ANGIOTENSIN I - CONVERTING ENZYME GENE POLYMORPHISM AND ACTIVITY IN PATIENTS WITH ISCHEMIC STROKE

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Abstract

The possible association of ACE polymorphism with ischemic stroke (IS) was evaluated in 65 patients with IS and 330 age and BMI-matched controls. ACE genotypes were determined by polymerase chain reaction (PCR). There was no significant difference in ACE genotype/allele frequencies between case and control group (p>0.05). Patients with D allele had 4.7 times higher risk for large vessel IS than healthy persons D allele possessors. Persons with D allele had 9.2 times higher risk for large vessel disease than small vessel disease. These data suggest a possible association of ACE gene polymorphism with pathogenesis of large vessel IS.

INTRODUCTION

Stroke is a complex, multifactorial disease. It is one of the leading causes of disability and death. About 80% of all strokes are ischemic and have a polygenic basis. Advances in genetic epidemiology have revealed that some genetic variants increase the risk for IS. Evidence suggests that genetic variation in the rennin angiotensin-aldosterone system (RAAS) contributes to the risk of ischemic stroke. Among the various sequence variations in RAAS, the insertion/deletion (I/D) polymorphism in angiotensin I- converting enzyme (ACE) is the most extensively studied.

ACE is a dipeptidyl carboxylase that converts angiotensin I into the antinatriuretic vasoactive angiotensin II, an octapeptide involved in vasoconstriction, aldosterone production, and norepinephrine release from sympathetic nerve endings and inactivates bradykinin, a vasodilator and natriuretic substance. ACE is encoded by a 21-kb 26 exons gene located on chromosome 17 at q23. A deletion polymorphism in the ACE gene consists of the absence of a 287-bp Alu repetitive sequence in reverse orientation near the 3’ end of intron 16. Mean ACE activity concentration in DD carriers are around twice those found in II carriers (1, 2).
The purpose of this study was: a) to report the distribution of ACE genotypes and alleles in patients with ischemic stroke (IS), b) to investigate the impact of ACE polymorphism on serum lipid and apolipoprotein concentrations, as well as on ACE activity and arterial blood pressure level.

**MATERIALS AND METHODS**

Blood samples were obtained from 65 unrelated subjects with acute IS (mean age 41). These were proven by computed tomography or magnetic resonance of the brain. Acute stroke patients were separated into two groups: one with large vessel disease (45 patients) and the other with small vessel disease (lacunar stroke) (20 patients). The age and BMI-matched control group consisted of 330 unrelated Serbian people. They did not show any signs of cerebrovascular disease from their health questionnaires and clinical examinations. Informed consent was obtained from each participant in the study; and all procedures were in accordance with the Helsinki Declaration.

DNA was extracted by Triton X-100 lysis, proteinase K digestion and phenol/chloroform extraction. The reaction pertaining to ACE was performed with 20 pmol of the sense strand-specific primer SA66, 7 pmol of the anti-sense strand-specific primer 167, and 1.5 pmol of another I allele sense strand specific primer ACE3, as previously described (3). Amplification products pertaining to I/D polymorphism were separated by electrophoresis in 1.8 % (w/V) agarose gels. Alleles were identified according to their expected lengths. All gels were visualized and analyzed by GDS8000 gel documentation system (Ultra Violet Products Inc, Upland). ACE activity was determined in serum samples using commercial test (Trinity, Biotech). Lipid and apolipoprotein concentrations were determined by standard methods.

**STATISTICAL ANALYSIS**

Results are presented as mean ± S.D. for continuous normally distributed variables, and as percent for categorical data. Comparisons between normally distributed continuous variables were performed by the Student’s t-test and ANOVA. If the distribution of quantitative variables was skewed, log-transformed values were used for the analysis. Association between categorical variables was tested by the chi-square test for contingency tables. Frequencies of genotypes/alleles were determined by the gene counting method. Analyses were conducted with the SPSS (version 11.1) software package.

**RESULTS**

According demographic and clinical data of stroke patients and controls, we found that stroke patients were more often female, smokers, and hypertensive compared with controls. ACE genotypes and allele distribution among cases and controls were also determined and presented in Table 1. There was no significant difference in the distribution of ACE gene variant between cases and controls.

**Table 1. Genotype and Allele Frequencies of ACE in Serbian Stroke Patients and Control Subjects.**

| ACE | Stroke patients | Control subjects |
|-----|-----------------|------------------|
| Genotypes | | |
| II | 8 (0.123) | 59 (0.179) |
| ID | 37 (0.569) | 168 (0.509) |
| DD | 20 (0.308) | 103 (0.312) |
| Allele frequency | | |
| I | 52 (0.400) | 286 (0.433) |
| D | 78 (0.600) | 374 (0.567) |

Table 2 shows the ACE genotype and allele distribution among patients with large vessel stroke and small vessel (lacunar) stroke. Patients with D allele had 9.21 (95% CI 1.67–50.96) times higher risk for large vessel disease than
lacunar stroke. Patients with D allele had 4.68 (95% CI 1.10–19.86) times higher risk for large vessel stroke than healthy persons D allele possessors.

Table 2. Genotype and Allele frequencies of ACE in subgroups of IS patients.

| ACE    | Genotip ACE | Large vessel IS | Lacunar stroke |
|--------|-------------|-----------------|----------------|
| Genotypes | I           | 2 (0,044)       | 6 (0,300)       |
|         | ID          | 28 (0,600)      | 9 (0,500)       |
|         | DD          | 15 (0,356)      | 5 (0,200)       |
| Allele frequency | I           | 32 (0,344)      | 21 (0,550)      |
|         | D           | 58 (0,656)      | 19 (0,450)      |

Patients-carriers of DD genotypes had significantly higher (p<0.05) tryglicerides, apolipoprotein B, higher ACE catalitic activity, and significantly lower (p<0.05) HDL-cholesterol and apolipoprotein AI concentrations compared with control group. Patients with IS had higher levels of systolic blood pressure, diastolic blood pressure, mean arterial pressure and pulse pressure regardless of ACE genotype they possess. There was no significant difference in serum lipids, apolipoproteins and blood pressure levels according ACE genotype in stroke patients group. Patients with DD genotype had significantly higher catalitic ACE activity than patients with II or ID genotype. (Table 3)
### Table 3. Lipid Profile, BP Levels and ACE Activity in Relation to ACE Genotype in IS Patients and Controls.

| ACE genotype | Stroke patients | Control subjects | p, Student t-test |
|--------------|-----------------|------------------|------------------|
|              | Srednja vrednost | SD               | Srednja vrednost | SD               |                   |
| Total cholesterol (mmol/L) |                 |                  |                  |                  |
| II           | 5,10            | 0,96             | 5,77             | 1,32             | >0,05             |
| ID           | 6,00            | 1,31             | 5,77             | 1,15             | >0,05             |
| DD           | 6,60            | 1,86             | 5,76             | 1,36             | >0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
| HDL-cholesterol (mmol/L) |                 |                  |                  |                  |
| II           | 1,08            | 0,30             | 1,37             | 0,37             | <0,05             |
| ID           | 1,18            | 0,55             | 1,38             | 0,36             | <0,05             |
| DD           | 1,10            | 0,52             | 1,45             | 0,39             | <0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
| LDL-cholesterol (mmol/L) |                 |                  |                  |                  |
| II           | 3,07            | 0,91             | 3,81             | 1,11             | >0,05             |
| ID           | 3,61            | 1,41             | 3,75             | 1,00             | >0,05             |
| DD           | 4,00            | 1,81             | 3,66             | 1,17             | >0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
| Triglycerides (mmol/L) |                 |                  |                  |                  |
| II           | 1,76            | 0,83             | 1,31             | 0,86             | >0,05             |
| ID           | 2,11            | 0,92             | 1,39             | 0,81             | <0,05             |
| DD           | 2,08            | 0,89             | 1,43             | 0,80             | <0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
| Apolipoprotein AI (g/L) |                 |                  |                  |                  |
| II           | 1,35            | 0,27             | 1,51             | 0,35             | >0,05             |
| ID           | 1,33            | 0,35             | 1,47             | 0,33             | >0,05             |
| DD           | 1,31            | 0,40             | 1,53             | 0,30             | <0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
| Apolipoprotein B (g/L) |                 |                  |                  |                  |
| II           | 1,25            | 0,31             | 1,09             | 0,39             | >0,05             |
| ID           | 1,40            | 0,55             | 1,19             | 0,42             | <0,05             |
| DD           | 1,57            | 0,67             | 1,02             | 0,29             | <0,05             |
| p (ANOVA)    | >0,05           | <0,05            |                   |                   |                   |
| Apolipoprotein E (g/L) |                 |                  |                  |                  |
| II           | 53,30           | 10,12            | 41,45            | 20,18            | >0,05             |
| ID           | 40,13           | 11,63            | 39,77            | 19,52            | >0,05             |
| DD           | 51,12           | 23,75            | 44,68            | 22,04            | >0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
| Lipoprotein (a) (g/L) |                 |                  |                  |                  |
| II           | 0,25            | 0,22             | 0,18             | 0,16             | >0,05             |
| ID           | 0,26            | 0,42             | 0,22             | 0,26             | <0,05             |
| DD           | 0,17            | 0,16             | 0,19             | 0,13             | <0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
| ACE (U/L)    |                 |                  |                  |                  |
| II           | 18,83           | 10,91            | 18,78            | 6,59             | >0,05             |
| ID           | 38,81           | 11,78            | 35,28            | 12,29            | >0,05             |
| DD           | 67,95           | 20,21            | 57,40            | 13,25            | <0,05             |
| p (ANOVA)    | <0,05           | <0,05            |                   |                   |                   |
| Systolic blood pressure (mmHg) |                 |                  |                  |                  |
| II           | 149,38          | 21,95            | 126,27           | 22,30            | <0,05             |
| ID           | 164,32          | 29,93            | 128,54           | 20,48            | <0,05             |
| DD           | 160,00          | 31,50            | 131,41           | 23,14            | <0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
| Diastolic blood pressure (mmHg) |                 |                  |                  |                  |
| II           | 96,88           | 15,34            | 81,10            | 12,83            | <0,05             |
| ID           | 97,30           | 13,57            | 81,49            | 11,06            | <0,05             |
| DD           | 96,50           | 16,23            | 83,33            | 12,26            | <0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
| Mean arterial pressure (mmHg) |                 |                  |                  |                  |
| II           | 114,37          | 16,08            | 96,16            | 15,29            | <0,05             |
| ID           | 119,64          | 18,26            | 97,17            | 13,47            | <0,05             |
| DD           | 117,67          | 20,97            | 99,36            | 14,85            | <0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
| Pulse pressure (mmHg) |                 |                  |                  |                  |
| II           | 52,50           | 16,26            | 45,17            | 13,71            | >0,05             |
| ID           | 67,03           | 19,91            | 47,05            | 13,40            | <0,05             |
| DD           | 69,50           | 17,33            | 48,08            | 16,19            | <0,05             |
| p (ANOVA)    | >0,05           | >0,05            |                   |                   |                   |
**DISCUSSION**

This is the first study in Serbia that has examined the effect of ACE I/D gene polymorphism on IS risk. No significant evidence was provided to support our hypothesis that ACE gene polymorphism may represent a susceptibility mutation for IS. Previous reports on ACE gene polymorphism and IS produced conflicting results as to the importance of ACE alleles in predisposition to IS. We compiled results from 7 meta-analyses and 53 case-control studies (Table 4).

Table 4. Genetic association studies of ACE I/D polymorphism and ischemic stroke.

| Study                          | Year | Methodology                       | Phenotype       | Result                             |
|--------------------------------|------|-----------------------------------|-----------------|------------------------------------|
| Sharma i sar. (4)              | 1994 | Case–control: 100 patients, 73 controls | IS              | Negative                           |
| Castellano i sar. (5)          | 1995 | Cross-sectional: 199 patients     | IMT             | Positive                           |
| Dessi i sar.(6)                | 1995 | Case–control: 193 patients, 147 controls | IS              |                                    |
| Markus i sar. (7)              | 1995 | Case–control: 100 patients, 137 controls | IS              | Positive                           |
| Ueda i sar. (8)                | 1995 | Case–control: 488 patients, 188 controls | IS              | Negative                           |
| Catto i sar. (9)               | 1996 | Case–control: 418 patients, 231 controls | IS              | Negative                           |
| Hosoi i sar. (10)              | 1996 | Case–sectional: 288 patients      | IMT NIDDM       | Positive                           |
| Kario i sar. (11)              | 1996 | Case–control: 138/90 patients, 90/104 controls | IS, lacunar stroke in hypertensive. Hypertensive/normotensive control | Positive |
| Margaglione i sar. (12)        | 1996 | Case–control: 101 pacijent, 109 controls | IS              | Positive                           |
| Pullicino i sar. (13)          | 1996 | Case–control: 60 patients, controls | Lacunar stroke  | Negative                           |
| Sertic i sar. (14)             | 1996 | Case–control: 50 patients, 25 controls | IS              | Negative                           |
| Agerholm-Larsen i sar. (15)    | 1997 | Case–referent: 184 pacijent, 5028 referenata | IS              | Negative                           |
| Agerholm-Larsen i sar. (15)    | 1997 | Case–referent: 268 patients, 4015 referenata | IS              | Negative                           |
| Doi i sar. (16)                | 1997 | Case–control: 181 patients, 271 controls | IS              | Positive                           |
| Nakata i sar. (17)             | 1997 | Case–control: 55 patients, 61 controls | IS              | Positive                           |
| Watanabe i sar. (18)           | 1997 | Cross-sectional: 169 patients     | CS/ lacunar stroke | Positive |
| Aalto Setala i sar. (19)       | 1998 | Cross-sectional: 234 patients     | IS              | Negative                           |
| Molyaka i sar. (20)            | 1998 | Case–control: 52 patients, 80 controls | IS              | Negative                           |
| Pfohl i sar. (21)              | 1998 | Case–control: 388 patients        | IS              | Negative                           |
| Seino i sar. (22)              | 1998 | Case–control: 26 patients, 28 controls | IS              | Positive                           |
| Shen i sar. (23)               | 1998 | Case–control: 44 patients, 62 controls | IS              | Positive                           |
| Xu i sar. (24)                 | 1998 | Case–control: 65 patients, 117 controls | IS              | Positive                           |
| Hejman i sar. (25)             | 1999 | Cross-sectional: 79 patients      | CVD             | Negative                           |
| Kostulas i sar. (26)           | 1999 | Case–control: 100 patients, 100 controls | IS/CS           | Negative                           |
| Notsu i sar. (27)              | 1999 | Case–control: 175 patients, 213 controls | IS              | Negative                           |
| Zee i sar. (28)                | 1999 | Nested case–control: 348 patients, 348 controls | IS/PICH         | Negative                           |
| Lin i sar. (29)                | 2000 | Case–control: 306 patients, 300 controls | IS              | Negative                           |
| Wei i sar. (30)                | 2000 | Case–control: 87 patients, 257 controls | IS              | Positive                           |
| Szolnoki i sar. (31)           | 2001 | Case–control: 664 patients, 199 controls | IS              | Negative (small vessel) Positive |
| Um i sar. (32)                 | 2001 | Case–control: 106 patients, 498 controls | IS              | Negative                           |
| Zhang i sar. (33)              | 2001 | Case–control: 152 patients, 72 controls | IS              | Positive                           |
| Zhang i sar. (34)              | 2001 | Case–control: 165 patients, 106 controls | IS              | Positive                           |
| Li i sar. (35)                 | 2002 | Case–control: 143 patients, 154 controls | IS              | Negative                           |
The first meta-analysis has evaluated the risk of stroke in 1196 subjects versus 722 controls from seven studies. It was concluded that the ACE genotype conferred a small but modest effect, with an odds ratio of 1.31 (95% CI 1.06–1.62), according to a dominant model of inheritance. A weaker association was seen under a recessive model (57). In a meta-analysis of Casas et al. (58) including 2990 predominantly white patients and 11 305 controls, the DD genotype was shown to confer a small but significant risk of ischemic stroke (odds ratio 1.21; 95% CI 1.08–1.35). From 2007-2009 five meta-analysis were published. Aiyarathnam et al. (59) investigated the association of ACE I/D in three ethnic groups of non-European descent (a total of 3572 Chinese individuals, 1601 Japanese individuals, and 2750 Korean individuals). The overall OR for the nine studies in the Chinese population was 1.90 (95% CI 1.23–2.93) and for six Japanese studies the OR was 1.74 (95% CI 0.88–3.42). The overall OR in the Asian group (Chinese and Japanese) was 1.82 (95% CI 1.28–2.60). Meta-analysis of Banerjee et al. (60) included six studies and did not detect a significant association with ACE gene insertion/deletion polymorphism. Xu et al. (61) identified statistically significant associations with IS and ACE I/D (OR = 1.87, 95% CI =1.45–2.42 (15 studies, 1935 patients/1485 controls). Tao i sar. (62) analyzed 29 studies in Chinese population (3654 patients/3058 controls) and found that ACE DD genotype is a risk factor cerebral infarction in Chinese population (OR 1.91 (95% CI 1.56–2.34), P<0.00001). Rao et al. (63) investigated the association of IS subtypes of subjects with the ACE I/D polymorphism published in 11 papers and gave ACE/DD OR 1.31 for small vessel stroke (95% CI 0.96–1.79, p = 0.09) compared to large vessel stroke (OR 1.02, 95% CI 0.82–1.26, p = 0.88) showed a preferential association for the DD genotype with small compared to large vessel disease.

Twenty three out of 53 studies (Table 4) examined the association of ACE gene polymorphism and ischemic stroke yielded a significant association with IS. Only a few studies have been done on the association of the ACE I/D polymorphism with lacunar infarction. Markus et al. (7) studied this polymorphism in a small series of lacunar infarction cases (n=18) in which there was a positive association between D allele and lacunar infarction; OR associated with the DD genotype for lacunar stroke was 5.60 (95% CI, 2.00 to 15.71). Study on a Japanese population found that,
in hypertensive patients only, the ACE D allele was associated with silent brain infarction (11). However, this result was not replicated in another study using a larger population of lacunar infarction patients (n=130) (9). Watanabe et al. (18) showed no association of the ACE polymorphism with SBI in a smaller population (n=36). Takami et al. (64) failed to show an association with number of lacunae in 134 Japanese patients.

Discrepancy in the obtained results can be explained by heterogeneity of study designs employed, differences in inclusion and exclusion criteria, ethnically different patient populations, small sample sizes, unmatched controls, combinations of ischemic and hemorrhagic strokes, different stroke subtypes and age of stroke onset, type of statistical evaluation, covariates, correction for multiple testing, and other factors that affect many publications.

There is less literature data about the association of serum lipids and apolipoproteins with ACE I/D polymorphism in patients with IS. The results of our study are consistent with two previously published studies. Del Ser et al. (65) showed that serum triglycerides concentration is elevated in stroke patients with DD ACE genotype. Sertić et al. (14) found significant association of triglycerides concentration and DD genotype (DD>ID=II). It can be hypothesized that the increased serum triglycerides level in DD ACE genotype could be a feature of an insulin resistance syndrome and determine atherogenic and thrombogenic risk (65).

Agerholm-Larsen et al. (66) determined the effect of ACE genotype on serum ACE activity in 900 women and men from the Copenhagen City Heart Study and found that ACE gene polymorphism explains 30-40% of variability in serum ACE activity and this effect was codominant, with DD subjects having the highest, ID subjects intermediate, and II subjects the lowest serum ACE activity. The relatively small number of published studies with contradictory results examined the association between ACE activity and ACE I/D polymorphism in patients with IS compared to the number of studies that examined only the association of ACE I/D polymorphism polymorphism with IS.

Our study showed expected trend in ACE activity (DD>ID>II) in patient and control groups. Patients with DD genotype had significantly higher ACE activity (p<0.05) compared with controls. This result is in accordance with croatian study (patients with angiographically proved cerebral atherosclerosis) (14). Dikmen et al. (46) examined Turkish acute stroke patients (185) although ACE activity was high in DD genotype, no difference was determined to ACE enzyme activity according to ACE genotypes in patients and controls. Some studies suggested that D allele of the ACE gene is a marker of an elevated circulation ACE level. Catto et al. (9) reported plasma ACE activity significantly lower in stroke patients (cerebral infarction or cerebral hemorrhage) than in controls, 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. Markus et al. (7) found that patients with IS and DD genotype has higher ACE activity compared with carriers of II genotype.

We expect that subsequent genome-wide association studies in large and well-characterized groups of patients of different ethnic origin will contribute substantially in the near future to a better understanding of the pathophysiology underlying ischemic stroke and permit the identification of new therapeutic targets aimed at stroke prevention and neuroprotection from ischemic injury.

**ACKNOWLEDGEMENTS**

This work was supported by the Ministry of Science of Serbia on the basis of contract No.145010.

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