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Effect of ACE gene polymorphism of Iraqi patients on ischemic stroke

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Abstract: Objectives: Evaluate the polymorphism of angiotensin converting enzyme gene as an independent risk factor for ischemic stroke in Iraqi population.
Design and methods: the study was conducted on (60) patients with acute ischemic stroke and (30) apparently healthy subjects were taken as control group. The ACE genotyping was performed using allele specific polymerase chain reaction.
Results: Genotypes of ACE gene determined by PCR technique were defined as DD, II and ID according to the presence of the D (deletion) and I (insertion) alleles. There was statistically significant difference in both the genotypic distribution and allelic frequency between the patients versus healthy controls (O.R. = 4.35 , $CI_{95\%}$ 1.05 - 18.03) of the DD genotype and D allele making them consider as an independent risk factors for ischemic stroke.
Conclusion: DD genotype and D allele of ACE gene can be considered as an independent risk factors for ischemic stroke, hence the significant association between DD genotype and D allele with ischemic stroke suggested by our study.
Key words : Ischemic stroke, Angiotensin converting enzyme gene , polymerase chain reaction , polymorphism.

Introduction

Ischemic stroke is death of brain tissue due to interruption of blood flow to a region of the brain, caused by occlusion of a carotid or vertebral artery or, less likely, a cerebral vein. The renin-angiotensin system (RAS) is a hormonal signaling mechanism implicated in the atherosclerosis and regulation of blood pressure[1]. Angiotensin-converting enzyme (ACE), a key enzyme in the renin-angiotensin system, plays important roles in vascular remodeling, atherosclerosis, and ischemic stroke[2]. It catalyses the conversion of inactive angiotensin I to active angiotensin II, which is known to be involved in vascular hypertrophy, vasoconstriction, and atherosclerotic processes[3].

The association between ACE gene polymorphism with ischemic stroke risk is an interesting field. The human ACE gene is located on chromosome 17q23, where an insertion/deletion polymorphism in intron 16 has been identified . This polymorphism is based on the presence (insertion, I) or absence (deletion, D) of a 300-bp DNA fragment. A co-dominant pattern of this polymorphism makes the DD genotype of ACE gene has been associated with highest serum ACE level and has been investigated as a potential susceptibility factor for

ischemic stroke. A large number of studies have reported the association between the I/D polymorphism of ACE gene and the risk of ischemic stroke, but the results were inconclusive[4].

Previously published meta-analyses reported significant associations between ACE I/D and risk of ischemic stroke[5].

There is substantial evidence suggesting a role of the rennin angiotensin system (RAS) in the development of hypertension and cardiovascular disease. Cerebrovascular endothelium has been shown to be rich in angiotensin-converting enzyme (ACE) by histochemical studies. Furthermore, in experimental stroke models in spontaneously hypertensive rats, ACE has been an important mediator of vascular changes. ACE has also been demonstrated in human studies to have an important role in the pathogenesis of white material lesions and lacunar infarcts[6].

Homozygous presence of the deletion polymorphism has been associated with higher plasma ACE activity[7].

The ACE gene polymorphism has been investigated for its possible association with essential hypertension, coronary artery disease, atherosclerosis of the carotid artery and cerebral white matter lesions in patients with essential hypertension, and the findings seem to vary between populations of different genetic and environmental backgrounds[8]. ACE gene polymorphism has also been associated with increased incidence of stroke in some populations, although contradictory results have been reported [(9,10)].

Methods

The patients group who subjected to this study were (60) persons in the age group ranging from 44 - 86 years , the mean ± standard deviation (SD) was (62.3 ± 11.29 years). This group comprised of males (60%), with their age ranging from 44 - 86 years old, the mean ± SD was (61.8 ± 11.4 years) , and females (40%) with age ranging from 45 - 85 years, and mean ± SD was (63.1 ± 11.3 years).

Thirty apparently healthy individuals were taken as a control group of the age ranging from 43 - 82 years, the mean ± standard deviation (SD) was (61.9 ± 10.7 years). This group comprised of males (63%) their age ranging from 46 - 82 years , mean ± SD was (60.4 ± 10.2 years), and females (37%) their age ranging from 43 - 81 years , mean ± SD was (64.5 ± 11.6 years) .

The age and sex of this group were matched to age and sex of patient group, where statistical analysis showed non significant differences in the age and sex between patient and control groups ($p > 0.05$).

For ACE genotyping , a set of primers including (forward primer : 5′ -CTG GAG ACC ACT CCC ATC CTT TCT-3′; and Reverse primer : 5′-GAT GTG GCC ATC ACA TTC GTC AGA T-3′) was used to amplify the gene which produced a DNA segment of 190 bp (in deletion polymorphism) and 490 bp (in insertion polymorphism) [(11, 12)].

PCR optimization was done as a first step by using a gradient temperature ranging from 65 ^{0}Cto 70 ^{0}Cwith 0.3 step differences in PCR wells. This is highly important to determine the optimum annealing temperature. After the determination of optimum annealing temperature (67 ^{o}C), the PCR reaction mixture consisted of 20-50 ng template DNA, 250 mM of each dNTP, 5 µl buffer, 1 U Taq DNA polymerase (Biooneer), 10 pmol of each primer and 30 mM $MgCl_2$ in 20 µl of total reaction volume.

Amplification reactions were carried out by using GTC Series thermocycler (Cleaver Scientific /UK) apparatus.

After determination of the optimum annealing temperature the following program was set in the thermocycler to amplify the target DNA fragments as shown in table (1).

Table (1) Amplification conditions of ACE genotyping

Cycles	Function	Time(min)	Temp.(C^O)	Stage
	Initial denaturation	5	94	1
30	DNA denaturation	1	94	2
	Primer annealing	0.5	67	
	Template elongation	0.5	72	
	Final elongation	5	72	3
Hold	Incubation	-	4	4

In order to distinguish between homozygous and heterozygous deletion polymorphism , another PCR amplification was made using a primers set as follows : (5′-TGG GAC CAC AGC GCC CGC CAC AC-3′ as a forward primer and 5′-TCG CCA GCC CTC CCA TGC CCA TAA-3′ as reverse primer) which produced a DNA segment of 335 bp in heterozygous deletion and no product in homozygous deletion polymorphism .

The annealing temperature (determined by gradient PCR) was (68.5 ^{o}C), with the other conditions of the PCR of the first amplification remain unchanged.

Results

The results of amplification of ACE gene were two alleles : D allele with molecular size of 190 bp and I allele with molecular size of 490 bp , as shown in figure (1) .

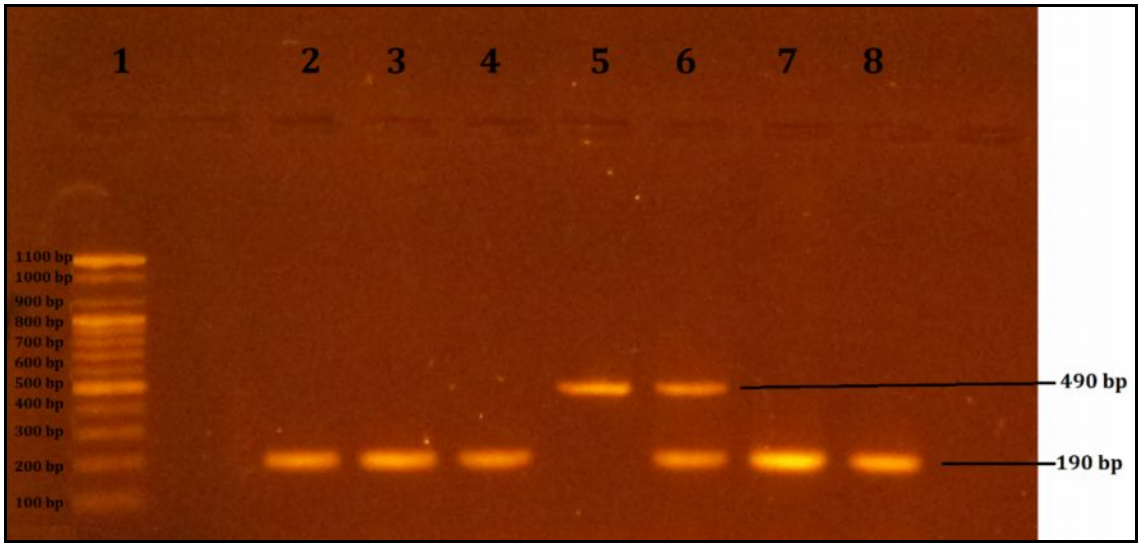

Figure (1) : Electrophoretic picture represents the ACE genotyping , where lane 1 is 100 bp DNA ladder , lane (2,3,4,7,8) have a band at 190 bp representing the D allele , lane 5 has a band at 490 bp representing homozygous I allele and lane 6 has heterozygous DI alleles.

All DD genotypes were re-amplified by using a second primer pair specific for the inserted sequence . Only the I allele produced a 335 bp fragment, while DD homozygotes showed no product, as shown in figure (2).

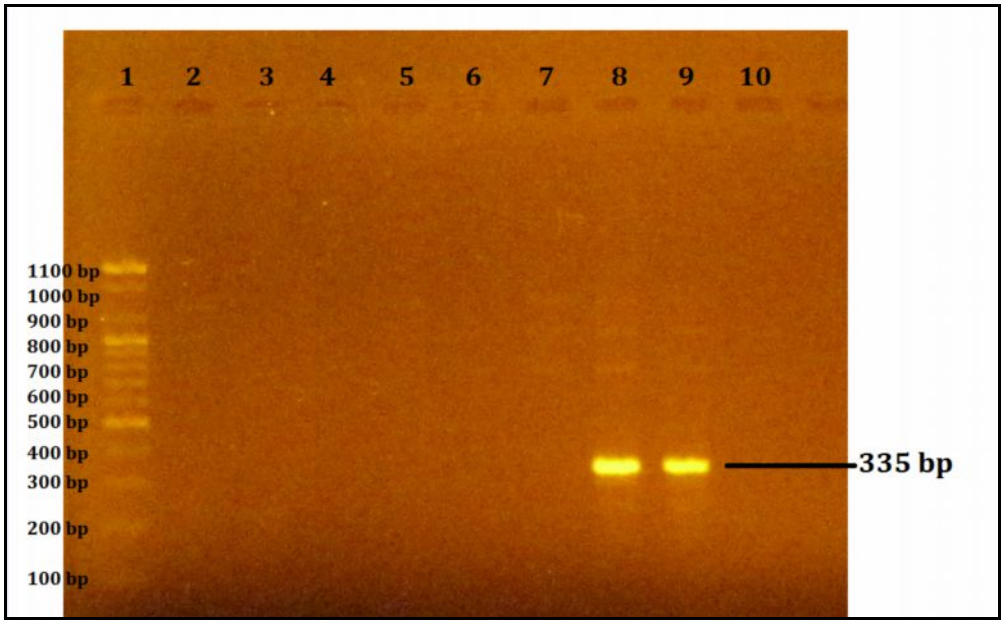

Figure (2) : Electrophoretic picture represents the confirming of homozygosity of DD genotype of ACE gene , where lane 1 is 100 bp DNA ladder , lanes (2,3,4,5) belongs to patients and lanes (6,7,8,9,10,) belongs to control subjects .

Lanes that have no product represent homozygous DD genotype, while those with product at (335 bp) represent heterozygous DI genotype such as lane 8 and 9 .

All subjects are categorized depending on the process of fragmentation of VEGF gene being (DD) for homozygous deletion polymorphism of ACE gene , (DI) for heterozygous polymorphism , and (II) for homozygous insertion polymorphism . Table (2) summarizes genotyping of study subjects according to ACE gene.

Table (2) Genotyping of ACE gene polymorphism with allele frequency .

Group	Genotype			Total	Allele frequency	
	DD	DI	II		D	I
Control	8 (27%)	16 (53%)	6 (20%)	30	53%	47%
Patient	29 (48%)	26 (43%)	5 (9%)	60	70%	30%
Total	37	42	11	90		

From the table above one can tell that heterozygous genotype (DI) is more frequent in control group , while DD is the abundant genotype of the patient group .

In order to evaluate the significance of these results , Chi square test was used to investigate the odds ratio (O.R.) and significance of genotyping and allele frequency , as shown in table (3) .

Table (3) ACE gene polymorphism characterization in ischemic stroke patients and control group .

Genotype	Patient	Control	Odds ratio	CI $_{95\%}$**
DD	29 (48%)	8 (27%)	**4.35***	**1.05 - 18.03**
DI	26 (43%)	16 (53%)	**1.95**	**5.1 - 7.5**
II	5 (9%)	6 (20%)	**Reference group**	
D	70%	53%	**2.7***	**1.16 - 3.7**
I	**30%**	**47%**		

* Significant difference ($P \leq 0.05$).
** CI $_{95\%}$: confidence interval at 95 % level .

Discussion

The DD genotype is known as an independent risk factor in several cardiovascular diseases such as hypertrophic cardiomyopathy[13], myocardial infarction[14]and ventricular hypertrophy[15], as well as chronic renal diseases such as IgA nephropathy[16], diabetic nephropathy[17], renal scarring[18] and congenital urological anomalies[19].

The results of the present study suggest an association between ischemic stroke and the presence of DD genotype and D allele in patient population. The results of prior studies of ACE polymorphism in stroke patients have been consistent with some groups reporting a positive association between the DD genotype and/or D allele and stroke, while others reported to the opposite. Doi*et al*. reported a significant association between the polymorphism of ACE gene and the incidence and mortality rate of ischemic stroke in patients age 60 years or younger in a Japanese population[20].

Also Kostulas*et al.* reported a positive correlation between ACE gene polymorphism and ischaemic cerebrovascular disease[21].

ACE could be involved in the pathogenesis of cerebrovascular disease by several biological mechanisms, including activation of angiotensin I and inactivation of bradykinin, resulting in decreased tissue perfusion, and stimulation of plasminogen activator inhibitor type I [22]. However, the role of ACE either as a direct mediator of or secondary following an acute cerebrovascular event is not fully understood[23].

The plasma ACE concentration is an important factor in increasing the risk of cardiovascular and cerebrovascular diseases, since long exposure to high levels of plasma ACE may result in vascular wall thickness and stiffness[24]. Early studies demonstrated a strong correlation between the D allele and levels of circulating, intracellular, and tissue activity of ACE [25-29]. Since both alleles have co-dominant effects on ACE levels, homozygous DD genotype result in the highest levels of the enzyme, while homozygous II genotype result in the lowest, and heterozygous DI genotype result in an intermediate level.

In present study, we found that variant genotypes of ACE I/D polymorphism were associated with higher stroke risk in Iraqi population.

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