Coexistence of Angiotensin II Type-1 Receptor A1166C and Angiotensin-Converting Enzyme D/D Polymorphism Suggests Susceptibility for Small-Vessel-Associated Ischemic Stroke

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Abstract

The renin–angiotensin system plays an important role in the maintenance of blood pressure homeostasis. The angiotensin-converting enzyme (ACE) converts angiotensin I into angiotensin II. Angiotensin II, which binds the angiotensin II type-1 receptor (AT1R), is a potent vasoconstrictor. On a pathophysiological basis, both ACE I/D and AT1R A1166C polymorphism lead to an enhanced activity of the angiotensin II-AT1R axis, thereby possibly contributing to circulatory disturbances. A mutually facilitatory effect may be presumed between the two polymorphisms. We examined whether this synergistic effect is involved in the evolution of different types of ischemic stroke. Genetic and clinical data on 308 consecutive patients with acutely developing ischemic stroke were analyzed. A total of 272 stroke and neuroimaging alteration-free subjects served as a control group. Univariate and logistic regression statistical approaches were used. The ACE D allele combined with the AT1R 1166C allele did not yield a risk of ischemic stroke. However, the co-occurrence of the homozygous ACE D/D and at least one AT1R 1166C allele was more frequent in the ischemic stroke group than in the control group (22.4 vs 11%, \(p < 0.005\), OR, 2.33; 95% CI, 1.46–3.7). After specific subgroup analysis, this synergistic association was even stronger for small-vessel ischemic stroke (OR, 3.44; 95% CI, 1.9–6.24; \(p < 0.0005\)). Multivariate logistic regression analysis of the data confirmed this association (adjusted OR, 3.54, 95% CI, 1.88–7.16; \(p < 0.0005\)). Our results demonstrate that ACE D/D and AT1R 1166C
Introduction

The renin–angiotensin system (RAS) plays an important role in the maintenance of blood pressure homeostasis (Agachan, 2003; Henderson, 2004; Miller, 2004). The angiotensin-converting enzyme (ACE) converts angiotensin I into angiotensin II. Angiotensin II, which binds the angiotensin II type-1 receptor (AT1R), is a potent vasoconstrictor and a stimulator of cardiac growth (Imai, 2005; Kuba, 2005; Nicholls, 2005). Both ACE and AT1R display polymorphisms that can alter their functions. The ACE D/D polymorphism, associated with an elevated angiotensin II level, has been demonstrated to play an important role in the development of ischemic stroke and leukoaraiosis (Szolnoki, 2002, 2003, 2004). The AT1R A1166C polymorphism, which leads to an enhanced responsiveness of the AT1R, has been suggested as a mild susceptibility factor for ischemic stroke (Rubattu, 2004; Brenner, 2005). As both polymorphisms are associated with an elevated angiotensin II level, has been demonstrated to play an important role in the development of ischemic stroke and leukoaraiosis (Szolnoki, 2002, 2003, 2004). The AT1R A1166C polymorphism, which leads to an enhanced responsiveness of the AT1R, has been suggested as a mild susceptibility factor for ischemic stroke (Rubattu, 2004; Brenner, 2005). As both polymorphisms are associated with an enhanced activity of the angiotensin II–AT1R axis, we postulated at a clinical level, a synergistic effect between them as regards the evolution of ischemic stroke. We also analyzed to what extent the different subtypes of ischemic stroke are related to the gene–gene interaction polymorphism were associated with the development of small-vessel ischemic stroke through a mutually facilitatory interplay between them. Genetic interactions might contribute to the altered functional network in renin–angiotensin system in vascular disorders.

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Index Entries: ACE I/D; AT1RA1166C polymorphism; angiotensin; network; receptor; stroke.

Methods

Study Population

The data on 308 consecutive patients with acutely developing ischemic stroke who had never suffered a previous stroke event were analyzed. These subjects had been admitted to our Department of Neurology and Neurophysiology between January 1998 and 2004 after being examined by an internist in the local emergency unit or by a family physician at their homes. All 308 subjects underwent a detailed clinical scrutiny, including the medical history, the family history, an evaluation of vascular risk factors, general physical and neurological examinations, urine analysis, extensive laboratory examinations, electrocardiography, extracranial and transcranial Doppler sonography of the brain-supplying arteries, transthoracic and/or transoesophageal echocardiography where appropriate, and magnetic resonance imaging (MRI) examinations within 2 d after the onset of the symptoms. All scans were read by an experienced investigator without knowledge of the clinical and laboratory data. The patients were enrolled immediately after the clinical neurological and MRI examinations. Subjects on whom MRIs could not be recorded or for whom the examined clinical parameters and risk factors could not be obtained with certainty in consequence of some technical cause or death were excluded from the study groups. Patients with atrial fibrillation and cardio-embolic source were also excluded in order to make the study groups more homogeneous.

Following evaluation of the clinical and radiological features, the patients were enrolled into one or other of three subgroups. Group 1 corresponded to large-vessel infarction (cortical or cerebellar lesions and/or brainstem infarcts or subcortical hemispheric infarcts more than 1.5 cm in diameter on the MRIs, with a cerebral cortical impairment or a brainstem or cerebellar dysfunction); group 2 corresponded to small-vessel occlusion (one or more subcortical hemispheric or brainstem infarcts with a diameter of less than 1.5 cm on the MRIs, with one of the features of the traditional clinical lacunar syndrome and without a cerebral cortical dysfunction); and group 3 corresponded to a mixed vascular pathology (one or more lacunar and large-vessel infarcts on the MRIs, with lacunar syndrome or cortical/cerebellar/brainstem dysfunction). This classification based on the clinical and radiological features was considered to be the most exact and quantifiable method with regard to the requirement that the subgroups reflect the main well-defined vascular pathologies and their overlapping, which may possibly be affected by the examined mutations.
As a control group, 272 stroke and neuroimaging alteration-free Caucasian Hungarian subjects were examined. The controls were randomly selected by using a sex-matched technique from general practice registers from the same locality as the stroke cases, with the requirement that they had negative brain MRI or computed tomography (CT) findings in order to avoid silent brain infarctions. They were healthy and believed to be free of cerebrovascular disease. Subjects with any previous clinical data suggesting a cerebrovascular or cardiovascular event (such as transient ischemic attack or angina pectoris) were excluded from the control group. Both the controls and the patients gave their informed consent to the clinical work-up and the DNA analysis. The study was approved by the local ethics committee.

**Assessment of Clinical Data**

The smoking and drinking habits and the presence of hypertension or diabetes mellitus were recorded in all groups. The serum cholesterol level, the serum triglyceride level, and the hematocrit were also measured and analyzed as important clinical parameters. Hypertension was diagnosed when the blood pressure repeatedly exceeded 140 mmHg systolic and/or 85 mmHg diastolic or when the patient was taking antihypertensive medication. Diabetes mellitus was diagnosed when the glucose level was at least 7.78 mM in a fasting state and/or at least 11.11 mM 2 h after a meal or 75 g oral glucose loading, according to the World Health Organization criteria 12 (WHO, 1985). Ischemic heart disease was diagnosed when a history of angina pectoris or acute myocardial infarction was present or if there was electrocardiogram evidence of coronary heart disease. Patients were classified as smokers if they had ever smoked more than five cigarettes per day for at least a year. Patients were considered to be moderately heavy drinkers if they drank 40 g of alcohol or more per day. The body mass index was calculated as the weight in kilograms divided by the square of the height in meters.

**DNA Analysis**

Genomic DNA was extracted from 200 µL of peripheral blood anticoagulated with EDTA by the desalting method (Miller, 1988). All blood samples were stored at –20°C until DNA isolation.

For AT1R (GenBank accession no. NT 005612) genotyping, a new primer set was constructed. The forward primer was 5’ AAGGCAATCCACTCAAA 3’, and the reverse primer was 5’ CAGGACAAAGCAGGCCTAGG 3’. PCR was performed with an MJ Research PTC-200 thermal cycler. The reaction volume was 50 µL, containing 1 µL of DNA (40–80 ng), 0.2 µM of each of the primers, 5 µL of reaction buffer (100 mM Tris-HCl; pH 9.0; containing 500 mM KCl; and 15 mM MgCl2), 200 µM of dNTP, and two units of Taq polymerase. The polymerase chain reaction (PCR) conditions were as follows: initial denaturation at 96°C for 120s, followed by 35 cycles of denaturation (96°C for 30s), annealing (53°C for 30s), and extension (72°C for 60s). PCR products were digested with 1.5 U of Dde I (New England Biolabs, Kvalitetex Scientific, Technological, Trading Ltd, Budapest, Hungary) at 37°C overnight. The restriction fragments were separated by electrophoresis on 3% agarose gels containing ethidium bromide and visualized by ultraviolet illumination. The AT1R 1166A allele results in 58 and 374 bp fragments, whereas the AT1R 1166C allele results in 58, 143, and 231 bp fragments.

The ACE polymorphism was examined by a PCR method developed in order to decrease the examination time and provide a better detection of heterozygotes (Somogyvari, 2001). This method consisted of fluorescent probe melting point analysis performed with fluorescently labeled oligonucleotide hybridization probes on the LightCycler™ instrument (Roche Diagnostics, Roche Kft, Budapest Hungary). The genetic examinations were carried out without a knowledge of the results of the clinical work-up.

**Statistics**

The clinical data were expressed as means ± SD where appropriate. The differences between the clinical parameters in the stroke group and the controls were assessed by using the χ² test or the Mann-Whitney test where appropriate. The stroke groups were tested against the control group for the frequencies of the different genotypes and their combinations by the χ² test. Logistic regression models were evolved to evaluate the importance of the co-occurrences of the ACE D and AT1R 1166C alleles in ischemic stroke. The main genotype categories examined were the homozygous AT1R 1166C/C and ACE D/D polymorphisms (with a score of 1 for the homozygous state, and of 0 for the
heterozygous state and lack of the given allele); and the presence of at least one of the ACE D and AT1R alleles (with a score of 1 for both the homozygous and heterozygous states, and of 0 for lack of the given allele). For all the ORs, the 95% confidence intervals (95% CI) were calculated. Logistic regression analyses were performed with the statistical package SYSTAT 10 (Chicago) for Windows.

As a consequence of the great number of statistical comparisons, there arose a chance of statistically significant associations being registered between ischemic stroke and a given combination pair of the genotypes by accident, without valid biological associations. In order to decrease this possibility, we randomly divided our groups into two identical-sized samples and carried out statistical calculations independently for them. We considered an association between ischemic stroke and the occurrence of a given genotype biologically valid if that association was calculated to be statistically significant (p < 0.05) in both independent samples (data not shown). This methodical approach could resolve the statistical problems that stem from exploratory multiple testing.

### Results

The clinical data are listed in Table 1. The AT1R 1166C allele did not occur significantly more frequently in the stroke subgroups (large-vessel, 51.3%; small-vessel, 51.7%; mixed type, 50.7%; overall, 51.3%) than in the controls (52.9%) (Table 2). The ACE D/D genotype meant a minor risk of small-vessel ischemic stroke (adjusted OR, 1.81; 95% CI, 1.1–3.12; p < 0.05) (Table 3). This result was not confirmed in the divided stroke groups (data not shown). Co-occurrence of the AT1R 1166C and ACE D alleles did not consist of a significant risk of ischemic stroke (Table 3).

A synergistic effect was found between the AT1R 1166C allele and the homozygous ACE D/D genotype in the small-vessel stroke subgroup (adjusted OR, 3.54; 95% CI, 1.88–7.16; p < 0.0005), the mixed vascular type (adjusted OR, 2.41; 95% CI, 1.2–5.1; p < 0.05), and the overall ischemic stroke group (adjusted OR, 2.42; 95% CI, 1.51–3.82; p < 0.005) (Table 3). The synergistic effect as shown earlier was not confirmed in the divided stroke groups for the mixed vascular type stroke group (data not shown).

### Table 1

Characteristics of Patients and Control Subjects

| Clinical features                  | Overall stroke group | Control group |
|-----------------------------------|----------------------|--------------|
|                                   | N = 308              | N = 272      |
| Sex (females/males)               | 148/160              | 129/143      |
| Age (yr)                          | 63.2 ± 11.5<sup>a</sup> | 53.7 ± 14.8 |
| BMI (kg/m²)                       | 26.9 ± 2<sup>a</sup> | 23.1 ± 3.1  |
| Cholesterol (mM)                  | 6.9 ± 1.5<sup>a</sup> | 5.2 ± 1.5   |
| Triglycerides (mM)                | 1.92 ± 0.9<sup>a</sup>| 1.26 ± 0.8  |
| Hematocrit (%)                    | 45 ± 9               | 45 ± 6      |
| Platelet count (x10<sup>9</sup>)  | 250 ± 53             | 251 ± 48    |
| Hypertension (%)                  | 50<sup>b</sup>       | 18          |
| Diabetes mellitus (%)             | 30.8<sup>b</sup>     | 4.8         |
| Smokers (%)                       | 31.2<sup>b</sup>     | 9.9         |
| Drinkers (%)                      | 11.7<sup>b</sup>     | 3.7         |
| Ischemic heart disease (%)        | 15.9<sup>b</sup>     | 6.6         |

<sup>a</sup>p < 0.001.

<sup>b</sup>p < 0.0005.

The overall stroke group was compared with the control group by the χ² test or the Mann-Whitney test where appropriate.

BMI, body mass index.
Because of the low number of the homozygous AT1R 1166CC genotype, its dose-dependent effect could not be evaluated statistically in combination with the ACE D allele and ACED/D genotype.

**Discussion**

In our population, we did not reveal a direct association between the AT1R A1166C polymorphism and ischemic stroke. The ACE D/D genotype was an independent minor risk factor for small-vessel infarcts. Because of the low significance level, this result of our present study would need to be confirmed. However, this association was consistent with earlier findings (Szolnoki, 2002).

The co-occurrence of the AT1R 1166C and ACE D alleles did not enhance the risk of ischemic stroke in any subtype as compared with the control group. The combination of the homozygous ACED/D genotype and at least one AT1R 1166C allele increased the risk of ischemic stroke. After specific subgroup analysis, this synergistic association was even stronger for small-vessel-associated ischemic stroke. Although there was a tendency to an increase in this genotype combination in large-vessel pathology-associated cerebral infarction, a significant interplay was not observed in this subtype.

A similar synergistic association has been described in ischemic heart diseases (Fukazawa, 2004). There is no exact explanation for this additive effect. On a pathophysiological basis, however,
both unfavorable polymorphisms result in an enhanced activity of the angiotensin II-AT1R axis. This enhanced activity can lead to an endothelial dysfunction and vasoregulation disturbances (De Ciuceis, 2005; Watanabe, 2005). The local vasoregulation of the cerebral blood flow takes place at a small-vessel level. A normal endothelial function is essential for this process. Accordingly, an endothelial dysfunction leads primarily to small-vessel circulatory disturbances. This association explains why the synergistic interaction we observed led to small-vessel infarction. The presence of the ACE D/D genotype produces an elevated ACE level, and thereby yields an increased level of the angiotensin II (Malik, 1997). There are data showing that the AT1R 1166C allele is associated with an increased angiotensin II responsiveness (Van Geel, 2000; Jones, 2003). Both the elevated level of the angiotensin II and the enhanced responsiveness of its target might comprise the molecular basis of the synergistic effect seen at the clinical level.

This interplay might have practical implications for everyday clinical practice. Increasing attention has recently been paid to primary stroke prevention, this centering on endothelial protection (Larose, 2004). Modern antihypertensive drugs such as ACE inhibitors and AT1R blockers protect against an endothelial dysfunction, and also lower blood pressure (De Gennaro, 2005; Higashi, 2005; Saavedra, 2005; Schmieder, 2005; Zhou, 2005). On a pathophysiological basis, they might directly counterbalance the unfavorable effects of both ACE D/D and AT1R 1166C polymorphisms (De Gennaro, 2005;
Higashi, 2005; Saavedra, 2005; Schmieder, 2005; Zhou, 2005). Identification of the population at a higher risk of ischemic stroke in consequence of the combination of ACE D/D and AT1R 1166C polymorphisms may help in the selection of patients for whom ACE inhibitors and /or AT1R blockers, either alone or in combination, should be chosen as specific preventive tools against ischemic stroke or an endothelial dysfunction. The association between the risky haplotype of RAS and drug responsibility necessitates further studies.

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