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The Evaluation of Angiotensin-converting Enzyme (*ACE*) Gene I/D and *IL-4* Gene Intron 3 VNTR Polymorphisms in Coronary Artery Disease

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Abstract. *Background/Aim: Genetic polymorphism is a strong risk factor for coronary artery disease (CAD). In the present study, our aim was to evaluate angiotensin-converting enzyme (ACE) gene I/D polymorphism and interleukin-4 (IL-4) gene Intron 3 variable number of tandem repeat (VNTR) polymorphism in CAD. Materials and Methods: One hundred and twenty-four CAD patients and one hundred and twenty-three controls were enrolled. Genomic DNA was isolated and genotyped using polymerase chain reaction (PCR) analyses. Results: The risk associated with inheriting the combined genotypes for the two polymorphisms were evaluated and it was found that the individuals who were P2P2-homozygous at IL-4 gene intron 3 VNTR and DD-homozygous at ACE gene I/D have a higher risk of developing CAD. Conclusion: Although, there is no correlation between IL4 VNTR polymorphism and ACE gene polymorphism and CAD, there is a strong association between CAD and co-existence of IL-4 VNTR and ACE gene polymorphisms in the Turkish population.*

Coronary artery disease (CAD) is a public health problem that has high morbidity and mortality rates accounting for up to 40% of all deaths in industrialized countries (1). Genetic polymorphism is a risk factor for CAD as well, as it may predispose to risk factors like hyperlipidaemia, obesity, hypertension, left ventricular hypertrophy, diabetes mellitus (DM), *etc*. (2). Mainly, it is known that CAD is a multi-factorial disease and includes gene–gene and gene–environment interactions (3). Nonetheless, the detection of possible genetic risk factors on the development of CAD is important for early diagnosis due to evaluating the individual cardiovascular risk profile.

In the pathogenesis of CAD, the main problem is atherosclerosis due to inflammation (4). Interleukin-4 (IL-4) is a potent cytokine secreted by T-helper 2 cells, eosinophils and mast cells. It plays a role in the formation of endothelial cell adhesion molecules, chemotaxis of immune cells and anti-inflammation. The *IL-4* gene has been mapped to the q arm of chromosome 5 in a cluster of cytokine genes. Variable number of tandem repeat (VNTR) polymorphism with a unit size of 70-bp sequence is a frequent polymorphism of *IL-4*. It is located in the third intron of the gene and it contains two alleles, P1 (183 bp) and P2 (253 bp) (5). In literature, *IL-4* VNTR polymorphism has been found to be related with many diseases, as rheumatoid arthritis (RA), bladder cancer, ischemic stroke, multiple sclerosis (MS), alopesi areata (AA) and diabetic neuropathy (6-9). In the present study, we hypothesized that the *IL-4* gene VNTR polymorphism may play a primary or secondary role in the pathogenesis of CAD due to its role on anti-inflammation.

The angiotensin-converting enzyme (ACE), which is one of the key components of the renin–angiotensin system (RAS), is a zinc metallopeptidase that participates in the formation of the vasoconstrictor angiotensin II (Ang II) and reduces the vasodilator bradykinin-2 (10). Besides, ACE has effects on glucose metabolism (11). The human *ACE* gene polymorphism that affects ACE activity is localized in the long arm of chromosome 17 and it includes 26 exons and 25 introns. The insertion or deletion (I/D) polymorphism includes a 287-bp fragment in intron 16. *ACE* D/D and I/I homozygotes and I/D heterozygotes are genotypes of I/D polymorphism (10). *ACE* gene I/D polymorphism may be a risk factor for CAD due to RAS activation.

To the best of our knowledge, there is no report to have investigated both *ACE* gene I/D polymorphism and *IL-4* gene VNTR polymorphism in CAD patients. In the present study, we aimed to experimentally explore these two polymorphisms

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Key Words: Coronary artery disease, IL4 VNTR polymorphism, *ACE* gene I/D polymorphism.

Table I. *Baseline clinical and demographic characteristics of the patients with CAD and healthy controls.*

Characteristics	CAD patients n=124	Healthy controls n=123	*p*-Value
Gender, male/female, n (%)	81/43 (65.3/34.7)	67/56 (54.5/45.5)	0.092
Age, years	59.88±8.185	58.75±10.617	0.349
HDL cholesterol (mg/dl)	44.61±12.375	44.97±11.442	0.815
LDL cholesterol (mg/dl)	114.99±38.664	130.77±35.599	0.002
Ever smoking, n (%)	18 (14.5)	22 (17.9)	0.494
History of hypertension, n (%)	87 (70.2)	62 (50.4)	0.002
History of diabetes mellitus, n (%)	39 (31.5)	28 (22.8)	0.152

Data were analyzed by analysis of variance and χ^2 test. Mean plus standard deviation values are presented for age, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol. CAD: Coronary artery disease.

for CAD patients in a Turkish population. We hypothesized that these polymorphisms may have an effects on CAD due to atherosclerosis or other risk factors like diabetes mellitus and hypertension.

Materials and Methods

Subjects. The study group consisted of 124 unrelated patients with CAD (81 male and 43 female; mean age: 59.88±8.185; years±standard deviation [SD]) and 123 (67 male and 56 female; mean age: 58.75±10.617 years) unrelated healthy controls. CAD patients were recruited consecutively and prospectively including those whom were treated and followed-up in the Cardiology Department of Gaziosmanpasa University Research Hospital, Tokat, Turkey. The diagnosis of CAD was confirmed by coronary angiography performed by experienced cardiologists and it was defined as the presence of a stenosis of greater than 50% in one or more of the main coronary arteries. All control subjects were confirmed free from coronary artery disease by either angiography or clinical symptom together with electrocardiogram (ECG) examinations. Exclusion criteria for both groups included the presence of congenital heart disease, cardiomyopathy, valvular diseases or autoimmune diseases. All participants, patients and healthy controls, were of Turkish origin from the inner Central Black Sea region of Turkey. The healthy controls were matched for age and gender with RAS patients (Table I). The study protocol was approved by the Local Ethics Committee of Gaziosmanpasa University, Faculty of Medicine and written informed consent was obtained from the study participants.

Genotyping. Genomic DNA was extracted from whole venous blood samples using a commercial DNA isolation kit (Sigma-Aldrich, Taufkirchen, Germany). The *ACE* gene I/D (rs5186) and the *IL-4* gene 70 bp VNTR (rs8179190) polymorphisms were analyzed by polymerase chain reaction (PCR). The PCR amplifications were carried out in a total volume of 25 μl reaction containing 100 ng of genomic DNA, 2.5 μl of 10X PCR buffer, 200 μM dNTP, 10 pM of each primer and one unit of Taq DNA polymerase. The *ACE* gene I/D polymorphism was analyzed by using forward (F) 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' and reverse (R) 5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3' primers. The amplification conditions consisted of an initial melting step of 5 minutes at 94˚C; followed by 30 cycles of 1 min at 94˚C, 1 min 45 sec at 60˚C and 1 min 30 sec at 72˚C; and a final elongation step of 5 min at 72˚C. In the absence of the 287 bp in intron 16 of the *ACE* gene, this PCR method resulted in a 190 bp product (D allele) and in the presence of the 287 bp, resulted a 477 bp product (I allele). In heterozygote samples, two bands (477 and 190 bp) were detected. For *IL-4* gene 70 bp VNTR polymorphism, amplification was carried out using F 5'- AGG CTG AAA GGG GGA AAG C-3', R 5'-CTG TTC ACC TCA ACT GCT CC-3' primers with initial denaturation at 95˚C for 5 min, 30 cycles of denaturation at 94˚C for 30 s, annealing at 58˚C for 45 s, extension at 72˚C for 1 min and final extension at 72˚C for 10 min. The PCR products were visualized on 3% agarose gel stained with ethidium bromide. The PCR product was of 183 bp for P1 allele and 253 bp for P2 allele. A second PCR was performed to confirm samples with no conclusive results.

Statistical analysis. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS Statistics, version 20; IBM, Address) and OpenEpi Info software package version 3.01 (www.openepi.com). The Chi-square (χ^2) test was used to evaluate the Hardy-Weinberg equilibrium (HWE) for the distribution of the genotypes of the patients and the controls. The relationships between *ACE* gene I/D and *IL-4* gene 70 bp VNTR polymorphisms and the clinical as well as demographic characteristics of patients were analyzed by using the χ^2 test, Fisher's exact or analysis of variance (ANOVA) statistics. The χ^2 test and Fisher's exact test were used to compare categorical variables appropriately; odds ratio (OR) and 95% confidence interval (CI) were used for the assessment of risk factors. All *p*-values were 2-tailed and p-values less than 0.05 were considered significant.

Results

The baseline clinical and demographic characteristics of CAD patients and controls are presented in Table I. Gender, age, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, smoking, history of hypertension and history of diabetes mellitus of RAS patients and controls were compared. Among these characteristics, LDL cholesterol and history of hypertension showed statistically significant differences between patients and controls (p=0.002 and p=0.002, respectively). Clinical and demographic characteristics of CAD patients (gender, age,

Table II. *Clinical and demographic characteristics of CAD patients stratified according to ACE gene I/D and IL-4 gene intron 3 VNTR polymorphisms.*

Characteristics	*ACE* gene I/D polymorphism					*IL-4* gene intron 3 VNTR polymorphism		
	Total n=124	DD n=58	ID n=55	II n=11	*p*-Value	P2P2 n=97	P1P2+P1P1 n=27	*p*-Value
Gender, male/female, n (%)	81/43 (65.3/34.7)	36/22 (62.1/37.9)	39/16 (70.9/29.1)	6/5 (54.5/45.5)	0.451	63/34 (64.9/35.1)	18/9 (66.7/33.3)	0.868
Age (years)	59.88±8.185	61.57±7.872	58.05±7.792	60.09±12.518	0.073	60.03±7.824	59.33±9.515	0.697
Disease duration (years)	4.50±3.106	4.62±3.139	4.13±2.760	6.00±4.726	0.314	4.64±3.195	4.04±2.804	0.425
HDL cholesterol (mg/dl)	44.61±12.375	45.92±12.762	44.08±12.505	39.62±8.175	0.385	44.21±11.394	46.00±15.568	0.543
LDL cholesterol (mg/dl)	114.99±38.664	108.59±36.605	121.89±40.460	113.62±37.970	0.242	116.815±39.610	108.56±35.187	0.369
Triglyceride (mg/dl)	166.36±90.750	154.96±85.301	178.72±101.389	163.62±42.298	0.442	167.63±95.234	161.91±74.505	0.791
History of hypertension, n (%)					0.884			0.061
Positive	87 (70.2)	41 (70.7)	39 (70.9)	7 (63.6)		72 (74.2)	15 (55.6)	
Negative	37 (29.8)	17 (29.3)	16 (29.1)	4 (36.4)		25 (25.8)	12 (44.4)	
History of diabetes mellitus, n (%)					0.562			0.102
Positive	39 (31.5)	20 (34.5)	17 (30.9)	2 (18.2)		34 (35.1)	5 (18.5)	
Negative	85 (68.5)	38 (65.5)	38 (69.1)	9 (81.8)		63 (64.9)	22 (81.5)	
Smoking, n (%)					0.121			0357
Yes	18 (14.5)	12 (20.7)	4 (7.3)	2 (18.2)		16 (16.5)	2 (7.4)	
No	106 (85.5)	46 (79.3)	51 (92.7)	9 (81.8)		81 (83.5)	25 (92.6)	

Data were analyzed by analysis of variance, χ^2 or Fisher's exact test. Mean plus standard deviation values are presented for all variables except gender, history of hypertension, history of diabetes mellitus and smoking. CAD: Coronary artery disease, ACE: angiotensin-converting enzyme, IL-4: interleukin-4, VNTR: variable number of tandem repeat, HDL: high-density lipoprotein, LDL: low-density lipoprotein. Also, briefly define/explain DD, ID, II, and P2P2, P1P2+P1P1.

disease duration, HDL cholesterol, LDL cholesterol, triglycerides, history of hypertension, history of diabetes mellitus and smoking) stratified according to *ACE* gene I/D and IL-4 gene intron 3 VNTR polymorphisms are shown in Table II. Any association was not found between clinical and demographic characteristics of CAD patients and the *ACE* gene I/D and the *IL-4* gene 70 bp VNTR polymorphisms ($p>0.05$) (Table II).

Allelic and genotypic distributions of the *ACE* gene I/D and the *IL-4* gene intron 3 VNTR polymorphisms are shown in Table III. Genotype and allele frequencies did not show any significant differences between patients and controls according to *ACE* gene I/D and the *IL-4* gene VNTR polymorphisms ($p>0.05$) (Table III).

We also examined the risk associated with inheriting the combined genotypes for the two polymorphisms (Table IV). Only the homozygosity for P2P2 at *IL-4* gene intron 3 VNTR and homozygosity for DD at *ACE* gene I/D encoded a significant *p*-value of 0.031. Thus, individuals who were P2P2-homozygous and DD-homozygous have a higher risk of developing CAD. The observed and expected frequencies of *IL-4* gene intron 3 VNTR polymorphism were in Hardy-Weinberg equilibrium in the control group but not in the patient group. However, the observed and expected frequencies of *ACE* gene I/D polymorphism were in Hardy-Weinberg equilibrium in both groups.

Discussion

In the current study, it was found that there was no correlation between CAD and *IL-4* gene VNTR polymorphisms in the Turkish population from the Central Black Sea region. It is known that anti-inflammatory cytokines play a key role due to prevention of atherosclerosis in the development of CAD. IL-4 is one of the anti-inflammatory cytokines also known for its effects on haematopoiesis, inhibition of nitric oxide synthase and superoxide, and anti-tumour activities (12). There are several published studies that have investigated the association between different genetic polymorphisms and CAD in the Turkish population, such as *APOE, NF-κB1A* promoter and *LOX-1* gene polymorphisms (13, 14, 15). To the best of our knowledge, there is no study on IL-4 VNTR polymorphism and CAD in Turkish population. Sobti *et al*. has studied the association of *IL-4* gene VNTR polymorphism with CAD in Indian population and, similar to our results, they reported that there was no correlation between this polymorphism and CAD. They suggested that IL-4 VNTR polymorphism acted minimally in atherogenesis. Differently from our results, they suggested that this polymorphism was associated with other risk factors like diabetes mellitus (DM), hypertension, family history and mental stress in CAD, indicating probably that this polymorphism was not a primary risk factor of CAD but could play a role as a secondary risk factor due to CAD (16). CAD

Table III. *Genotype and allele frequencies of IL-4 and ACE gene polymorphisms in patient and control groups.*

Gene (polymorphism)	CAD patients n=124 (%)	Healthy controls n=123 (%)	*p*-Value	OR (CI 95%)
IL-4 (intron 3 VNTR)				
Genotypes				
P2P2	97 (78.2)	89 (72.4)	0.074	
P1P2	22 (17.8)	33 (26.8)		
P1P1	5 (4.0)	1 (0.8)		
P2P2 : P1P2+P1P1	97 (78.2) : 27 (21.8)	89 (72.4) : 34 (27.6)	0.286	0.73 (0.40-1.31)
Alleles				
P2	216 (87.1)	211 (85.8)	0.667	0.89 (0.53-1.5)
P1	32 (12.9)	35 (14.2)		
ACE (I/D)				
Genotypes				
DD	58 (46.8)	43 (35.0)	0.132	
ID	55 (44.3)	63 (51.2)		
II	11 (8.9)	17 (13.8)		
DD+ID : II	113 (91.1) : 11 (8.9)	106 (86.2) : 17 (13.8)	0.220	0.61 (0.26-1.36)
DD : ID+II	58 (46.8) : 66 (53.2)	43 (35.0) : 80 (65.0)	0.059	0.61 (0.37-1.02)
Alleles				
D	171 (69.0)	149 (60.6)	0.051	0.69 (0.48-1.00)
I	77 (31.0)	97 (39.4)		

Data were analyzed by the χ^2 or Fisher's exact test. CAD: coronary artery disease, ACE: angiotensin-converting enzyme, IL-4: interleukin-4, VNTR: variable number of tandem repeat.

occurs from accumulation of lipids, calcium and inflammatory cells throughout the wall of coronary arteries. This accumulation causes narrowing in lumen and reduction in blood flow (17). Inflammation plays a key role on pathogenesis of CAD. IL-4 is an anti-inflammatory cytokine. In a study by Jha *et al*., IL-4 serum levels were found higher in a CAD group compared to the normal group and they suggested that serum levels of vascular inflammation markers might help determine patients with high risk for CAD (18). Unfortunately, the relation with genetic polymorphism of IL-4 VNTR and serum levels of *IL-4* is not as yet completely clear. Although it is known that IL-4 has a role in the pathogenesis of CAD, the present study does not support the effects of *IL-4* VNTR polymorphism in CAD patients in the studied Turkish population.

In the present study, no correlation was found between CAD and *ACE* gene I/D polymorphism in the Turkish population from the Central Black Sea region. Ozturk *et al*. has reported that the D allele may affect pulse pressure (PP) in patients with first acute anterior myocardial infarction in *ACE* gene I/D polymorphism. Also, in this study, they found no correlation between *ACE* gene I/D polymorphism and risk factors like hypercholesterolemia, DM, hypertension and obesity (19). Similar to our results, Agirbasli *et al*. suggested that there is no significant association between *ACE* gene I/D polymorphism and CAD in Turkish population (3). Inversely, Guney *et al*. reported that the DD genotype and the D allele may be related with the severity of CAD in Turkish population. They suggested that the *ACE* I/D polymorphism may be used as a risk factor for CAD (20).

Table IV. *Comparative analysis of combined genotypes of CAD patients and controls.*

	Patient (n=124)		Control (n=123)		*p*-Value
Genotypes	n	%	n	%	
intron 3 VNTR - I/D					
P2P2-DD	46	37.1	30	24.4	0.031
P2P2-ID	42	33.9	47	38.2	0.477
P2P2-II	9	7.3	12	9.8	0.482
P1P2-DD	9	7.3	13	10.6	0.361
P1P2-ID	11	8.9	15	12.2	0.394
P1P2-II	2	1.6	5	4.1	0.440
P1P1-DD	3	2.4	0	0	-
P1P1-ID	2	1.6	1	0.8	-

Data were analyzed by the χ^2 or Fisher's exact test. CAD: Coronary artery disease, VNTR: Variable number of tandem repeat. The results that are statistically significant are typed in bold.

It was found that patients with the P2P2 genotype at *IL-4* gene intron 3 VNTR and DD genotype at *ACE* gene I/D were susceptible for developing CAD in the current study. This

result indicates that the co-existence of both genetic polymorphisms increase the probability of CAD in Turkish population. The combination of these gene polymorphisms is important. CAD is a multi-factorial disease; thus, the interactions of several genetic polymorphisms may be susceptible to developing CAD *via* different pathways that cannot be presently evaluated due to the lack of similar studies.

In conclusion, there is no correlation between *IL4* VNTR polymorphism and *ACE* gene polymorphism and CAD. Additionally, there is a strong association between CAD and co-existence of IL-4 VNTR and ACE gene polymorphisms in the Turkish population studied. Further studies that include serum levels of IL4 and ACE are required to explain the exact mechanism of this cooperation in the pathogenesis of the disease. Finally, genetic factors are related to many complex diseases such as CAD, however it is not true to charge only one genetic polymorphism. It is better to find interaction of different genetic factors with each other in the pathogenesis of complex diseases.

Conflicts of Interest

The Authors have no conflicts of interest regarding the current study.

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