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ARE THE ANGIOTENSIN-CONVERTING ENZYME GENE AND ACTIVITY RISK FACTORS FOR STROKE?

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ABSTRACT - Stroke is a multifactorial disease in which genetic factors play an important role. This study was carried out to determine angiotensin-converting enzyme (ACE) gene polymorphism in Turkish acute stroke patients and to establish whether there is an association of angiotensin-converting enzyme gene I/D polymorphism with clinical parameters. In this study 185 patients and 50 controls were recruited. We have investigated the association among the allelic distribution of the insertion/deletion (I/D) polymorphism of the ACE gene identified by polymerase chain reaction. Distribution of ACE gene I/D genotypes and allele frequencies in patients were not significantly different from controls. D allele frequencies were 57.8% in patients versus 53.0% in controls and I allele 42.2% versus 47% respectively. History of hypertension, stroke, renal, heart and vessel diseases incidence and age, gender, systolic-diastolic blood pressures and creatinine levels were significantly high in patients. But these results and ACE activities had no significant differences among the ACE genotypes in patients and controls. Our results suggest that the ACE gene polymorphism is not associated with the pathogenesis of stroke in Turkish stroke patients.

KEY WORDS: ACE gene, PCR, polymorphism, stroke.

São fatores de risco para acidente vascular cerebral o gene e a atividade da enzima conversora de angiotensina ?

RESUMO - O acidente vascular cerebral (AVC) é doença multifatorial em que fatores genéticos desempenham papel importante. Este estudo foi desenvolvido para verificar o polimorfismo do gene da enzima conversora da angiotensina (ECA) em pacientes turcos com AVC agudo e estabelecer se existe associação do gene I/D da ECA com parâmetros clínicos. O estudo foi realizado com 185 pacientes e 50 controles. A associação entre a distribuição alélica da inserção / deleção (I/D) do polimorfismo do gene da ECA foi estudada pela reação em cadeia da polimerase. A distribuição dos genótipos I/D do gene da ECA e suas freqüências não apresentaram significância estatística quando comparados os pacientes e controles. As freqüências dos alelos D foram 57,8% nos pacientes versus 53% nos controles e dos alelos I 42,2% versus 47% respectivamente. Antecedentes de hipertensão, AVC, doença renal, doenças cardíacas, idade, gênero, pressão arterial sistólica e diastólica e níveis de creatinina foram significantemente elevados no grupo dos pacientes. No entanto estes resultados quando comparados com a atividade e o polimorfismo do gene da ECA não apresentaram diferenças estatísticas entre o grupo de pacientes e controles. Nossos resultados sugerem que o polimorfismo do gene da ECA não é associado com a patogênese do AVC em paciente turcos.

PALAVRAS-CHAVE: gene da ECA, PCR, polimorfismo, acidente vascular cerebral.

Stroke represents a leading cause of death, in most countries together with coronary artery disease[1]. Stroke, especially ischemic stroke, can be presenting feature of a number of single-gene disorders. The etiology of these disorders is multifactorial. Classical form of inheritance cannot be demonstrated, however evidence suggests the importance of genetic factors[2,3]. The similarity in the pathophysiology of myocardial infarction with cerebral infarction prompted investigators to study the role of the angiotensin-converting enzyme (ACE) gene (GenBank accession no: NM 000789.2) polymorphism in stroke[4]. In vitro autoradiography and immunohistochemical studies have mapped ACE within the brain, with high concentration of ACE being found in nigrostiatal pathway and basal ganglia[5].

ACE (dipeptidyl carboxy peptidase I, EC 3.4.15.1) is a peptidyl dipeptide hydrolase belonging to the class of zinc metalloproteases which main functions are to convert angiotensin I into angiotensin II, and to inactivate bradykinin. It is assumed that this step of the renin-angiotensin system is not limiting in plasma, and indeed these are no indication that plasma ACE levels are directly related to blood pressure lev-

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els. However, the local generation of angiotensin 1 and the degradation of bradykinin might depend on the level of ACE expressed in tissues[6-11]. Angiotensin-converting enzyme gene is localized on the band 17q 23 of 17th chromosome in human. The human ACE gene contains 26 exons interrupted by 25 introns and spans approximately 21 kb of DNA[10]. The ACE I/D polymorphism detected by polymerase chain reaction (PCR) was evident as a 490 bp product in the presence of the insertion (I allele) and as a 190 base pair (bp) fragment in the absence of the insertion (D allele). Thus, each DNA sample is presented in one of three possible patterns after electrophoresis: a 190- bp band (genotype DD), both a 190- and a 490-bp band (genotype ID), or a 490-bp band (genotype II)[1]. The mechanisms underlying positive associations between the ACE I/D alleles and disease are not yet clear[12]. Any possible association of the ACE genotype with stroke pathogenesis should be important, particularly since hypertension is a major risk factor for stroke[5]. Some authors reported that there was a significant association between ACE gene polymorphism and brain infarction of lacunar type in Caucasian[13,14]. Also it was found that there was a significant association between ACE gene polymorphism and ischemic stroke in Japanese hypertensive patients[1], although other investigators could not detect the association[15-17].

In this study, we aimed to investigate the ACE gene alleles frequencies, the association of ACE gene polymorphism with stroke patients applying to the Research Hospital of Osmangazi University.

METHOD

This study included 185 acute stroke patients (102 males, 83 females; mean±SE age, 62.99±0.91 years) and 50 controls (15 males, 35 females; mean±SE age, 57.10±1.28 years) recruited from Osmangazi University, Medical Faculty, Neurology Department. The study population were genetically homogene Turkish native. Acute stroke patients separated to six subgroups according to CT, MR and neuroradiological analyses results. These groups are large vessel disease, small vessel disease (lacune), cardioembolism, transient ischemic attacks (TIA), other ischemic strokes and hemorrhage. In both patient and control groups, hypertension was defined as either a systolic blood pressure ≥140 mmHg and diastolic blood pressure ≥90 mmHg[18], or current treatment with antihypertensive drugs. Control subjects were consecutively selected among people without personal and family history of stroke. Ethical approval for this study was obtained from the Ethics Committee of the University of Osmangazi.

Polymorphism determination – DNA was extracted from 10 ml of venous blood, anticoagulated with 1.6 mg/mL EDTA, by salt method and stored at +4°C[19].

Amplification of DNA was performed by PCR with 1 μL of DNA extract and thermostable taq polymerase (Sigma D-6677) according to the Marre et al.[20]. The PCR was performed in a thermal cycling (Eppendorf Mastercycler Personal). Oligonucleotide sequences of the PCR primers for D/I alleles were

5'-CTGGAGACCACTCCCATCCTTTCT-3' and

5'-GATGTGGCCATCACATTCGTCAGAT-3'

The DNA was amplified for 30 cycles with denaturation at 92°C for 40 seconds, annealing at 56°C for 40 seconds, and extension at 72°C for 40 seconds. Oligonucleotide sequences specific for I alleles primers were

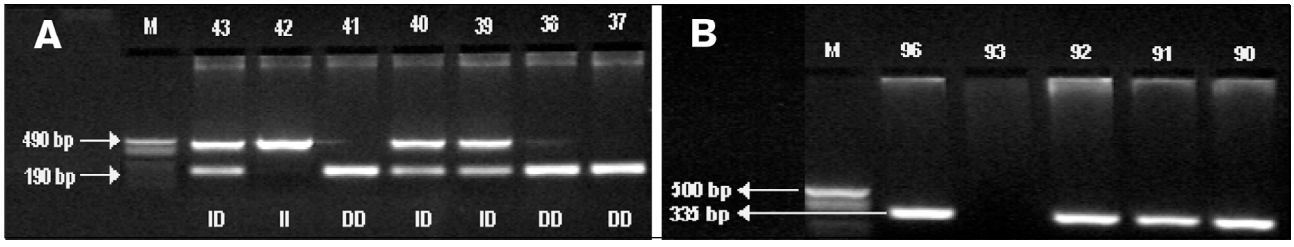

Fig 1. A) Determination of ACE genotypes (DD, ID, II) by PCR using ACE primers. B) Determination of ACE II genotype by PCR using insertion specific ACEX primers (M; marker).

Table 1. Distributions of ACE genotypes between patients' subgroups and controls.

Genotypes	Stroke patients							Controls
	Ischemic Subgroups					Total	Hemorrhage	
	Large vessel	Small vessel	Cardioembolic	TIA	Others	Ischemic		
II (n)	6	7	5	2	1	21	8	12
ID (n)	19	35	18	3	1	76	22	23
DD (n)	16	12	9	3	4	44	14	15

5'-TGGGACCACAGCGCCCGCCACTAC-3' and
5'-TCGCCAGCCCTCCCATG CCCATAA-3'[18].

DNA was amplified for 30 cycles with denaturation at 92ºC for 40 seconds, annealing at 63ºC for 40 seconds, and extension at 72ºC for 40 seconds. The PCR products were separated by electrophoresis on 2% agarose gel containing 4 μL ethidium bromide (50 μg/mL) and were visualized by using CCD camera. Results were evaluated with the gel analysis software (LabWorks) (Fig 1).

Enzyme assay – Plasma was stored –80ºC, and ACE activities were determined by using ACE ELISA Kit, (Chemicon International, USA). ACE levels were measured in patient and control subjects not taking ACE inhibitors.

Statistical analysis – Statistical analysis were performed using SPSS (Statistical Package for Social Sciences) software package, version 10.0 for Windows (SPSS Inc., Chicago, III. USA). Values are expressed as means ±SE. Alleles and genotype frequencies between patients and control subjects were compared by chi-square test with Hardy-Weinberg predictions. Some physiologic and clinical parameters of patients and controls were analysed by chi-square and t-test. According to the ACE genotypes, the ACE activities, some physiologic and clinical parameters of patients and controls were compared by ANOVA and chi-square test. less than 0.05 was considered statistically significant.

RESULTS

Distributions of ACE genotypes between patients subgroups and controls are given in Table 1.

ACE genotypes distributions and I/D alleles frequencies are shown in Table 2. The distribution of ACE

Table 2. Frequencies of ACE genotype and alleles in stroke and control subjects.

Groups	n	Genotypes			I Allele n (%)	D Allele n (%)
		II n(%)	ID n(%)	DD n(%)		
Total stroke patients	185	29 (15.7)	98 (53.0)	58 (31.3)	156 (42.2)	214 (57.8)
Controls	50	12 (24.0)	23 (46.0)	15 (30.0)	47 (47.0)	53 (53.0)

Genotype and allele frequencies were compared with χ^2 test, not statistically significant, p>0.05

Table 3. Some physiologic and clinical parameters of patients and controls.

		Stroke patients (n=185)	Controls (n=50)	Statistical analysis
Age (years±SE.)		62.99±0.91	57.10±1.28	p<0.01
Gender (Male/Female)		102/83	15/35	p<0.01
Serum creatinine (mg/dL)		1.05±0.05	0.78±0.02	p<0.01
HDL-C (mg/dL)		44.66±1.08	48.20±1.29	p>0.05
Total cholesterol (mg/dL)		182.43±4.00	206.26±6.38	p<0.01
Triglyceride (mg/dL)		98.61±5.02	145.88±8.48	p<0.001
Systolic BP. (mmHg)		152.11±2.43	124.66±1.23	p<0.001
Diastolic BP. (mmHg)		88.38±1.21	82.36±0.76	p<0.05
ACE Activity (ng/mL)		(n=39) 403. 06±37.83	(n=39) 361.13±26.81	p>0.05
Hypertension	n (%)	129 (69.7)	15 (30.0)	p<0.001
Diabetes	n (%)	40 (21.6)	11 (22.0)	p>0.05
Renal disease	n (%)	15 (8.1)	–	p<0.05#
History of stroke	n (%)	37 (20.0)	–	p<0.01
Heart disease	n (%)	74 (40.0)	8 (16,0)	p<0.01
Vessel disease	n (%)	39 (21.0)	–	p<0.01

χ^2 test for categorical variables , unpaired t test for continuous variables (values; mean±SE); #Fisher's exact test.

Table 4. According to the ACE genotypes, some physiologic and clinical parameters of patients and controls

		Stroke patients			Controls		
		II	ID	DD	II	ID	DD
Age (years±SE.)		65.4±2.07	63.4±1.14	61.0±1.93	61.6±1.96	55.5±2.18	55.9±1.97
Gender (M/F)		22/7	50/48	30/28	1/11	8/15	6/9
Serum creatinine (mg/dL)		1.13±0.13	1.05±0.08	1.04±0.06	0.76±0.07	0.79±0.03	0.80±0.70
HDL-C (mg/dL)		41.3±2.42	45.7±1.44	44.7±2.13	50.5±2.63	48.6±1.90	45.8±2.43
Total cholesterol (mg/dL)		180.79±10.54	181.91±5.28	184.12±7.58	195.92±14.62	212.48±9.30	205.00±12.67
Triglyceride (mg/dL)		110.41±14.28	95.81±6.38	97.43±9.54	119.50±13.43	156.61±12.67	150.53±16.88
Systolic BP (mmHg)		155.55±5.26	152.93±3.39	149.00±4.54	126.08±1.77	123.91±1.91	124.67±2.61
Diastolic BP (mmHg)		91.31±2.95	88.21±1.49	87.21±2.58	83.75±1.63	81.87±1.21	82.00±1.25
Hypertension	n (%)	18 (62)	69 (70)	42 (72)	5 (41.6)	7 (30.4)	3 (20.0)
Diabetes	n (%)	6 (20,6)	20 (20,4)	14 (24.1)	4 (33,3)	3 (13.0)	4 (26.6)
Renal disease	n (%)	4 (13,7)	9 (9,2)	2 (3.4)	–	–	–
History of stroke	n (%)	7 (24,1)	20 (20,4)	10 (17.2)	–	–	–
Heart disease	n (%)	9 (31,0)	42 (42,8)	23 (39,6)	2 (16.6)	3 (13.0)	3 (20.0)
Vessel disease	n (%)	7 (24,1)	21 (21,4)	11 (18,9)	–	–	–

χ^2 test for categorical variables , ANOVA for continuous variables (values; mean±SE); p values from χ^2 and ANOVA tests that examined in patients and controls according to ACE genotypes; not statistically significant=p>0.05.

Table 5. ACE activity in relation to genotypes in stroke patients and controls.

Groups	Genotypes	n	ACE activity (ng/mL)	Statistical analysis
Stroke patients	II	13	335.02±34.76	F=1.249
	ID	13	330.05±50.38	p>0.05
	DD	13	418.31±51.68	
Controls	II	13	389.71±68.00	F=0.63
	ID	13	358.41±59.56	p>0.05
	DD	13	461.08±37.83	

Values are mean ± SE; ANOVA test was used to compare the ACE activities according to ACE genotypes in patient and control groups; not statistically significant=p>0.05.

genotypes in patients with stroke were as follows: II, 29 (15.7%); ID, 98 (53.0%); and DD, 58 (31.3%), which was not significantly different from the distribution in control subjects: II, 12 (24%); ID, 23 (46%); and DD, 15 (30%). ACE gene I/D allele frequencies were 42.2% I and 57.8% D in patients; and 47% I and 53% D in controls. The patients and control populations were in Hardy-Weinberg equilibrium.

Some physiologic and clinical parameters of patient and control groups are presented in Table 3. The stroke patients age, gender, creatinine, systolic and diastolic blood pressures, hypertension, renal disease, history of stroke, heart and vessel disease distributions were significantly higher than those of the control subjects. But, total cholesterol and triglyceride levels were significantly lower in stroke patients as compared with controls.

According to the ACE genotypes, some physiologic and clinical parameters of patients and controls are shown in Table 4. In stroke patients and controls, therewere no significant differences in age, gender, creatinine, HDL-C, total cholesterol, triglyceride, systolic and diastolic blood pressures, hypertension, diabetes, renal disease, history of stroke, heart and vessel diseases among the ACE genotypes (p>0.05).

ACE activities in relation genotypes in stroke pa-

tients and controls are shown in Table 5. ACE activities were not significantly different according to ACE genotypes in patient and control groups (p>0.05).

DISCUSSION

ACE is predominantly located on capillary endothelial cells of vascular beds, on cells of absorptive epithelia such as those of the renal proximal tubule, and on other epithelia including those of the brain. ACE activity is detectable in plasma and, despite large interindividual differences, its levels are very stable within an individual. ACE converts angiotensin I into the antinatriuretic vasoactive angiotensin II, an octapeptide involved in vasoconstriction, aldosterone production, and norepinephrine release from sympathetic nerve endings. On the other hand, infusion of angiotensin II results in an in vivo substantial increase in the circulating levels of plasminogen activator inhibitor-1. ACE also inactivates bradykinin, a vasodilator and natriuretic substance[21].

In this study, we demonstrated that there was no statistically significant difference between ACE genotypes and I/D allele frequencies in the stroke patients and healthy persons.

Recent studies have shown that especially D polymorphism in the ACE gene may be a potent risk factor for ischemic stroke and myocardial infarction in human[5,21-23]. Also, some studies showed a positive association between lacunar stroke and ACE polymorphism[5,14]. But several studies have suggested a weak or no significant association between ACE gene polymorphism and stroke[1,4,5,13-15,20,24]. Stephen et al. have reported that the ACE gene I/D polymorphism is not associated with the blood pressure and cardiovascular benefits of ACE inhibition[25].

In our study, I/D allele frequencies were closely determined each other in normal subjects and acute stroke patients. D allele frequency was 57.8% in acute stroke patients versus 53% in controls and I allele 42.2% versus 47% respectively. Literature have research shown that D allele frequency given in the studies conducted on hypertension and ACE gene polymorphism in Turkey were 57.5%[26] and 51.7%[19] in hypertension patients. But D allele frequency is different in other countries, for example in Greece 57.0%[22], in Swedish 62.0%[23], in Japan 47.0%[1] in stroke patients. So in a meta analyses study[4], the difference of D allele frequencies was determined to range from 50 to 72%. Also, our findings of genotype frequency are similar to those of a study[14] involving patients of cerebrovascular disease. We found that ACE genotype frequencies were II=15.7%, ID=53% and DD=31.3% in stroke patients. Markus et al. also found that they were II=17.8%, ID=46.5% and DD= 35.5% in stroke patients[14].

In our study, although ACE activity was high in DD genotype, no difference was determined to ACE enzyme activity according to ACE genotypes in patients and controls. Also, in another our study, we found that hypertension patients were no significant differences in the ACE activities whether the inhibitors were used or not[19]. Some studies suggested that D allele of the ACE gene is a marker of an elevated circulation ACE level[5,14,27]. Catto et al. reported plasma ACE activity significantly lower in stroke patients than in controls[5], and levels of ACE activity were significantly lower during the acute phase of stroke but were similar to level of control activity after 3 months[5]. Reduced ACE activity may be a feature of the acute event, although ACE has not previously been reported in the acute phase of cerebral infarction[5,28].

In our study, the stroke patients age, gender, creatinine, systolic and diastolic blood pressures, hypertension, renal disease, history of stroke, heart and vessel disease distribitions were significantly higher than those of the control subjects. But, total cholesterol and triglyceride levels were significantly lower in stroke patients as compared with controls. Some studies[29-32], which are in an agreement with us, have found that hypertension, smoking, atrial fibrillation, ischemic coronary disease, peripheral vascular disease, heart failure and diabete disease were found to be higher in the ischemic stroke patients than controls. Madonna et al.[33] reported that smoking, hypertension, diabetes and hyperlipidemia rates were higher in ischemic stroke patients than control subjects, but there was no significant difference statistically. In another study[34], it was found that diabetes, hypertension, history of stroke were significantly higher in peripheral artery disease. On the other hand, age, sex, diabetes, smoking, hypercholesterol level were no significant difference between patient and control groups in hypertension coronary heart disease[35] and stroke disease[36].

The results of this study demonstrate norelationship between the ACE I/D polymorphism and stroke. ACE gene polymorphism, especially DD genotype, could not be a risk factor for acute stroke. Our findings suggest that an important contribution of ACE gene to stroke is unlikely and that the ACE I/D genotype will not be a useful tool for risk assessment or prognostication.

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