THE INTERACTION OF APOLIPOPROTEIN E AND ANGIOTENSIN I-CONVERTING ENZYME DNA POLYMORPHISMS WITH HYPERTENSION ON EARLY ISCHEMIC STROKE RISK

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Abstract
Combinations of multiple predisposing polymorphisms and their interactions with modifiable factors may result in synergistic effects on early ischemic stroke risk. We evaluated the potential interaction of apolipoprotein (apo) E and angiotensin I-converting enzyme (ACE) gene polymorphisms and hypertension on early ischemic stroke risk in Serbian population. We analyzed 65 stroke patients (mean age 35 yrs) and age- and body mass index matched 330 controls. ACE genotypes were determined by polymerase chain method (PCR) and apoE genotypes by PCR appended by HhaI restriction fragment-length polymorphism/MADGE analysis. Odds ratios (ORs) for stroke were 1.35 (95% confidence interval (CI) 0.50–3.62) in subjects with one studied polymorphism and 3.78 (95% CI, 1.28–11.18) in those with two. Compared with nonhypertensive subjects bearing no polymorphisms, ORs were 2.73 (95% CI 0.32–17.55) and 4.80 (95% CI 0.50–28.12) for nonhypertensive subjects with one and two polymorphisms, 8.53 (95% CI 1.04–62.47) and 30.00 (95% CI 3.21–186.45) for hypertensive. These data suggest a gene-dose effect of the examined gene variants and a synergistic effect of these polymorphisms and hypertension in the pathogenesis of early ischemic stroke.

INTRODUCTION
As one of the leading causes of death within both the developed and developing world, stroke is a worldwide problem. It primarily affects elderly people, but about 20% of strokes occur in younger population. The genetic etiology of ischemic stroke is polygenic. An increased risk of stroke may be the result of the interactions of a gene with environmental or behavioral factors. The studies investigating the role of the genetic components in the pathogenesis of ischemic stroke might be made more effective by analyzing: (a) the interactive effects of multiple candidate polymorphisms; (b) their interactions with conventional predisposing factors; and (c) younger age groups. Numerous case-control studies have investigated the role of genetics in the pathogenesis of stroke, with varied and often contradictory results (1). In particular, the polymorphism of the apoE gene, and I/D polymorphism of the angiotensin I-converting enzyme (ACE) are among the most frequently studied. The published reports have investigated these
markers as isolated gene mutations. Also, the differing degrees of susceptibility to ischemic stroke in patients with hypertension must be taken into account. However no data are available to date on effect of apo E and I/D polymorphism ACE and their potential interactions with hypertension in young subjects with ischemic stroke in our population.

The purpose of this study was: a) to access the synergistic effects of two polymorphisms of apoE and ACE gene on early ischemic stroke risk and b) to evaluate their potential interactions with hypertension.

MATERIALS AND METHODS
Blood samples were obtained from 65 unrelated subjects with first-ever acute ischemic stroke younger than 40 years (mean age 35 yrs). These were proven by computed tomography or magnetic resonance of the brain. 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. Personal data (age, sex, weight, height and arterial blood pressure) were obtained from all participants. Blood pressure measurements were determined and arterial hypertension was defined as systolic blood pressure of at least 140 mmHg, or the diastolic blood pressure of at least 90 mmHg, or use of antihypertensive agents. The diagnosis of diabetes mellitus was established according to WHO criteria. Cigarette smokers were categorized as current smokers or nonsmokers. Hypercholesterolemia was considered present if cholesterol serum levels ≥ 6.2 mmol/L or if the subject was undergoing treatment with cholesterol-lowering drugs. Body mass index (BMI; kg/m2) was also calculated. Presence of obesity was considered when BMI was ≥30 kg/m2.

DNA was extracted by Triton X-100 lysis, proteinase K digestion and phenol/chloroform extraction. The assay conditions and restriction isotyping/MADGE analysis for apoE genotyping were performed as published previously (2). 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).

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. 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. Because carriers of the ε34 genotype and ε4 allele turned out to be associated with a higher risk of ischemic stroke in bivariate analysis, the relationship between the ε34 genotype (ε4 carriers) and stroke risk was examined relative to the ε33 genotype (non-ε4 carriers) and expressed in terms of ORs, adjusted for sex, age, smoking habit, blood pressure and cholesterol levels by a logistic regression model. The same was done with ACE DD genotype and D allele. Binary variable was determined for each polymorphism (“yes” or “no” based on the status of “carrier” or “noncarrier” apo ε4 allele and ACE D allele). An individual genetic score (GS) was calculated based on the number of such genetic markers (from 0 to 2) in each subject. Therefore, the entire study group was stratified into 3 categories corresponding to a GS of 0, 1, and 2, respectively. The 6 x 2 table approach (4) was used to estimate the additive interaction between the GS and hypertension. Nonhypertensive subjects carrying no polymorphism (GS=0) were the reference category and were compared with nonhypertensive subjects with 1 polymorphism (GS=1), nonhypertensive with 2 polymorphisms (GS=2), hypertensive subjects with no polymorphism, hypertensive subjects with 1 polymorphism and hypertensive subjects with 2 polymorphisms. The Rothman’s synergy (S) measure was computed (5). The S index is the ratio of the observed effect with joint exposure divided by the effect predicted for joint exposure assuming additivity of the effects. No interaction corresponds to S=1, whereas S>1 (S<1) can be interpreted as a measure of relative increase.
(decrease) in the effect among those exposed to both factors. Analyses were conducted with the SPSS (version 11.1) software package.

**RESULTS**

Demographic characteristics and prevalence of some risk factors of the participants are presented in Table 1. Stroke cases were more often hypertensive, smokers and hypercholesterolemic compared with controls.

Table 1. Demographic and clinical characteristics of stroke patients and controls.

| Characteristics      | Stroke Patients (%) | Control Subjects (%) | Crude OR | 95% CI         |
|---------------------|---------------------|----------------------|----------|----------------|
| Sex (male)          | 27.7                | 50                   | 0.38     | 0.21–0.69      |
| Current smokers     | 66.2                | 45.8                 | 2.31     | 1.32–4.04      |
| Hypertension        | 69.2                | 28.0                 | 3.88     | 2.19–6.88      |
| Diabetes Mellitus   | 2.3                 | 0.0                  | …        | …              |
| Hypercholesterolemia| 33.8                | 32.0                 | 1.59     | 0.90–2.83      |
| Obesity             | 11                  | 5                    | 0.34     | 0.10–1.13      |

ApoE and ACE genotypes and allele distribution among cases and controls were also determined and presented in Table 2. A significant increase of stroke risk was associated with the apoE ɛ4 carrier status (OR, 10.77; 95% CI, 5.92–19.61). In contrast, there was no significant difference in the distribution of ACE gene variant between cases and controls.

Table 2. Allele frequencies of apoE and ACE in Serbian stroke patients and control subjects.

| Allele frequency | Stroke patients | Control subjects | Crude OR | 95% CI         |
|------------------|-----------------|------------------|----------|----------------|
| Apolipoprotein E |                 |                  |          |                |
| ɛ2               | 6 (0.046)       | 87 (0.132)       | 0.34     | 0.14–0.81      |
| ɛ3               | 102 (0.785)     | 513 (0.777)      | 1        |                |
| ɛ4               | 22 (0.169)      | 60 (0.091)       | 1.84     | 1.08–3.14      |
| Non-ɛ4 carriers  | 45 (0.750)      | 273 (0.827)      | 1        |                |
| ɛ4 carriers      | 20 (0.250)      | 57 (0.173)       | 10.77    | 5.92–19.61     |
| ACE               |                 |                  |          |                |
| I                 | 52 (0.400)      | 286 (0.433)      | 1        |                |
| D                 | 78 (0.600)      | 374 (0.567)      | 1.14     | 0.782          |
| Non-D carriers    | 58 (0.892)      | 271 (0.821)      | 1        |                |
| D carriers        | 7 (0.108)       | 59 (0.179)       | 0.55     | 0.24–1.27      |

Hypertensive persons carrying the ɛ33 genotype had an increased risk of stroke compared with nonhypertensive with the same genotype (OR, 3.85; 95% CI 1.86–7.96). The combination of the ɛ34 genotype and hypertensive status was associated with 8-times-greater odds of ischemic stroke (OR, 8.19; 95% CI 3.38–19.85). The combined effect of the ɛ34 genotype and increased arterial blood pressure on stroke risk was greater than that predicted by assuming additivity of effects (S=2.42). In contrast, there was no evidence of DD genotype effect among subjects with/without hypertension.

When the prevalence of combinations of two studied polymorphisms was analyzed, a cumulative effect was observed. The presence of one polymorphism increased the risk of stroke 1.3-fold (OR 1.35; 95% CI 0.50–3.61), and presence of two polymorphisms increased the risk of stroke 3.8-fold (OR 3.78; 95% 1.28–11.18) compared with subjects bearing no polymorphisms (Table 3).
Table 3. Prevalence and odds ratios of genetic markers among cases and controls.

| GS | Stroke patients | Controls | OR (95% CI) |
|----|----------------|----------|-------------|
| 0  | 5              | 41       | 1           |
| 1  | 41             | 249      | 1.35 (0.50–3.61) |
| 2  | 18             | 39       | 3.78 (1.28–11.18) |

Table 4 summarizes the results of the analyses undertaken to find potential interactions between genetic background and hypertension. Compared with the reference category of nonhypertensive individuals carrying none of the studied polymorphisms, the risk of stroke was increased almost 3-fold among nonhypertensive subjects with one polymorphism (OR, 2.72; 95% CI 0.31–17.55) and 4.8-fold among nonhypertensive subjects with two polymorphisms (OR, 4.80; 95% CI 0.49–28.11). The presence of hypertension resulted in a 8-fold increase of risk when combined with one polymorphism (OR, 8.52; 95% CI 1.04–62.46) and in 30-fold increase of risk when combined with two polymorphisms (OR, 30.00; 95% CI 3.20–186.44).

Table 4. 6x2 Table for genetic score–hypertension interaction.

| Nonhypertensive with GS=0 | Nonhypertensive with GS=1 | Nonhypertensive with GS=2 | Hypertensive with GS=0 | Hypertensive with GS=1 | Hypertensive with GS=2 |
|---------------------------|---------------------------|---------------------------|------------------------|------------------------|------------------------|
| Cases 1                   | Controls 30               | Cases 14                  | Controls 154           | Cases 4                | Controls 25            |
| OR (95%)                  | OR (95%)                  | OR (95%)                  | OR (95%)               | OR (95%)              | OR (95%)              |
| 1                         | 2.72 (0.31–17.55)         | 4.80 (0.49–28.11)         | 10.90 (1.04–88.46)     | 8.52 (1.04–62.46)      | 30.00 (3.20–186.44)    |
| Nonhypertensive with GS=1 |                          |                          |                        |                        |                        |
| Cases 4                   | Controls 25               | Cases 11                  | Controls 95            | Cases 14               | Controls 14            |
| OR (95%)                  |                          |                          | OR (95%)              | OR (95%)              | OR (95%)              |
| 1                         |                          |                          | 8.52                  | 30.00                  |                        |
| (0.31–17.55)              |                          |                          | (1.04–62.46)          | (3.20–186.44)          |                        |

DISCUSSION

The genetic risks implicated in ischemic stroke have been reported over last decade. There is evidence those genetic factors that are minor or insignificant when present alone can not only exert an additive effect, but also facilitate the effects of other common clinical risk factors at a clinical phenotype level. Their co-occurrence in the same person can raise a significant relative risk of an ischemic stroke event. However no data are available to date on interactive effects of apoE and ACE gene polymorphisms and their interaction with modifiable factors on the risk of ischemic stroke in our population.

A complex network of interactions between genetic factors and environmental and clinical risk factors (hypertension, diabetes mellitus, smoking, alcohol drinking etc.) that lead to the development of ischemic stroke has been determined in a series of older stroke patients in only few studies. Szolnoki et al. (6–8) demonstrated that unfavorable genetic factors such as the Leiden V and methylenetetrahydrofolate reductase (MTHFR) 677TT mutations and the apoA4 and ACE DD genotypes, which alone are not major risk factors, can modify and exert a synergistic effect on certain clinical risk factors and can multiply the relative risk of ischemic stroke. APO A4 allele had yielded a more general synergistic effect with hypertension (OR for hypertension + APOA4: 15.6) (8).

Only few studies examined the interactions between genetic mutations and clinical risk factors in the development of early ischemic stroke as in this study. Our results are consistent with previous studies that suggest the importance of the cumulative effect of several polymorphisms in combination with other cardiovascular risk factors on the occurrence of myocardial infarction in young adults (9–11) as well as with recent observations from series of ischemic stroke patients not stratified by age (8, 12, 13). Pezzini et al. (14) examined the cumulative effect of prothrombotic
and proatherogenic gene variants (20210A variant of prothrombin gene, the 1691A variant of factor V gene, the TT677 genotype of the MTHFR gene, and the ω1-carriership of the apoE gene) and their potential interactions with modifiable risk factors (including hypertension) in the pathogenesis of cerebral ischemia in young adults. They found that compared with nonhypertensive subjects bearing no polymorphisms, ORs were 1.91 (95% CI, 1.28–2.87) and 3.68 (95% CI, 1.64–8.26) for nonhypertensive subjects with 1 and 2 polymorphisms, and 3.28 (95% CI, 1.01–10.7) and 10.79 (95% CI, 1.01–115.40) for hypertensive.

The obtained results suggest a gene dose effect of the studied polymorphisms on the risk of ischemic stroke in young adults and a potential synergistic interaction of these genetic factors with hypertension. In particular, an increased risk of disease was associated with the presence of one of the genetic markers and was even more pronounced in subjects with two, particularly in the subgroup of individuals who were hypertensive. This study suggests more aggressive treatment of arterial hypertension should be especially recommended in young subjects with a predisposing genetic background for ischemic stroke.

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**References**

1. Stanković S, Majkić-Singh N. Advances in the genetic basis of ischemic stroke. Jug Med Biochem 2008; 2: 5-9.
2. Stanković S, Glišić S, Alavantić D. Apolipoprotein E gene DNA polymorphism in Serbian population. Jug Med Biochem 1999; 18: 9–15.
3. Stanković A, Ilić N, Žunić Z, Glišić S, Alavantić D. Association of the insertion/deletion polymorphism at the angiotensin I-converting enzyme locus with arterial blood pressure: population-based study. Jug Med Biochem 1999; 18(4): 1417.
4. Botto LD, Khoury MJ. Facing the challenge of gene-environment interaction: the two-by-four table and beyond. Am J Epidemiol 2001; 153: 1016–20.
5. Rothman KJ. Synergy and antagonism in cause-effect relationship. Am J Epidemiol 1974; 99: 385–8.
6. Szolnoki, Z, Somogyvári F, Kondacs A, Szabó M, Fodor L. Evaluation of the roles of the Leiden V mutation and ACE I/D polymorphism in subtypes of ischemic stroke. J Neurol 2001; 248: 756–61.
7. Szolnoki Z. Evaluation of the interactions of common genetic mutations in stroke. From: Methods in Molecular Medicine, Vol. 104: Stroke Genomics: Methods and Reviews Edited by: S. J. Read and D. Virley © Humana Press Inc., Totowa, NJ. 2004, pp. 241–9.
8. Szolnoki Z, Somogyvári F, Kondacs A, Szabó M, Fodor L, Bene J, Melegh B. Evaluation of the modifying effects of unfavourable genotypes on classical clinical risk factors for ischemic stroke. J Neurol Neurosurg Psychiatry 2003; 74: 1615–20.
9. Inbal A, Freimark D, Modan B, Chetrit A, Matetzky S, Rosenberg N, Dardik R, Baron Z, Seligsohn U. Synergistic effects of prothrombotic polymorphisms and atherogenic factors on the risk of myocardial infarction in young males. Blood 1999; 39: 2186–90.
10. Doggen CJ, Cats VM, Bertina RM, Rosendaal FR. Interaction of coagulation defects and cardiovascular risk factors: increased risk of myocardial infarction associated with factor V Leiden or prothrombin 20210A. Circulation 1998; 97: 1037–41.
11. Siscovick DS, Schwartz SM, Rosendaal FR, Psaty BM. Thrombosis in the young: effects of atherosclerotic risk factors on the risk of myocardial infarction associated with prothrombotic factors. Thromb Haemost 1997; 78: 7–12.
12. Glueck CJ, Fontaine RN, Wang P. Interaction of heritable and estrogeninduced thrombophilia: possible etiologies for ischemic optic neuropathy and ischemic stroke. Thromb Haemost 2000; 85: 256–9.
13. Szolnoki Z, Somogyvari F, Kondacs A, Szabo M, Fodor L. Evaluation of the interactions of common genetic mutations in stroke subtypes. J Neurol 2002; 49: 1391–7.

14. Pezzini A, Grassi M, Del Zotto E, Archetti S, Spezi R, Vergani V, Assanelli D, Caimi L, Padovani A. Cumulative effect of predisposing genotypes and their interaction with modifiable factors on the risk of ischemic stroke in young adults. Stroke 2005; 36: 533–9.