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ORIGINAL ARTICLE

Association of ACE gene I/D polymorphism and ACE levels with hemorrhagic stroke: comparison with ischemic stroke

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Abstract In the present study, we investigated the association of insertion/deletion polymorphism of ACE gene with genetic predisposition to hemorrhagic stroke and also determined the mean ACE activity levels in ischemic and hemorrhagic stroke patients. Two hundred hemorrhagic stroke, 200 ischemic stroke patients and 200 gender and age matched controls were recruited for the study. We found statistically significant difference in the genotypic distribution between hemorrhagic patients and controls for dominant, co-dominant and recessive models. Significant difference was observed in the allelic frequencies between hemorrhagic patients and controls. Multiple logistic regression analysis confirmed these findings [adjusted OR for DD genotype was 2.46 (95 % CI 1.43–4.21) and $p = 0.001$] and [adjusted OR for ID genotype was 5.45 (95 % CI 2.6–10.4) and $p = 0.001$]. We have already established the association of this polymorphism in ischemic stroke patients. Comparing hemorrhagic with ischemic stroke, we found a significant difference in genotypic distribution between the two [for II vs. DD, $\chi^2 = 4.75$; $p = 0.03$, OR = 0.5 (95 % CI 0.27–0.93) and for DD vs. ID, $\chi^2 = 5.1$; $p = 0.02$, OR = 1.8 (95 % CI 1.1–3.3)]. Our results indicate that DD genotype and D allele are important risk factors for the development of stroke. Individuals harboring DD genotype of ACE I/D polymorphism are more predisposed to hemorrhagic stroke than ischemic stroke. Further, the mean ACE activity level was found to be significantly higher in hemorrhagic and ischemic stroke in comparison with controls, but there was no significant difference in the levels found between the two types of stroke.

Keywords Hemorrhagic stroke · Ischemic stroke · Angiotensin converting enzyme · Insertion/deletion polymorphism · Genotypes

Introduction

Hemorrhagic stroke is known to have a mortality rate four times higher than ischemic stroke [1] and both genetic and environmental factors are known to contribute to its progression [2]. This form of brain stroke accounts for 7–27 % of all strokes worldwide [3]. Genetic evidence shows that most stroke cases are clustered within family and the chances of developing hemorrhagic stroke increases by having a first-degree relative with hemorrhagic stroke. Approximately 10 % of patients have been recorded to have a positive family history of hemorrhagic stroke [4]. Previous studies have identified a substantial number of candidate genes, contributing to the risk of hemorrhagic stroke, but due to lack of reproducibility there is uncertainty about the nature and number of genes contributing to the risk of hemorrhagic stroke [5]. Angiotensin converting enzyme (ACE) is a zinc metallopeptidase that converts the inactive decapeptide,

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angiotensin I to the active octapeptide and potent vasoconstrictor angiotensin II [6]. It is the main active product of the rennin angiotensin aldosterone system (RAAS), and plays a vital role in the development of hypertension and cerebrovascular diseases including stroke [7].

The gene encoding ACE has been mapped to 17q 23.3, is 21 kilo bases (kb) long and comprises 26 exons and 25 introns. Rigat [8] in 1990 found the polymorphism involving the presence/insertion or absence/deletion (I/D) of a 287-bp Alu sequence of DNA in intron 16 of the gene that influences the levels of the circulating enzyme. Most of the studies on association of insertion deletion polymorphism of ACE have been focussed on ischemic stroke [9–14] with very few reports on hemorrhagic stroke from different regions of the world and especially from India [15–18]. Nevertheless, the correlation of ACE gene polymorphism with pathogenesis of stroke still remains controversial [19–21]. Thus, a comparative study between the two types of stroke in a particular race/ethnicity could be highly useful in deciphering any genetic level differences in pathogenesis of ischemic stroke and hemorrhagic stroke.

Thus, in the present study, we investigated the association between I/D polymorphism of ACE gene and hemorrhagic stroke in a South Indian population from Andhra Pradesh. The genotypic distribution and allelic frequencies were compared with ischemic stroke patients. In addition to this, the ACE activity was evaluated in ischemic and hemorrhagic stroke patients in comparison with healthy controls.

Materials and methods

Subjects

Two hundred hemorrhagic stroke patients (males:females = 148:52) presenting with new stroke evaluated in the neurology department of Nizam's Institute of Medical Sciences, Hyderabad (AP, India) between June 2006 and June 2013 were enrolled for the study. The study was approved by the ethical committee of the study hospital as well as the institutional ethical committee. All the patients were examined by a qualified stroke neurologist and hemorrhagic stroke was differentiated by computed tomography (CT) scans and magnetic resonance imaging (MRI). All the patients underwent CT scan as well as MRI. Patients with major cardiac, renal, hepatic, endocrinological disorders, skeletal disorders and cancerous diseases were excluded from this study. As a control group, 200 healthy individuals matched for sex and age (males:females = 148:52) were recruited from the same geographic area with no clinical evidence of any cerebrovascular disease. Information on demographic characters and risk factors were collected using a structured questionnaire. Hypertension, alcohol use, diabetes and smoking were defined as reported previously [12]. All the subjects included in the study were above the age group of 18 years and samples were collected only after obtaining their written informed consent. For evaluating the ACE activity the serum samples stored at −80 °C of confirmed ischemic and hemorrhagic stroke patients were used.

DNA isolation and genotyping

Five milliliters of blood was collected in EDTA tubes and genomic DNA was extracted from blood samples using standard phenol–chloroform method. The polymorphism in ACE gene was detected using PCR technique. The primers used for the amplification of the intron 16 polymorphism are forward: 5′-CTGGAGACCACTCCCATCCTTTCT-3′ and reverse: 5′-GATGTGGCCATCACATTCGTCAGAT-3′. The amplified PCR products were separated on 2 % agarose gel. The presence of 190-bp fragments represented the D allele and the presence of 490-bp fragment represented the I allele. However, mistyping of I/D heterozygotes as DD homozygotes may occur due to the preferential amplification of the D allele and inefficient amplification of the I allele. Hence, to increase the specificity of DD genotyping, confirmatory PCR was done with insertion-specific PCR primers forward: 5′-TGGGACCAC AGCGCCCGCCACTAC-3′ and reverse: 5′-TCGCCAG CCCTC CCATGC CCATAA-3′. This reaction yielded a 335-bp amplified product in the presence of I allele and no product in samples having DD genotype. In addition to this, the polymorphism was confirmed by subjecting 40 % of samples to sequencing.

ACE activity

For estimation of ACE levels, 3 ml of blood was collected in clot activators for serum separation. ACE activity was determined by a semi-auto analyser (ERBA, Chem-7, Transasia Biomedicals Ltd, Ringanwada, Daman, India) using a kit provided by Trinity Biotech (IDA, Business Park, Bray, Ireland). Here, the substrate was *N*-(3-(2-furylacryloyl)-L-phenyl alanyl glycylglycine (FAPGG), which was hydrolysed to furylacrylolphenyl alanine and glycylglycine. Hydrolysis of FAPGG resulted in decreased absorbance at 340 nm. The ACE activity in the samples was determined by comparing the sample reaction rate to that obtained with the ACE calibrator.

Statistical analysis

Hardy–Weinberg equilibrium was tested for the ACE gene polymorphism and association between genotypes and

hemorrhagic stroke was examined by odds ratio with 95 % confidence interval (CI) and Chi-square analysis using Open EPI6 software (Open Epi Version 2.3.1 from Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA). Allelic frequencies were calculated according to the number of different alleles observed and the total number of alleles examined. A stepwise multiple logistic regression (MLR) analysis was performed using SPSS18. The independent variables were decoded as the following dummy variables: genotype, 0 for II genotype, 1 for ID genotype and 2 for DD genotype; groups; sex, 1 for male and 2 for female; smoking 1 for smokers and 2 for non-smokers; alcoholism 1 for alcoholics and 2 for non-alcoholics; hypertension, 1 for hypertension and 2 for normotension; for diabetes 1 for presence and 2 for absence, for stroke family history 1 for presence of history and 2 for absence of history; for total cholesterol 1 for normal levels and 2 for high levels; for HDL 1 normal levels and 2 for high levels; for LDL 1 for normal levels and 2 for high levels; for TG 1 for normal and 2 for high levels; for VLDL 1 for normal levels and 2 for high levels. Statistical significance was defined as $p < 0.05$.

Results

Two hundred hemorrhagic stroke patients and 200 controls from the same demographic area were recruited for the study and all the subjects were of South Indian origin, from Andhra Pradesh, India. The demographic and clinical data of the hemorrhagic stroke patients and controls have been summarized in Table 1. The mean age of hemorrhagic stroke patients was 54.2 years and that for controls was 54.9 years. Profiles of patients for the various risk factors revealed hypertension in 56 %, diabetes in 33 %, smoking in 37 %, alcohol use in 41 % and family history of stroke in 5 % of patients. In the control group 40 % had hypertension, 28 % were diabetic, 30 % smokers, 29 % were alcoholic and only 1 % had family history of stroke. Stroke patients also had higher blood pressure and higher serum triglycerides ($p < 0.05$).

The ACE genotypes and allelic frequencies observed in hemorrhagic stroke patients have been given in Table 2. The frequency of the DD genotype as well as D allele was high among the hemorrhagic patients as compared to controls (Table 2). The DD genotype and D allele associated significantly with the disease using dominant, recessive and co-dominant genotype models (Table 3). The results were further confirmed by carrying out a stepwise MLR analysis. The DD and ID genotype were significantly associated with the disease. For DD genotype [adjusted odds ratio = 2.46, 95 % CI (1.43–4.21); $p = 0.00$] and for ID genotype [adjusted odds ratio = 5.45, 95 % CI (2.59–10.44); $p = 0.001$]. We have already established the role of ACE I/D polymorphism in the development of ischemic stroke in this ethnic group [12]. Therefore, we compared the genotypic distribution and allelic frequencies of hemorrhagic stroke with ischemic stroke group (already published). A significant difference was observed in the genotypic distribution between the two groups (Tables 4, 5). The frequency of DD genotype was very high in hemorrhagic stroke in comparison with ischemic stroke patients.

Table 1 Demographic features of study population

Characteristics	Hemorrhagic stroke patients ($n = 200$)	Control subjects ($n = 200$)	p value
Mean age	54.2 (3.7)	54.9 (1.65)	
Male:female	148:52	148:52	
Hypertension	112 (56 %)	80 (40 %)	<0.01
TC (mg/dl)	179.5 (39.33)	174.1 (41.63)	NS
HDL (mg/dl)	53.5 (18.7)	80.9 (21.07)	NS
TG (mg/dl)	125.4 (68.9)	78.2 (32.89)	<0.01
Diabetics	66 (33 %)	56 (28 %)	<0.01
Smokers	74 (37 %)	60 (30 %)	<0.01
Alcohol users	82 (41 %)	58 (29 %)	<0.01
Family history of stroke	10 (5 %)	2 (1 %)	<0.01
Family history of hypertension	68 (34 %)	42 (21 %)	<0.01

TC total cholesterol, *HDL* high-density lipoprotein, *TG* triglycerides, *NS* nonsignificant

The mean ACE activity level of ischemic stroke patients, hemorrhagic stroke patients and controls was found to be 52.8 ± 3.2, 53.2 ± 4.5 and 42.6 ± 2.9 u/L, respectively. There was a significant difference in the ACE activity between ischemic stroke patients and controls ($p < 0.001$) and also between hemorrhagic stroke patients and controls ($p < 0.001$). However, there was no significant difference in ACE levels between the two types of stroke ($p = 0.30$).

Discussion

Angiotensin converting enzyme gene, a component of rennin angiotensin aldosterone system (RAAS) has been proposed as an independent genetic factor for hypertension and other cardiovascular diseases [14, 22]. ACE converts angiotensin I to angiotensin II and thus produces arteriolar constriction and a rise in systolic and diastolic blood pressure [8]. Hypertension has been established as the major risk factor for hemorrhagic stroke [23]. Several

Table 2 Distribution of ACE genotypes and allele frequencies of study population

Group	ACE genotypes				Allele frequencies		
	II	ID	DD	Total	I	D	Total
Patients, *n* (%)	49 (24.5 %)	99 (49.5 %)	52 (26 %)	200	197 (0.49)	203 (0.51)	400
Control, *n* (%)	112 (56 %)	70 (35 %)	18 (9 %)	200	294 (0.74)	106 (0.26)	400

studies have reported the association of I/D polymorphism with ischemic stroke and hemorrhagic stroke. However, these results may vary depending upon the ethnicity of the study population [24].

Ariesen et al. [25] have found a positive association between hypertension and intracerebral hemorrhage (ICH). Studies revealing association of ACE (I/D) polymorphism and hemorrhagic stroke are very few. However, the contribution of ACE in the development of hemorrhagic stroke cannot be ignored. In the present study I/D polymorphism within intron 16 of ACE gene (i.e., DD genotype) was found to be significantly associated with hemorrhagic stroke, suggesting a possible role of D allele in predisposing individuals to hemorrhagic stroke.

In a recent meta-analysis carried out by Sun et al. [26], including 805 cases and 1,641 controls from 8 case–control studies including both Asians and Caucasians revealed that the DD homozygote carriers among Asians are more prone to the increased risk of hemorrhagic stroke as compared to Caucasian subjects. This study suggested the dominant genetic model of the polymorphic variant I/D to be associated with 58 % increase in susceptibility to ICH. Recently in a meta-analysis carried out by Huang et al. [27] including 744 cases and 1,411 controls suggested that ICH cases have significantly lower frequency of II genotype among the Asians.

In our study also hemorrhagic stroke subjects were found to have a significantly higher frequency of DD and ID genotypes in comparison with controls and the frequency of II genotype was low in the hemorrhagic stroke patients. This is in agreement with a previous study from North India, where the ACE DD genotype was reported to contribute 7.4 times higher risk of ICH [15]. Moreover, cardiovascular diseases are also known to be affected by ACE I/D polymorphism which has been well established among Asians and Caucasians by a recent meta-analysis carried out by Chen et al. [28]. However, the limitation of the present study is that we did not have information about the cardiovascular state of the subjects involved. Since cardiovascular and cerebrovascular diseases have similar pathophysiology, we could have better understood the phenotypic effects of ACE I/D polymorphism and the intricate play between cardiovascular and cerebrovascular system if we had information about the cardiovascular state of all the study subjects too.

The D allele of ACE gene has also been reported to be associated with 28–47 % of the variance of ACE activity in both circulation and in tissues [8, 29, 30], but it has also been suggested that circulating ACE level is unrelated to intracerebral ACE activity [31]. In our study, we observed a significantly higher level of ACE activity in patients in comparison with the controls, but not between the two types of stroke. High levels of plasma ACE is known to increase vascular wall thickness and stiffness which leads to cerebrovascular risk [32]. Further higher ACE activity leads to high amount of vasoconstrictor angiotensin II which results in smooth muscle arteriolar proliferation, death of smooth muscle cell wall and collagen deposition

Table 3 Analysis of ACE genotypes and alleles among hemorrhagic stroke patients and controls

Genotype	OR (95 % CI)	χ^2	*p* value
II vs. DD	0.15 (0.08–0.28)	37.9	<0.01
II vs. ID	0.31 (0.19–0.48)	26.3	<0.01
DD vs. ID	6.6 (3.51–12.43)	37.9	<0.01
II vs. ID + DD	0.26 (0.17–0.4)	41.2	<0.01
Dominant			
ID vs. II + DD	1.82 (1.21–2.72)	8.6	<0.003
Co-dominant			
DD vs. II + ID	3.6 (1.99–6.33)	19.9	<0.01
Recessive			
D vs. I	2.9 (2.12–3.84)	49.5	<0.01
I vs. D	0.35 (0.26–0.47)	49.5	<0.01

OR odds ratio, *CI* confidence interval

Table 4 Distribution of ACE genotypes and allele frequencies in hemorrhagic and ischemic stroke group

Study group	ACE genotypes				Allele frequencies		
	II	ID	DD	Total	I	D	Total
Hemorrhagic group, *n* (%)	49 (24.5 %)	99 (49.5 %)	52 (26 %)	200	197 (0.49)	203 (0.51)	400
Ischemic group, *n* (%)	47 (29 %)	90 (56 %)	25 (15 %)	162	184 (0.57)	140 (0.43)	324

Table 5 Comparison of ACE genotypes between hemorrhagic and ischemic stroke group

Genotype	OR (95 % CI)	χ^2	*p* value
II vs. DD	0.5 (0.27–0.93)	4.75	0.029
II vs. ID	0.95 (0.58–1.5)	0.04	0.83
DD vs. ID	1.9 (1.1–3.3)	5.1	0.02

[33, 34]. These changes result in hypertension, worsen atherosclerosis and eventually contribute to ICH [32].

The difference in ACE activity between patients and controls might also be on account of therapeutic differences in use of ACE-inhibitors, AT1 antagonists and diuretics since there were hypertensive subjects in all the groups and hypertension is a major clinical factor known to substantially alter ACE levels. It would have been interesting to study the effects of therapeutics which alter the ACE activity levels. However, the lack of data on therapeutic profile of study subjects is a limitation of the study. Additionally, angiotensin II acts via its specific receptor subtypes (AT1, AT2) and AT2 is known to antagonize actions of AT1 [35]. Moreover, local tissue rennin angiotensin system (RAS) is expressed on different tissues including brain and is far complex than circulating RAS which is influenced not only by angiotensin II, but also by the activities of receptors and other products of angiotensinogen metabolism that mostly exerts opposite effects to Ang II action [36].

The association between intron 16 ACE I/D polymorphism and the development of ICH has already been established in Asians and Caucasians [16, 17, 37–39]. However, in contrast to these studies, findings by Dardiotis et al. [18], were unable to resolve the effect of the ACE gene with ICH in two independent ethnically different cohorts. The discrepancies in these results might be on account of ethnic differences. Additionally, findings by Chen et.al (2008), which used a haplotype-based analysis in ACE gene variants, i.e., A −240T and Alu I/D reported T-D haplotype to be associated with spontaneous deep intracerebral hemorrhage (SDICH) only in female patients. The study also reported the major role and minor role played by the environmental risk factors and ACE polymorphisms, respectively, in progression of SDICH [38]. We have already reported a significant association of ACE I/D polymorphism with ischemic stroke and its subtypes [12]. In the present study, we compared the ACE polymorphism in hemorrhagic stroke. The DD genotype was found to be significantly high in hemorrhagic stroke patients in comparison with ischemic stroke.

Therefore, in conclusion the present study shows that genetic heterogeneity of ACE (I/D) gene is significantly associated with risk of hemorrhagic stroke in South Indian Population from Andhra Pradesh. Further, the frequency of DD genotype was significantly high in hemorrhagic stroke group. This suggests that individuals bearing DD genotype are at a higher risk of hemorrhagic stroke in comparison with ischemic stroke. Significantly higher ACE activity was observed in hemorrhagic stroke and ischemic stroke patients in comparison with healthy controls. However, there was no significant difference in the ACE activity between the two types of stroke. Identifying ACE I/D polymorphism might help for a better understanding of the pathophysiology underlying hemorrhagic stroke and would permit the development of therapeutic strategies for the management of stroke.

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