotensin-converting enzyme; I/D, Insertion/deletion

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