The I/D polymorphism of the angiotensin converting enzyme gene as a risk factor for ischemic stroke in patients with essential hypertension in Kyrgyz population

Kırgız toplumunda esansiyel hipertansiyonlu hastalarda iskemik inmenin risk faktörü olarak anjiyotensin dönüştürücü enzim geninde I/D polimorfizminin rolü

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Objectives: We investigated the association of the I/D polymorphism of the angiotensin converting enzyme (ACE) gene with essential hypertension (EH) and ischemic stroke in Kyrgyz male subjects.

Study design: The study included a total of 313 Kyrgyz men, including 180 patients with uncomplicated EH, 69 patients with EH complicated by ischemic stroke, and 64 healthy age-matched controls. All the subjects underwent I/D genotyping and determination of serum ACE activity. Ambulatory blood pressure (BP) monitoring and carotid ultrasound (51 patients) were also performed in patients with EH.

Results: The mean ACE concentration was 23.3±0.7 mU/ml/min for II, 32.2±0.9 mU/ml/min for ID, and 38.8±2.3 mU/ml/min for DD genotypes. The ID and DD genotypes were associated with significantly higher ACE levels compared to the II genotype ($p<0.01$ and $p<0.0001$, respectively). The frequency of the DD genotype in EH patients with ischemic stroke was more than two-fold greater than those with uncomplicated EH (0.31 *vs* 0.13, $p<0.02$), and nearly four-fold greater than the control group (0.31 *vs* 0.09, $p<0.02$). Patients with ischemic stroke had the highest frequency of the D allele compared to EH patients without stroke and controls (0.56 *vs* 0.36 and 0.29 respectively, $p<0.001$). Patients with the DD genotype differed significantly from those with the II or ID genotypes with greater variability of systolic and diastolic BP, more common abnormal night BP profile, and increased carotid intima-media thickness.

Conclusion: In the Kirghiz population, the presence of the DD genotype is associated with higher ACE levels and increased risk for ischemic stroke as a complication of EH.

Key words: Cerebrovascular accident; DNA/analysis; genotype; hypertension, pulmonary/genetics; Kyrgyzstan; peptidyl-dipeptidase A/genetics; polymorphism, genetic; risk factors.

Amaç: Bu çalışmada, Kırgızistan'lı erkek bireylerde anjiyotensin dönüştürücü enzim (ACE) genindeki I/D polimorfizminin esansiyel hipertansiyon (EH) ve iskemik inme ile ilişkisi araştırıldı.

Çalışma planı: Çalışmaya toplam 313 Kırgız erkeği alındı. Bunların 180'i EH nedeniyle komplikasyon gelişmemiş hasta, 69'u EH yanı sıra iskemik inme gelişen hasta ve 64'ü yaşça uyumlu sağlıklı kontrol idi. Tüm olgularda I/D genotiplemesi yapıldı ve serum ACE aktivitesi belirlendi. Ayrıca, EH'li hasta gruplarına ambulatuvar kan basıncı izlemi ve karotis ultrasonografisi (51 hasta) yapıldı.

Bulgular: Ortalama ACE konsantrasyonu II genotipinde 23.3±0.7 mU/ml/dk, ID genotipinde 32.2±0.9 mU/ml/dk, DD genotipinde 38.8±2.3 mU/ml/dk bulundu. ID ve DD genotipli olgularda ortalama ACE düzeyi, II genotipli olgulara göre anlamlı derecede yüksekti (sırasıyla $p<0.01$ ve $p<0.0001$). İskemik inme gelişen EH'li hastalarda DD genotipi sıklığı, komplikasyonsuz EH'li hastalardan iki kattan fazla (0.31 ve 0.13, $p<0.02$), kontrol grubundan ise yaklaşık dört kat fazlaydı (0.31 ve 0.09, $p<0.02$). Bu grupta D alleli sıklığı (0.56), komplikasyonsuz EH'li hastalara (0.36) ve kontrol grubuna (0.29) göre anlamlı derecede yüksek bulundu ($p<0.001$). Ayrıca, DD genotipli hastalar, II ve ID genotipli hastalardan, sistolik ve diyastolik kan basınçlarında daha fazla değişkenlik, daha sık anormal gece kan basıncı profili ve karotis arterde daha fazla intima-media kalınlığı açısından anlamlı farklılık gösterdi.

Sonuç: Kırgız toplumunda DD genotipinin varlığı, daha yüksek ACE düzeyi ve EH'nin komplikasyonu olarak iskemik inme riskinde artışla sonuçlanmaktadır.

Anahtar sözcükler: Serebrovasküler olay; DNA/analiz; genotip; hipertansiyon, pulmoner/genetik; Kırgızistan; peptidil-dipeptidaz A/genetik; polimorfizm/genetik; risk faktörü.

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Cardiovascular diseases are the leading cause of mortality in the Kyrgyz Republic, with more than 47% of all deaths and a dramatic and progressive increase. Mortality from cardiovascular diseases showed an increase by 16% from 261.8 events in 1991 to 304.6 events in 2000 among 100,000 individuals.

Analysis of statistical data show that not all cardiovascular diseases equally contribute to the death rate in the Kyrgyz Republic. Among them, arterial hypertension (AH) is one of the most widespread diseases. The prevalence of AH in Kyrgyzstan showed a sudden increase between 1980 and 1990. Twenty years ago, the frequency of AH in Kyrgyzstan was 24% among urban adult citizens and 18% among rural adult residents. An epidemiological study including more then 2,500 individuals showed in 2000 that about 38.4% of all adult population suffer from increased blood pressure (BP).[1] About one million of adult population of the republic have increased BP, i.e. every three residents older than 18 years.

Among numerous complications of AH, brain stroke is the leading cause of morbidity and mortality. This problem is especially specific to the Kyrgyz Republic because hypertensive Kyrgyzs are more prone to ischemic stroke than are Caucasians. According to a survey of the World Health Organization, Kyrgyzstan holds the first place in the Eurasian region with respect to mortality due to ischemic stroke with 60.67 in 100,000 individuals, compared to 48.57 in the former USSR, 45.33 in Ukraine, and 12.71 in European Union countries.[2]

Recent epidemiological studies involving twins, siblings, or families have provided strong support for a genetic influence on stroke.[3,4] However, genetic determinants of stroke are still largely unknown. There are some examples of mutation in specific genes that cause rare Mendelian forms of stroke, but none of these occur on the background of atherosclerosis and hypertension, therefore, these genes are probably not involved in the common forms of ischemic stroke.[5,6]

In most cases, the development of ischemic stroke is determined by polymorphism in several genes as well as interactions of genetic and environmental factors. In such cases, analysis of candidate genes is used. Among candidate genes investigated to date, the following genes have received substantial attention: genes of renin-angiotensin system,[7] eNOS gene,[8] genes affecting hemostasis proteins and homocysteine metabolism,[9,10] genes of lipid metabolism,[11] and genes of apoptosis.[12] One that may influence both the development of essential hypertension (EH) and its outcome is the angiotensin converting enzyme (ACE) gene, which is responsible for the generation of angiotensin II, a potent vasoconstrictor and trophic hormone and a key drug target in the management of hypertension and its complications, from angiotensin I.[13,14]

Circulating ACE level is a highly heritable trait and several genetic polymorphisms affecting ACE levels have been identified. Most investigators have focused on the insertion/deletion (I/D) polymorphism. The D allele is associated with increased ACE activity, but data on the relationship of this allele with susceptibility to stroke are conflicting. A comprehensive meta-analysis of genetic studies in ischemic stroke involving white males reported no increased allele frequency associated with stroke compared to healthy controls, but indicated the need to study other ethnic groups.[15]

In Kyrgyz population, it was previously demonstrated that subjects with the DD-genotype had higher levels of ACE, and that there was a significant association between the I/D polymorphism of the ACE gene and high-altitude pulmonary hypertension.[16]

Considering the presence of ethnic differences in gene distribution and the significant role of ACE in BP regulation and its tissue effects, the aim of this study was to investigate the association of the I/D polymorphism of the ACE gene with EH and ischemic stroke in an ethnic- and gender-homogeneous population of Kyrgyz male subjects. In this respect, a genotyping study was conducted in groups of patients with uncomplicated EH and hypertension complicated by ischemic stroke, as well as in normotensive subjects without stroke. In addition, we investigated the association of genetic polymorphism of the ACE gene with activity of enzyme in serum in a large group of unrelated Kyrgyz men.

PATIENTS AND METHODS

Subjects. This study was approved by the institutional review committee. All participants (350 males with EH) were Kyrgyz by descent, aged 40 to 65 years. Among those who gave informed consent for participation and provided blood samples for DNA analysis, a total of 249 individuals were selected for genotyping. Hypertension was diagnosed if resting clinical BP was >140/90 mmHg on two occasions (WHO 1999).

All patients were divided into two groups. One group was comprised of 180 untreated patients with uncomplicated EH, and the other group consisted of 69 patients with EH complicated by ischemic stroke, confirmed by computed tomography of the brain. Exclusion criteria were acute myocardial infarction, heart failure, atrial fibrillation, valvular heart disease, diabetes mellitus, and secondary hypertension.

The control group was composed of 64 healthy age-matched Kyrgyz men from the same local population, who had normal BP (<130/85 mmHg) on three occasions.

Allele and genotype frequencies of the ACE gene and the correlation of the I/D polymorphism with serum ACE activity were studied by the case-control method in 219 unrelated Kyrgyz men aged 18 to 80 years.

Ambulatory blood pressure. Twenty-four hour BP and heart rate monitoring was conducted (Tonoport IV device, Marquette Hellige, Freiburg, Germany). Blood pressure and heart rate were measured every 15 min from 06:00 to 24:00 hours, and every 30 min from 24:00 to 06:00 hours. Twenty-four hour average systolic and diastolic BP, time index (percentage of increase in BP at 24 hours), and variability (standard deviation) of both systolic and diastolic BP and daily index (percentage fall in the mean arterial pressure at night compared to daytime) were analyzed. Depending on the variability and the decrease in the mean nocturnal BP compared to the mean daytime BP, the patients were classified as dippers (fall ≥10% to 20%), extreme dippers (≥20%), non-dippers (≥0% to <10%), and night-peakers (<0%).

Carotid ultrasound. Examination of the carotid artery was performed in 51 patients with the Acuson Sequoia 512 ultrasound system (Siemens Ultrasound, Mountain View, CA, USA) equipped with a 7-MHz linear-array transducer. The carotid artery was identified by combined B-mode and Doppler/pulse-wave Doppler ultrasound. The image was synchronized with the R wave on the electrocardiogram and stored on a SVHS videotape. Vessel diameter, thickness, peak systolic velocity (Vs), end-diastolic velocity (Vd), and the resistive index - the relationship of the difference between Vs and Vd to Vs and systolic to diastolic ratio - were recorded.

ACE genotyping. DNA was extracted from venous blood samples using Nucleon BACC3 kits (Amersham Pharmacia Biotech, Sweden). The ACE I/D genotype was determined by the polymerase chain reaction (PCR) method with the oligonucleotide primers 5'-CTGGAGACCACTCCCATCCTT-3' and 5'-GTGGCCATCACATTCGTCAGAT-3', based on a previously described method.[17] Amplificated products were separated by electrophoresis in 2% agarose gel stained with ethidium bromide. The 490-bp and 190-bp fragments corresponded to the biallelic I (insertion) and D (deletion) polymorphisms, respectively.

ACE activity. ACE serum activity was assayed by hydrolysis of the specific substrate Z-Phe-His-Leu-OH as previously described[18] and was calculated using the following equation:

$$\text{ACE activity}\left(\frac{\text{nmol}}{\text{ml}} \times \text{min}\right) = \frac{\text{Fluorescence of probe} \times 10^4}{\text{Fluorescence of standard} \times 30 \text{ min}}$$

Statistical analysis. The data were expressed as means ± standard deviation. Clinical data and ACE serum activity were compared between groups using the two-sample Student's t-test. Allele and genotype distributions in the groups were compared using contingency tables and nonparametric the chi-square test and z-test. A p value of less than 0.05 was considered significant.

RESULTS

No significant differences were found between patients with uncomplicated EH and those having EH complicated by ischemic stroke with regard to systolic and diastolic BP, total cholesterol levels, and smoking status (p>0.05) (Table 1).

ACE activity. The mean ACE concentrations for different ACE genotypes were as follows: 23.3±0.7

Table 1. Clinical characteristics of the participants (mean±SD)

	Control group (n=64)	Uncomplicated EH (n=180)	Complicated EH (n=69)
Age (years)	53.7±10.7	54.9±5.5	56.6±9.6
Duration of disease (years)	–	8.4±2.6	9.8±3.3
Systolic blood pressure (mmHg)	116.9±8.7	163.2±17.1*	168.5±21.3*
Diastolic blood pressure (mmHg)	70.6±6.2	104.4±8.3*	103.3±12.1*
Total cholesterol (mmol/l)	5.3±0.8	5.8±0.6	5.7±0.8

ES: Essential hypertension; *p<0.001 for comparison between the two patient groups and the control group.

Table 2. Genotype and allele frequencies for the ACE gene polymorphism in different populations

	Genotype (%)			Allele (%)		
Population	II	ID	DD	I	D	Reference
Kyrgyz	42.5	44.7	12.8	64.8	35.2	This study
Japanese	35.0	55.0	10.0	63.0	37.0	Tsutaya et al., 1997[20]
Asiatic	39.8	41.8	18.3	61.0	39.0	Sagnella et al., 1999[21]
African	18.4	50.5	30.9	44.0	56.0	Sagnella et al., 1999[21]
European	18.4	49.6	32.0	43.0	57.0	Sagnella et al., 1999[21]

mU/ml/min for II, 32.2±0.9 mU/ml/min for ID, and 38.8±2.3 mU/ml/min for DD. The ID and DD genotypes were associated with significantly higher ACE levels compared to the II genotype ($p<0.01$ and $p<0.0001$, respectively).

The ACE I/D polymorphism in the Kyrgyz population. To assess the ACE I/D allele and genotype frequencies, DNA typing was carried out in 219 unrelated Kyrgyz males aged 18 to 80 years. It was found that the Kyrgyz population resembled the Southeast Asian populations[19,20] in that the frequency of the I allele was significantly higher than the D allele (64.8% *vs* 35.2%, respectively). The frequencies of the genotypes II, ID, and DD were 42.5%, 44.7%, and 12.8%, respectively, and the population was in Hardy-Weinberg equilibrium in this respect (Table 2).

DNA genotyping to seek possible associations between the I/D polymorphism of the ACE gene and EH with or without ischemic stroke showed no significant differences in I and D allele frequencies between healthy control subjects and patients with uncomplicated EH ($p>0.05$). However, in EH patients with ischemic stroke, the DD genotype frequency was more than two-fold greater than those with uncomplicated EH (0.31 vs 0.13, $p<0.02$), and nearly four-fold greater than the control subjects (0.31 *vs* 0.09, $p<0.02$) (Table 3). Similarly, EH patients with ischemic stroke had the highest frequency of the D allele (Table 3).

Ambulatory blood pressure. 24-hour average systolic and diastolic BP did not differ in hypertensive patients with different I/D genotypes. However, variability of systolic and diastolic BP was significantly greater in the DD genotype group compared to patients with other genotypes, which may be regarded as an unfavorable prognostic factor for increased risk for target organ damage in EH patients. Variability of systolic BP was 17.2±3.5 mmHg in DD patients compared to 14.7±3.6 mmHg in ID patients ($p<0.05$) and to 14.9±3.8 mmHg in II homozygote patients ($p<0.05$). Diastolic BP was 14.7±2.6 mmHg in DD patients, being significantly higher than ID heterozygote patients (12.7±3.0 mmHg, $p<0.03$) and II homozygote patients (12.7±3.4 mmHg, $p<0.05$).

Although there were no intergroup differences with regard to the average daily index of BP, qualitative analyses of BP profile revealed an abnormal daily BP rhythm in the form of non-dipping, night peaking, or extreme dipping in most of the patients with the DD genotype (83%), which are the most unfavorable disturbances of BP profile for cardiovascular complications.[21] The prevalence of an abnormal BP profile was significantly lower in the II and ID genotypes (44.2%, $p<0.01$; and 56.1%, $p<0.05$, respectively).

Carotid ultrasound. Of 51 patients who underwent ultrasound examination of the carotid artery, patients with the DD genotype had a significantly greater

Table 3. Genotyping of the ACE I/D polymorphism in patients with essential hypertension and in healthy controls

	Healthy controls (C) (n=64)		Essential hypertension (1) (n=180)		Hypertension with stroke (2) (n=69)				
	Absolute meaning	Frequency	Absolute meaning	Frequency	Absolute meaning	Frequency	*p* for C-1	*p* for C-2	*p* for 1-2
II	35	0.50	69	0.41	24	0.20	NS	<0.001	<0.001
ID	21	0.41	91	0.46	29	0.49	NS	NS	NS
DD	8	0.09	20	0.13	16	0.31	NS	<0.02	<0.02
I allele	91	0.71	229	0.64	61	0.44	NS	<0.001	<0.001
D allele	37	0.29	131	0.36	77	0.56	NS	<0.001	<0.001

NS: Not significant.

carotid intima-media thickness compared to those with the II or ID genotypes, suggesting a more pronounced remodeling. Intima-media thickness of the right carotid artery was 0.069±0.010 cm in DD patients compared to 0.057±0.012 cm in II homozygote ($p<0.01$), and to 0.056±0.010 cm in ID heterozygote patients ($p<0.02$). The corresponding figures for the left carotid artery were 0.062±0.003 cm, 0.056±0.008 cm, and 0.055±0.008 cm, respectively ($p<0.01$).

DISCUSSION

Essential hypertension and ischemic stroke are multifactorial diseases with significant contribution of genetic component. It is known that many genes are involved in the development of these diseases, including genes of the renin-angiotensin system. In this study, we examined the relationship between the ACE gene I/D polymorphism and EH with and without stroke as a complication in an ethnically homogeneous group of Kyrgyz males.

In common with other Asian populations, the D allele is less frequent in the Kyrgyz population. However, our findings showed that this allele was significantly more frequent in patients having EH with or without stroke, compared to healthy controls. Moreover, the DD genotype was significantly more common in patients who had ischemic stroke as a complication of EH.

The association between the I/D polymorphism of the AGE gene and hypertension remains controversial. Some earlier studies reported an association,[22] whereas some others did not find a link.[23] Interestingly, this association may differ between ethnic groups. Positive associations have been reported in studies of Asians and blacks but not Caucasians. Our study was conducted in a relatively homogeneous population of Kyrgyz men belonging to the Asian race and provided corroboration for the probability of race differences for the presence of an association between the I/D polymorphism of the AGE gene and the development of hypertension.

Our data suggest that the DD genotype may be associated with increased risk for stroke in Kyrgyz patients with hypertension. According to a meta-analysis by Staessen et al.[24] the DD genotype increases the risk of stroke in 94%. In contrast, Zee et al.[25] found no association between genetic polymorphism of the AGE gene and ischemic stroke in patients with hypertension.

Ambulatory blood pressure monitoring provides greater insight into the hypertensive phenotype than isolated clinical measurements.[26] In particular, it provides data on blood pressure variability. Greater blood pressure variability is associated with an increased risk for target organ damage in hypertension.[21] Consistent with the association with stroke, we found that Kyrgyz hypertensive patients with the DD genotype exhibited greater variability of blood pressure. Another important daily BP characteristic is the index of BP with decreases at night (daily index). It is known that persons with insufficient decreases in BP at night (non-dippers) have increased risk for brain stroke and myocardial infarction, and patients with excessive decreases in BP at night (extreme dippers) have increased risk for acute and chronic cerebrovascular diseases.[27] Thus, abnormal decreases in BP at night may be considered as a risk factor for the development of cardiovascular complications of EH. We found that the frequency of abnormal decreases in BP at night (non-dippers, night-peakers, and extreme dippers) among patients with the DD genotype was significantly higher than in patients with other genotypes. These disturbances in BP profile may represent a risk factor for stroke development in patients having the DD genotype. Our data are in good agreement with the data of Julve et al.[28] who reported no correlation between the genotypes of the ACE gene and average BP, but found that patients with the DD genotype had a higher BP at night.

Hypertension is associated with changes throughout the systemic vascular bed. One of the most accessible sites for clinical studies is the carotid aorta where increased intima-media thickness correlates with atherosclerosis and stroke.[29,30] We found a significant association between the DD genotype and increased intima-media thickness, suggesting the adverse effects of higher circulating angiotensin II levels. Benetos et al.[31] also showed significant associations of the D allele and DD genotype with intimal thickness of the carotid arteries.

Association between the I/D polymorphism and ACE concentrations has been reported in several studies.[32-34] We found that patients with the DD genotype had a higher level (1.7-fold) of ACE activity than those with the II genotype. The evidence that the D allele is associated with higher ACE levels provides mechanistic support to the associations between the genotype, greater BP variability, increased carotid intimal thickness, and the risk for stroke. A higher ACE activity would result in higher angiotensin II concentrations, which in turn have

direct effects on vascular resistance, vascular smooth muscle cell proliferation, and atherogenesis.[35]

In conclusion, in the Kyrgyz population, the presence of the DD genotype is characterized by higher ACE levels that predispose patients to complications of EH, in particular ischemic stroke. Thus, night hypertension, greater BP variability, and increased carotid intimal thickness are common features in patients with the DD genotype of the ACE gene. These clinical features are unfavorable for the development of EH complications and require adequate antihypertensive and antiaggregant therapy combined with statin administration.

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