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Angiotensin-Converting Enzyme, Angiotensin II Receptor, Apolipoprotein E and Endothelial Constitutive Nitric Oxide Synthase Gene Polymorphisms in Dilated Cardiomyopathy

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Summary

Genetic factors are hypothesized to contribute to the dilated cardiomyopathy (DCM) susceptibility, even in sporadic cases. Abundant reports have investigated the association between various gene polymorphisms and the phenotypic expression of DCM. The aim of the present study is to assess the effect of four candidate gene polymorphisms (1166 A/C polymorphism of the angiotensin II type 1 receptor (AGTR1) gene, I/D polymorphism of angiotensin converting enzyme (ACE) gene, endothelial nitric oxide synthase (ec-NOS) and apolipoprotein E (APOE genes) on the pathogenesis of dilated cardiomyopathy.

We studied 76 consecutive patients (mean age 58±12) with DCM and 88 healthy age- and sex-matched control subjects (mean age 59±12). All patients were assessed by 2-dimensional echocardiography and all had left ventricular dilatation (end diastolic diameter >55 mm) and impaired systolic function (ejection fraction <40%). All patients were catheterized. Patients having normal coronary arteries were classified as 'idiopathic' and the remaining group as 'ischemic' DCM. Patients with specific heart muscle disease, isolated right ventricular dilatation and valvular or pericardial disease were excluded. Deoxyribonucleic acid (DNA) was isolated from blood samples, and genotypes were determined by specific polymerase chain reaction (PCR) and separation of amplified fragments by agarose gel electrophoresis.

We compared genotypes and allele frequencies, echocardiographic measurements, biochemical variables in our patients and control group. Age, sex, body mass index differences were statistically non-significant. APO E genotypes and allele frequencies were significantly different in patients with DCM. Multiple regression analyses demonstrated lack of independent association. Subgroup analysis revealed that the four candidate gene polymorphisms were not associated with ischemic or idiopathic DCM.

Conclusions: No significant association exists between dilated cardiomyopathy and polymorphism of the AGTR1, ACE, ecNOS and APOE gene polymorphisms. ***(Türk Kardiyol Dern Arş 2004; 32: 295-301)***

Key words: *AGTR1 gene, ACE gene, ecNOS gene, APOE gene, polymorphism, dilated cardiomyopathy*

Özet

Dilate Kardiyomiyopatili Hastalarda Anjiotensin Dönüştürücü Enzim, Anjiotensin II Reseptör, Apolipoprotein E ve Endotelyal Konstitütif Nitrik Oksit Sentaz Geni Polimorfizmi

Amaç: Dilate kardiyomiyopati (DCM) sol yada her iki ventrikülün sistolik fonksiyonlarının bozulması ve genişlemesi ile karakterize bir hastalıktır. Ailesel kökenli DCM'nin tanımlanmasından sonra ailesel olmayan DCM

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olguları için de genetik faktörlerin rol oynayabileceği düşünülmüş ve konuyla ilgili araştırmalar yapılmıştır. Makalemizde ülkemizde yaşayan DCM'li olgularda, hastalığın patofizyolojisinde rol oynaması muhtemel dört aday genin polimorfizmleri araştırılmıştır (Angiotensin dönüştürücü enzim (ACE) I/D polimorfizmi, angiotensin II reseptör (AGTR1) 1166 A/C polimorfizmi, apolipoprotein E (APOE) ve endotelyal konstitütif nitrik oksit sentaz (ecNOS) geni polimorfizmi).

Ortalama yaşı 58±12 olan ardışık 76 hasta ve yaş ortalaması 59±12 olan 88 kontrol grubu çalışmaya alındı. Bütün hastalara ve kontrol grubuna ekokardiyografik çalışma yapıldı. DCM tanısı için ekokardiyografik olarak end-diastolik çapın >55mm ve ejeksiyon fraksiyonunun <%40 altında olması kriter alındı. Yine hasta grubuna koroner anjiografi yapılarak hastalar iskemik ve idyopatik DCM gruplarına ayrıldı. Spesifik kalp kası hastalığı, izole sağ ventrikül genişlemesi, kapak ve perikard hastalığı olanlar çalışma dışında tutuldu. Hasta ve kontrol grubunun kan hücrelerinden deoxiribonükleik asit (DNA) spesifik polimeraz zincir reaksiyonu (PCR) yöntemi ile genetik analiz yapıldı. Gen distribüsyonu ki kare testi ile değerlendirildi. Bağımsız risk için multivariate regresyon analizi uygulandı. 0,05 altındaki p değerleri istatistiksel olarak anlamlı kabul edildi.

Sonuçlar: Hasta ve kontrol grubunun allel sıklıkları, ekokardiyografi, biyokimya analizi sonuçları ve demografik verileri karşılaştırıldı. APO E gen allel sıklığına ait dağılım her iki grupta farklılık gösterse de regresyon analizi sonuçlarına göre bu farklılığın bağımsız bir risk oluşturmadığı gözlendi. ***(Türk Kardiyol Dern Arş 2004; 32: 295-301)***

Anahtar kelimeler: *AGTR1 geni, ACE geni, ecNOS geni, APOE geni, polimorfizm, dilate kardiyomiyopati*

Dilated cardiomyopathy is a myocardial disease characterized by impaired systolic function and dilation of the left or both ventricles [1]. Despite recent advances in medical and surgical therapies, it remains an important cause of mortality and morbidity and is a leading indication for heart transplantation. The dilated cardiomyopathies are a heterogeneous group of disorders with different inheritance patterns, including autosomal dominant (~23%), X-linked (~5%), autosomal recessive, and mitochondrial transmission. Approximately 30% of all DCM is thought to be inherited, while 70% is sporadic. A high percentage of sporadic cases appear to be due to acquired disease, including myocarditis or coronary artery disease and about 50% of them are idiopathic in origin [1].

Several genetic loci have been identified in rare monogenic forms of the disease. These findings led to the hypothesis that genetic factors might also be involved in sporadic forms of the disease. All genes coding for proteins involved in biochemical or physiological abnormalities of cardiac function are potential candidates for idiopathic DCM.

The angiotensin I-converting enzyme (ACE) insertion/deletion (ID) [2], the angiotensinogen, the aldosterone synthase (CYP11B2), the tumor necrosis factor-alpha, the transforming growth factor beta 1, the endothelial nitric oxide synthase (ecNOS), the brain natriuretic peptide genes [3] and endothelin receptor type A gene [4] were some of the polymorphisms in the genes that carry the potential to influence disease pathogenesis.

In the present study, we sought to investigate the role of polymorphisms of four candidate genes; 1166 A/C polymorphism of the AGTR1 gene, I/D polymorphism of ACE gene, ec-NOS and APOE genes in the development and progression of DCM in Turkish population.

METHODS

Ethics approval for the study was obtained from the Duzce and Cerrahpasa Medical School committees, and each subject signed written informed consent for the study.

76 patients with DCM (LV ejection fraction LVEF <40% at the initial assessment and LV end diastolic diameter LVEDD >55 mm) as determined using transthoracic echocardiography were included in the study. Patients were excluded from participation in the study if they had the following criteria: primary valvular disease, clinical or echocardiographic features consistent with an obstructive, hypertrophic or

restrictive cardiomyopathy, pericardial disease, primary hepatic, renal, neurological, pulmonary or endocrine disease, and arrhythmias that could alter LVEF. All patients were performed cardiac catheterization. Patients having normal coronary arteries were classified as 'idiopathic' and the other group as 'ischemic' DCM.

A control group of 88 apparently healthy unrelated age and sex matched subjects were randomly recruited from the general population. These subjects had a clinical history recorded; general examinations performed, and excluded if they had diabetes mellitus, hypertension or a family history of coronary artery disease. All the subjects were screened by transthoracic echocardiography for the presence of DCM or other exclusion criteria.

Genotyping: Genomic DNA was prepared from white blood cells by phenol extraction. Polymorphisms investigated were an insertion (I)/deletion (D) polymorphism in intron 16 of the ACE gene; a nucleotide substitution (A+1166C) in the AGTR1 gene, 27-bp repeats in intron 4 of the ecNOS gene and three different alleles (E2, E3 and E4) encoded by APOE gene. Extracted DNA was subjected to PCR under standard conditions by using primer pair's specific to the analyzed region.

Reactions were performed in a total volume of 50 µl containing 1 µg of genomic DNA, 40 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 1.25 U of *Taq* DNA polymerase, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 10 mmol/L Tris-HCl (pH 8.3). The thermocycling procedure was performed with a gene amplificatory system and consisted of initial denaturation at 94°C for 4 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 2 minutes, and a final extension at 74°C for 7 minutes. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

The ACE gene insertion/deletion (I/D) polymorphism was detected by a polymerase chain reaction (PCR) technique using oligonucleotide primers flanking the insertion sequence and insertion-specific primer pairs as previously described (5).

PCRs demonstrating the single base substitution from A1166 to C1166 of AGTR1 gene were carried out using primers upstream 5'TTGAGGTTGAGTGACATGTTCGA-3' and downstream, 5'CGGTTCAGTCCACATAATGCA-3'.

Endothelial constitutive ecNOS genotypes were determined by the PCR with oligonucleotide primers (upstream, 5'-AGGCCCTATGGTAGTGCCTTT-3'; downstream, 5'-TCTCTTAGTGCTGTGGTCAT-3') that flank the region of the 27-bp direct repeat in intron 4 as described previously, with minor modifications. The large allele, ecNOS4b, contained 5 tandem 27-bp repeats of the consensus sequence [GAAGTCTAGACCTGCTGC(A/G)GGGGTGAG]; the first 3 repeats contained A and the last 2 contained G as the 19th base of the 27-bp repeat. The smaller allele, ecNOS4a, contained 4 repeats; the first 2 repeats had A and the last 2 had G as the 19th base of the repeat. PCR analysis of genomic DNA generated fragments of 393 or 420 bp, corresponding to the ecNOS4a and ecNOS4b alleles, respectively.

The upstream primer used in the genotyping of the APOE locus was 5'-TCCAAGGAGCTGCAGGCGGCGCCA-3' and the downstream primer was 5'-ACAGAATTCGCCCCGGCCTGGTACACTGCCCA-3'. The 227-base pair (bp) polymerase chain reaction products were separately digested by restriction enzymes and resolved by electrophoresis in 2% agarose gel.

Statistical Analysis: Data were analyzed using the SPSS statistical software. Allele frequencies were estimated by gene counting. Hardy-Weinberg equilibrium was tested by chi-square analysis. Genotype distributions were compared between cases and controls by unpaired Student's t test. Yates corrections were taken into account. The relative risk was estimated from an odds ratio calculation. Association between polymorphisms and case/control status was also tested by means of multivariate logistic regression analysis controlling for age, gender and different covariates. A p value <0.05 was considered statistically significant. Continuous data were expressed as mean±S.D.

RESULTS

We studied 76 consecutive patients (mean age 58±12) with DCM and 88 healthy age and sex matched control subjects (mean age 59±12). Characteristics of patients and control subjects are presented in Table 1. Compared to control subjects, patients had a higher ratio of tobacco and alcohol consumption, and a higher preva-

Tablo 1. Characteristics of the study population

	Patients with DCM (n:76)	Control (n:88)	P value
Age (years)	58,4±12,1	58,8±11,9	0,625
BMI (kg/m^2)	25,3±4,4	25,5±4	0,578
Male (%)	78,9	71,2	0,278
Smokers (%)	71,1	43,2	**<0,01**
Alcohol history (%)	27,6	5,7	**<0,01**
DM history (%)	19,7	0	**<0,01**
Hypertension (%)	39,5	0	**<0,01**
Obesity (%)	10,5	4,6	**<0,01**
Family history of CAD (%)	35,5	0	**<0,01**

(DCM: dilated cardiomyopathy, BMI: body mass index, DM: diabetes mellitus, CAD: coronary artery diseae. Age and BMI are expressed as mean ± SD)

lence of diabetes mellitus, hypertension and family history of coronary artery disease. 35 patients had no significant stenosis in their coronary arteries and classified as idiopathic DCM. The rest 41 patients were grouped as ischemic DCM. The echocardiographic examination of the DCM patients revealed a mean left ventricular ejection fraction (LVEF) of 27,24 ± 13,75%, and the mean end-diastolic diameter was 7,25 ± 0,93 mm.

Distribution of genotypes of the four candidate genes among DCM patients and control subjects are presented in Table 2. There was no deviation from Hardy-Weinberg equilibrium for any of the polymorphisms considered. Polymorphisms of ACE, AGTR1 and ecNOS genes exhibited no significant difference of genotype distribution. The allele frequencies of ACE *I*, AGTR1 *a*, ecNOS *b* and APO *E3* were 0,33/0,79/0,83/0,74 in DCM group and 0,29/0,73/0,87/0,90 in control group respectively. The allele frequencies of ACE, AGTR1 and ecNOS genes did not differ significantly between cases and controls when Yates corrections were taken into account.

However the distribution of the APOE 22/23/24/33/34/44 genotypes and E2/3/4 allele frequencies were significantly different among patients and control group.

We further investigated a dominant and recessive model for APOE gene polymorphism where the major E3 allele was the reference allele. Dominant model did not show any significant difference between patients and controls where as the recessive trait for E3 demonstrated an association with DCM (Table 3).

Multivariate logistic regression analyses were performed to adjust risk factors, in which age, gender, diabetes, hypertension, hyperlipidemia, family history of coronary artery disease, smoking history, alcohol intake were independent variables and DCM was dependent variable remained non-significant. We further investigated whether polymorphisms were associated with the etiology of DCM, but again there were no significant differences between subgroups.

DISCUSSION

The present study indicate that polymorphisms of ACE, AT, APO E and ecNOS genes are not related with DCM in Turkish population. The rationale behind the selection of the genes that were investigated in our study was the possibility of association of these polymorphisms with DCM. Many investigations reported that the proteins encoded by these genes play important roles in the patophysiology of heart failure.

It is well known that the renin-angiotensin system is a key molecular system in the pathogenesis of heart failure. Blood ACE level largely changes with the insertion (I) or deletion (D) of 287 base pairs in intron 16 of the gene for ACE [5]. ACE tissue activity also is affected by ACE gene polymorphism [6]. In 1992, Cambien et al [7] first reported the D allele of this polymorphism as a potential risk factor for the development of myocardial infarction. Since then,

Table 2. Distribution of genotypes of the four candidate genes among DCM patients and control subjects

Genotypes	DCM (total) (n:76)	DCM (ischemic) (n:41)	DCM (idiopathic) (n:35)	Control (n:88)
ACE II	8 (10%)	4 (10%)	4 (11%)	11 (12%)
ACE ID	33 (43%)	16 (39%)	17 (49%)	28 (32%)
ACE DD	35 (54%)	21 (51%)	14 (40%)	49 (56%)
P Value	0,309	0,701	0,207	
AR aa	46 (60%)	23 (56%)	23 (65%)	46 (53%)
AR ac	29 (38%)	17 (41%)	12 (34%)	37 (42%)
AR cc	1 (2%)	1 (2%)	0	5 (5%)
P Value	0,25	0,7	0,205	
NOS aa	1 (1%)	0	1 (3%)	0
NOS ba	24 (32%)	13 (32%)	11 (32%)	23 (26%)
NOS bb	51 (67%)	28 (68%)	23 (65%)	65 (74%)
P Value	0,398	0,511	0,223	
APO e23	12 (16%)	6 (15%)	6 (17%)	7 (8%)
APO e24	0	0	0	2 (2%)
APO e33	41 (54%)	21 (51%)	20 (57%)	73 (83%)
APO e34	19 (25%)	12 (29%)	7 (20%)	6 (7%)
APO e44	4 (5%)	2 (5%)	2 (6%)	0
P Value	**<0,001**	**<0,001**	**0,007**	

(DCM: dilated cardiomyopathy, ACE: angiotensin converting enzyme, AR: angiotensin II receptor, NOS: nitric oxide synthase, APO:apolipoprotein)

many articles have reported an association of the D allele with the development of left ventricular hypertrophy [8] and hypertrophic cardiomyopathy [9]. The I/D polymorphism of the ACE gene was the first genetic factor reported to be associated with non-familial idiopathic DCM [10]. But, this finding failed to be confirmed in later studies [2,4,11] and in the present study.

AGTR1 polymorphism may be involved in the susceptibility of DCM because angiotensin effects on receptor level. The AGTR1 has been shown to be selectively down-regulated in failing left ventricle from patients with end-stage heart failure due to idiopathic DCM, suggesting that the failing human heart is exposed to increased concentrations of angiotensin-II [12]. The polymorphism investigated in the present study had been previously suggested to be involved in susceptibility to myocardial infarction [13]. However we did not find any association between DCM and AGTR1 polymorphism.

The human ecNOS gene is located on chromosome 7q35-36 and comprises 26 exons that span 21 kilobases [14]. The polymorphic regions in the ecNOS gene may influence the amount of nitric oxide synthase enzyme transcripted, leading excessive production of nitric oxide depressing myocardial contractility. Nitric oxide is a powerful vasoactive molecule produced from L-arginine by three distinct nitric oxide synthase enzymes. Two constitutively present enzymes are found in neuronal and endothelial cells, respectively. The endothelial isoform NOS3 is also constitutively expressed in cardiac myocytes [15] and its enhanced basal production has been reported in patients with heart failure [16]. However, the Cardigene study group [4] could not confirm the hypothesis that ecNOS polymorphism leads DCM. We did not also find any association between ecNOS polymorphism and DCM in Turkish population. One of the reason and our investigation could not confirm this hypothesis. The reason might be the presence of multiple polymorphic regions in the gene and one should investigate all of them separately before coming up with a certain conclusion.

ApoE is synthesized endogenously in foam cells and after stimulation by extracellular lipid-free apoA-1. This facilitates cholesterol efflux

Table 3. Odds ratios for DCM assuming a dominant and recessive genetic model for E3 allele

DOMINANT MODEL FOR E3 ALELE				
	Allele frequency	**OR**	**95% CI**	**P value**
DCM (total) vs. control	0.94/0.98	2.38	0.42-13.4	0.548
Ischemic DCM vs. control	0.95/0.98	2.2	0.3-16.2	0.803
Idiopathic DCM vs. control	0.94/0.98	2.6	0.35-19.1	0.684
RECESSIVE MODEL FOR E3 ALLELE				
DCM (total) vs. control	0.54/0.83	4.1	2.03-8.49	**<0.001**
Ischemic DCM vs. control	0.51/0.83	4.6	2-10.5	**<0.001**
Idiopathic DCM vs. control	0.57/0.83	3.65	1.52-8.71	**0.006**

(DCM: dilated cardiomyopathy, OR: odds ratio, CI: confidance interval)

from lipid-laden foam cells, within the intima of lesion, into circulation via HDL-containing apoA-1. Exogenous apoE can assist in cholesterol transport from lesional foam cells as an extracellular, free cholesterol acceptor.

ApoE also directly modifies macrophage and T-lymphocyte-mediated immune responses to inflammatory atherosclerosis. The production of apoE in macrophages is regulated by inflammatory cytokines, interferon-, and tumor necrosis factor-α [17].

In humans, apo E exists in 3 allelic forms: E2 (Cys112-Cys158), E3 (Cys112-Arg158), and E4 (Arg112-Arg158). In mixed white populations, the allele frequencies for E2, E3, and E4 are approximately 0.08, 0.77, and 0.15, respectively [18].

The 2 allele is associated with lower LDL-C levels, and the 4 allele is associated with higher levels relative to the 3 allele. This observation led to the hypothesis that apo E may play an important role in the development of coronary heart disease. It has been estimated that carriers of the 4 allele have a 1.4-times-higher risk of developing coronary heart disease than 3 carriers [19]. In the present study we speculated that APOE polymorphism can play a role especially in the pathophysiology of the "ischemic" form of DCM. We observed an increased E4 distribution among DCM patients (both in idiopathic and ischemic forms) however we could not confirm that it is an independent risk factor.

Finally, the authors admit that the sample size of the present study is relatively low. But we should remind the readers that it is the first paper in the literature investigating these four candidate genes in DCM patients. We hope our findings will encourage investigators to design larger scale studies.

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