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Abstract

We examined the association of the NG011648 polymorphism (insertion/deletion) of the angiotensin-converting enzyme (ACE) gene with ischaemic stroke occurrence, subtype of ischaemic stroke and ischaemic stroke patients' gender. Patients with first ever ischaemic stroke were recruited prospectively in a period of 18 months. Controls were matched with the patients for age, gender, and known risk factors for stroke. Demographic data, medical history, and vascular risk factors were collected. Genotypes were determined by polymerase chain reaction (PCR) and restriction enzyme analysis. Stroke and control groups were compared in regard to the prevalence of the NG011648 polymorphism. One hundred and seventy-six patients with ischaemic stroke and 178 controls were recruited and genotyped for NG011648 polymorphism (I/D) of the ACE gene. No significant difference in allele and genotype distributions emerged between control and patient groups, nor in the two subtype groups of lacunars and large artery atherosclerosis. After the data were stratified by gender, a low incidence of II homozygosity in female patients versus female controls ($p = 0.05$) and male patients ($p = 0.013$, Z score: -2.49) was found. Our results indicate that I/D polymorphisms may have a role in stroke onset, in respect to gender, with a possible favourable effect of II genotype in females.

Keywords

ACE, gender dimorphism, genes, ID polymorphism, stroke.

Introduction

Brain infarction is a multifactorial disorder, influenced by both environmental and genetic factors. In addition to recognised risk factors such as hypertension, dyslipidaemia, diabetes, smoking, obesity and advanced age, many studies are in favour of a genetic component in stroke, since there are higher concordance rates in monozygotic than in dizygotic twins, ethnic differences and familiar aggregation of stroke.[1,2] Genetic factors may act by predisposing or modulating the effect of risk factors such as hypertension.

The renin–angiotensin system (RAS) has a central role in hypertension and atherosclerosis.[3] Angiotensin-1 converting enzyme (ACE) is the rate-limiting enzyme of RAS, involved in vascular remodelling and atherosclerosis.[4,5] It catalyses the conversion of inactive angiotensin I to active angiotensin II, which induces vasoconstriction, and inactivates bradykinin. Angiotensin II and bradykinin are the two peptides involved in the modulation of vascular tone and the proliferation of smooth muscle cells.[6] The role of genetic polymorphisms in the function of RAS has recently been recognised.[5,6]

Plasma ACE concentration is an important factor in cardiovascular and cerebrovascular risk profiling, since chronic exposure to high levels of plasma ACE may result in vascular wall thickness and stiffness.[7] A single copy gene, encoding ACE, is located on chromosome 17q (rs NG011648) and has two alleles, depending on the insertion (I) or the deletion (D) of 287 bp Alu sequence in intron 16. This allelic variation is known to determine 47% of the variance of plasma ACE.[8]

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Regarding cerebrovascular disease, ACE gene I/D polymorphism has been investigated in association with stroke, with magnetic resonance abnormalities in the brain and with the plaque formation in the carotid artery.[5,7,9-13] There are studies that demonstrate a positive association of the DD genotype or D allele with ischaemic stroke, stroke severity, lacunars or increased intima media thickness.[5,7,9,11-13]

The aim of the study was to evaluate the association between NG011648 polymorphism (I/D polymorphism) of ACE gene with ischaemic stroke occurrence, subtype of ischaemic stroke and ischaemic stroke patients' gender.

Subjects and methods

Study participants

Following approval of the study protocol by the Institutional Review Committee and in compliance with the Helsinki Declaration, we recruited consecutively all the patients who were hospitalised with first ever ischaemic stroke within a period of 18 months, from December 2006 to May 2008, in the Stroke Reference Centre of Northwest Greece**,** a well-defined area with a homogeneous population and limited recent immigration.

An informed consent was read and signed by eligible subjects. An experienced neurologist carried out neurological assessment at admission and stroke was defined according to the WHO definition and confirmed by brain imaging showing a recent corresponding brain infarct. All patients underwent a brain Computerised Tomography (CT) at the acute phase, and ischaemic lesions not shown by CT scan at the acute phase were revealed by Magnetic Resonance Imaging (MRI), performed within 15 days from the onset of stroke.

Additionally to CT scan at the acute phase and MRI when needed, further evaluation included triplex imaging or CT angiography of extracranial or/and intracranial arteries, ECG, echocardiography (transthoracic or transoesophageal) and assessment of prothrombotic syndromes and autoimmune disorders, according to patient-specific clinical criteria.

Stroke subtype classification was based on TOAST (Trial of ORG 10172 in Acute Stroke Treatment)[14] criteria. Patients recruited in the study were stroke patients with small artery occlusions, known as lacunars (small, subcortical, hypodense lesions with a diameter less than 15 mm and appropriate clinical lacunar syndrome) and patients with large artery atherosclerosis (hypodense lesions with a diameter > 15 mm and > 50% stenosis in the appropriate intracranial or extracranial artery). Patients with known coronary artery disease**,** cardioembolic stroke patients, stroke patients of other specific causes and stroke patients of undetermined cause were excluded in order to make the study group more homogeneous.

The control group comprised subjects with no stroke or history of stroke and normal CT brain scan referred to the neurology outpatient clinic for reasons unrelated to stroke. A standard neurological examination documented the absence of stroke and a standardised questionnaire assessed the absence of previous ischaemic cerebrovascular events. The controls were unrelated to study patients, and matched to the study group for gender, age and known risk factors for stroke (hypertension, dyslipidaemia, diabetes mellitus and smoking).

Demographic characteristics, biochemical profile and established risk factors for stroke were recorded for both patients and controls at inclusion. Arterial hypertension was defined as present if it was clearly documented (>140/90 mmHg) or under treatment. Hypercholesterolaemia was considered present if fasting cholesterol levels were found > 220 mg/dl, or treated with lipid regulating drugs. Diabetes mellitus was noted if fasting glucose > 126 mg/dl or if there was current use of antidiabetic medication. Smoking was recorded as current if active or ceased within the last 6 months.

Whole blood samples from both patient and control groups were used for isolation of peripheral blood leukocytes for the genetic analysis.

Genetic analyses

Whole blood samples from both patients and controls were used for isolation of peripheral blood leukocytes for genetic analysis. Genomic deoxyribonucleic acid (DNA) from each individual was isolated using a standard NaCl extraction procedure. The DNA samples were genotyped for I/D polymorphism of ACE gene according to Rigat *et al.*[15] The forward and reverse primers were respectively: 5′- CTGGAGACCACTCCCATCCTTCTC -3′ and 5′- GATGTGGCCATCACATTCGTCAGAT-3′.

The final products, one of 490 bp with the insert (I allele) and one of 190 bp without the insert (D allele), were separated on 2% agarose gel. All samples were run in duplicates with positive controls, negative controls and blanks. The resulting genotypes were DD, ID and II. Regarding alleles, we grouped the subjects on the basis of carrying D allele or I allele of the ACE gene (those carrying the ID or DD genotype *vs.* carrying II genotype, and those carrying the ID or II genotype *vs.* carrying the DD genotype, respectively).

Statistical analyses

Binary data were described as percentages while continuous data were expressed as the mean ± standard deviation (SD). We calculated the proportion for each genotype, that is, DD, ID, and II, as well as the proportion for each allele,

that is, D, and I, in cases and in controls. Chi-square test was used to test the agreement of genotype frequencies with Hardy–Weinberg equilibrium expectations.

According to the study design, we evaluated possible differences in homozygous genotypes, either DD or II, and allele proportions between cases and controls. It was also evaluated whether the type of stroke was related to either of the homozygous genotypes (DD or II) or either of the alleles. We calculated the odds ratio (OR) and 95% confidence interval (CI) for the following comparisons: DD versus (ID + II), and II versus (ID +DD). We also calculated the OR with 95% CI for the comparisons between the alleles, that is, D versus I.

We also performed separate analyses to investigate whether gender may act as an effect modifier on the potential relationship between the ACE gene polymorphism and stroke. For each of the genotype and allele comparisons [DD *vs.* (ID + II); II *vs.* (ID +DD); D *vs.* I], we calculated the OR with CI for female participants and for male participants separately. To evaluate whether the OR for each comparison differs significantly between female and male participants, we calculated the *Z* score as a ratio where the nominator is the difference of normalised effects and the denominator is the square root of the variance of the difference.

We also analysed any association of the genotypes and alleles with hypertension and performed regression analysis to identify any associations and interactions between hypertension, the polymorphism and the gender on the occurrence of stroke.

We used the statistical software StatXact 3.0 to conduct the analysis. *p*-values for *Z* scores were calculated in Intercooled Stata, version 8.2 (Stata Corp., College Station, TX, USA). All *p*-values were two tailed. We considered as statistically significant a p-value < 0.05.

Results

One hundred and seventy-six patients, 116 males and 60 females, and 178 controls, 118 males and 60 females, were finally recruited into the study. Patients' mean age was 58.6 ± 9.2 years (age range: from 40 to 70 years)**.** The mean age of controls was 57.9 ± 8.6 years.

According to stroke subtype, the lacunar group comprised 87 (49.4%) patients, whereas the large artery atherosclerosis group included 89 patients (50.6%) (table 1). Stroke patients and control subjects did not differ in age, gender distribution and conventional vascular risk factors, as shown in table 1.

Genotype frequencies were in Hardy–Weinberg equilibrium ($p > 0.05$) in both the patient group and the control group.

Statistical analysis included all stroke patients together in comparison with controls and each of the two stroke subtypes separately. There was no difference in genotypes and alleles distribution between all stroke patients and controls (table 2). Similarly, there was no association in genotypes and alleles between lacunar stroke patients and controls, nor between large artery atherothrombosis stroke patients and controls (tables 3 and 4).

Table 1. Characteristics of patients and controls

Data	Cases $N = 176$	Controls $N = 178$
Male, *n* (%)	116 (65.9)	118 (66.3)
Female, *n* (%)	60 (34.1)	60 (33.7)
Age, mean (± SD)	58.6 (9.2)	57.9 (8.6)
Hypertension, *n* (%)	144 (81.8)	130 (73.0)
Hypercholesterolaemia, *n* (%)	111 (63.0)	99 (55.6)
Diabetes mellitus, *n* (%)	57 (32.3)	47 (22.4)
Smoking, *n* (%)	74 (42.0)	65 (36.5)
Small artery occlusion, *n* (%)	87 (49.4)	–
Large artery atherosclerosis, *n* (%)	89 (50.6)	–

Table 2. Genotype and allele distribution in patients and controls

Genotypes Alleles	All cases *n* (%) $N = 176$	Controls *n* (%) $N = 178$	OR (95% CI)	*p*-value
DD *vs.* (ID + II)				
DD	61 (34.7)	59 (33.1)	1.07	0.7
ID + II	115 (65.3)	119 (66.9)	0.69–1.66	
II *vs.* (ID + DD)				
II	29 (16.4)	29 (16.3)	1.04	0.9
ID + DD	147 (83.6)	149 (83.7)	0.57–1.78	
Alleles (D *vs.* I)				
D	208 (59.1)	208 (58.4)	1.02	0.8
I	144 (40.9)	148 (41.6)	0.76–1.38	

Analysing separately the two genders, the D allele and the DD genotype tended to be more frequent in female cases and I allele was more frequent in males but these differences were not statistically significant (table 5). Likewise, no significant association was found regarding the two alleles and the DD genotype. Some noteworthy, statistically significant findings emerged when correlations were made for the II homozygosity. II genotype was less frequent in female patients compared with female controls ($p = 0.05$) and there was a statistically significant difference between female and male patients, since the relative *p*-value for the relative OR between female and male patients was 0.013, for *Z* score: -2.49 (table 5).

Regarding hypertension, no statistically significant difference emerged when cases with hypertension were compared with cases without hypertension (table 6). Further regression analysis between hypertension, genotypes, alleles and genders on the occurrence of stroke did not disclose any further association or interaction.

Table 3. Genotype and allele distribution in lacunar (small vessel disease) cases in comparison with controls

Genotypes Alleles	Lacunar cases *n* (%) *N* = 86	Controls *n* (%) *N* = 178	OR (95% CI)	*p*-value
DD *vs.* (ID + II)				
DD	31 (36.0)	69 (38.8)	0.89 (0.50, 1.57)	0.7
ID + II	55 (64.0)	109 (61.2)		
II *vs.* (ID + DD)				
II	14 (16.3)	28 (15.7)	1.04 (0.48, 2.19)	1.00
ID + DD	72 (83.7)	150 (84.3)		
Alleles (D *vs.* I)				
D	103 (59.9)	219 (61.5)	0.93 (0.63, 1.38)	0.8
I	69 (40.1)	137 (38.5)		

Table 4. Genotype and allele distribution in large artery atherosclerosis cases in comparison with controls

Genotypes Alleles	Large artery atherosclerosis cases *n* (%) *N* = 90	Controls *n* (%) *N* = 178	OR (95% CI)	*p*-value
DD *vs.* (ID + II)				
DD	30 (33.4)	69 (38.8)	0.79 (0.45, 1.39)	0.4
ID + II	60 (66.6)	109 (61.2)		
II *vs.* (ID + DD)				
II	15 (16.6)	28 (15.7)	1.07 (0.50, 2.22)	0.9
ID + DD	75 (83.4)	150 (84.3)		
Alleles (D *vs.* I)				
D	105 (58.3)	219 (61.5)	0.88 (0.60, 1.29)	0.5
I	75 (41.7)	137 (38.5)		

Table 5. Genotypes and alleles distribution in respect to gender

Genotypes Alleles	Female cases *n* (%) *N* = 60	Female controls (*N* = 60)	OR (95% CI)	*p*-value	Male cases *n* (%) (*N* = 116)	Male controls (*N* = 118)	OR (95% CI)	*p*-value
DD *vs.* (ID + II)								
DD	26 (43.3)	20 (33.3)	1.53 (0.68, 3.43)	0.35	35 (30.2)	39 (33.0)	0.88 (0.49, 1.58)	0.7
ID + II	34 (56.7)	40 (66.7)			81 (69.8)	79 (97.0)		
II *vs.* (ID + DD)*								
II	2 (3.0)	9 (15.0)	0.20 (0.02, 1.02)	0.05	27 (23.3)	20 (16.9)	1.92 (0.95, 3.91)	0.07
ID + DD	58 (97.0)	51 (85.0)			69 (76.7)	98 (83.1)		
Alleles (D *vs.* I)								
D allele	84 (70.0)	71 (59.0)	1.61 (0.91, 2.85)	0.11	124 (53.4)	137 (58.0)	0.83 (0.57, 1.22)	0.3
I allele	36 (30.0)	49 (41.0)			108 (46.6)	99 (42.0)		

*p-value = 0.013 for the relative OR between females and males, for Z score: -2.49

Table 6. Genotype and allele distribution in cases with and without hypertension

Genotypes Alleles	Hypertension *n* (%) *N* = 144	No hypertension *n* (%) *N* = 32	OR (95% CI)	*p*-value
DD *vs.* (ID + II)				
DD	50 (34.7)	11 (34.4)		
ID + II	94 (65.3)	21 (65.6)	1.2 (0.53–2.74)	0.8
II *vs.* (ID + DD)				
II	26 (18.0)	3 (9.3)		
ID + DD	118 (82.0)	29 (90.6)	1.2 (0.45–3.28)	0.9
Alleles (D *vs.* I)				
D	168 (58.3)	40 (62.5)		
I	120 (41.7)	24 (37.5)	1.15 (0.67–1.99)	0.7

Discussion

In the present study we evaluated the distribution of ID polymorphism of ACE in acute ischaemic stroke patients and controls, as well as in different groups of lacunars and large artery atherosclerosis subtypes and patients' gender, without finding any association of the polymorphism with stroke occurrence and stroke subtype. No difference in the proportion for each genotype and allele was disclosed between patients with and without hypertension and no associations and interactions between hypertension, the polymorphism and the gender on the occurrence of stroke was found.

There are studies demonstrating negative results[16-19] regarding the association of I/D polymorphism with stroke, with possible reasons being the different selection criteria of patients, the different criteria of stroke, the racial differences and the absence of gender-related comparisons.

Although our study was in accordance with these negative studies,[16-19] regarding the frequency of alleles and genotypes in patient and control groups in general, important findings surfaced when the population was analysed for gender. More specifically, females with stroke more rarely had the II genotype ($p = 0.013$) and I allele and more frequently the DD genotype and the D allele.

The RAS genes have been shown to influence ACE expression and affect blood pressure phenotypes and vascular remodelling.[1,20,21] Recent studies demonstrate that the RAS genotypes' influence on the ACE plasma concentration and the molar ratio of Ang II to Ang-(1–7) has a gender dimorphism.[22-24] This sexually dimorphic trait provides evidence to explain why gene regulation of RAS, ACE concentration and circulating levels of angiotensin II are affected by the gonadal steroids testosterone and oestrogen in reproductive age and oestrogen replacement therapy in older females.[22,24,25]

There is evidence that allele I of ACE gene is associated with lower ACE levels, whereas D allele is associated with increased ACE activity.[26,27] Few studies demonstrate gender-related comparisons with genes, regarding multifactorial disorders as stroke, and refer to oestrogen receptor genes.[28,29] Regarding ACE gene, a limited number of studies demonstrate certain differences regarding gender, such as the higher levels of Ang-I and Ang-II in DD females in a series of healthy individuals[22] and the higher levels of renin and prorenin levels in DD women.[24] In this second study, the renin and prorenin levels were higher in males compared with females, independently of genotypes and alleles. This finding implies that the D allele and DD genotype, responsible for high ACE concentrations in women, lose strength as a causative factor in male patients, whose levels are independent of genotypes.[24] Furthermore, in another study, there was a favourable effect of D allele in males (OR = 0.53, CI 95% = 0.29–0.97, $p = 0.04$) but not in females with venous thromboembolism,[30] whereas in a ramipril efficacy study, ACE inhibition was renoprotective in males with the DD genotype.[31]

This gender dimorphism was confirmed in our study, showing a trend of unfavourable effect of D allele and DD genotype only in female patients and not in males, in accordance with the aforementioned studies and a statistically significant lower incidence of II genotype in female patients. This low II genotype incidence implies that there is a favourable effect of this genotype only in females. This favourable effect of II genotype in women was described in a postmenopausal healthy female athletes group, where II genotype positive female athletes had ~25% greater cardiac output than ACE DD genotype female athletes.[32]

The relationship between stroke and the I/D polymorphism is still being debated. The conflicting results of the studies may be explained by the absence of gender-related associations in most of them. Certain genotypes may exert an influence on disorders, as stroke, which can be seen only when the population is stratified according to gender.

Although the small number of patients, the small number of females and the absence of ACE measurement limit the value of our findings, our results indicate that I/D polymorphisms may have a role in stroke development, as far as gender is concerned. This is the first, to our knowledge, study to demonstrate gender-related findings in stroke, regarding I/D polymorphism. The strength of our study is that it has been conducted in a well-defined area with a homogeneous population.

In conclusion, we found a statistically significant decrease in the incidence of II genotype in females with stroke compared with males with stroke, whereas the DD genotype (and the D allele) was only moderately more common among females with stroke compared with males with stroke. Large-scale multicentre controlled prospective studies are warranted to further explore the effects of ACE polymorphisms on gender susceptibility to stroke.

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Conflict of interest

The authors report no potential conflicts of interest.

Author contributions

Study concept and design: SMark, SG, IG. Acquisition of data: SMark, CK, IB, SMara. Analysis and interpretation of data: SMark, CK, IG, SMara, AT. Drafting of the manuscript: SMark, SG. Critical revision of the manuscript for important intellectual content: SG, IG, AK. Statistical analysis: SMark, AT. Study supervision: IG, AK.

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