TABLE 1. Demographic Data of the Studied Population

	Nonsmokers	Smokers	*P**
Participants, n	166	344	
Age, y	56.4±8.1	57.4±7.1	0.16
BMI, kg/m^2	24.2±2.7	23.6±2.8	0.01
MBP, mm Hg	95.7±10.5	95.0±12.6	0.52
T-chol, mmol/L	5.38±0.91	5.23±0.94	0.08
HDL-C, mmol/L	1.31±0.36	1.36±0.40	0.20
TGs, mmol/L	1.39±0.78	1.57±1.08	0.04
Urinary IsoP, ng/mg creatinine†	1.11±0.63	2.11±1.25	<0.0001
CVR, mm Hg · min · mL^{-1}	1.74±0.37	1.77±0.44	0.46
CBF, mL · min^{-1} · 100 g tissue^{-1}	57.3±11.1	56.5±13.0	0.51
Hypertension, %	27.7	32.8	0.24
DM, %	10.2	17.2	0.04
Hypercholesterolemia, %	6.0	8.2	0.40

BMI indicates body mass index; T-chol, total cholesterol; HDL-C, HDL cholesterol; TGs, triglycerides; and DM, diabetes mellitus. Values are mean±SD or as indicated.

*By Student *t* test or by χ^2 test.

†Urine samples of male participants followed up in 2000 (34 nonsmokers and 79 smokers) were used in the measurement.

Health Science for a health-screening examination between 1995 and 2000 were recruited into the present study. Although the participants were from a local population, they were not taken as a population-based cohort by the precise epidemiological definition. In the interview, participants were asked about histories of smoking, medication for hypertension, diabetes mellitus, and hypercholesterolemia. Participants were considered to have these diseases when medical doctors had already diagnosed them. Blood pressure or fasting blood glucose measured on site was not included in the diagnostic criteria. Plasma was collected after overnight fasting to measure total cholesterol, HDL cholesterol, and triglyceride levels. Nitrite/nitrate (NOx) was measured in plasma by using commercial kits after nitrate was reduced to nitrite (Cayman Chemical Co). With the use of commercial kits (Cayman Chemical Co), urinary F_2-isoprostane (IsoP) was measured in 113 consecutive male participants who had visited the institute in 2000. IsoP was considered a good biochemical marker of oxidative stress in vivo.[16] Blood pressure was measured 3 times after at least 15 minutes of rest, just before the CBF measurement. The mean of the 3 measurements was taken as the representative blood pressure.

Periventricular hyperintensity (PVH) and brain infarction, including subcortical silent brain infarction, were monitored by MRI (0.2 T, Siemens). Diagnostic criteria for silent brain infarction and PVH have been described previously.[17–19] CBF was measured by the ^{133}Xe inhalation method as described.[19] CBF was represented as blood volume perfusing 100 g of brain tissue per minute. CBF measurements were performed in a quiet room with the subjects resting and their eyes closed. CVR was calculated as mean arterial blood pressure (MBP)/CBF.

For detailed analyses, subjects with brain infarction, including silent brain infarction, or with high grade PVH (≥3, thick PVH surrounding the lateral ventricle with or without marked extension into the white matter) were excluded because of the possibility of secondary decrease of CBF under such pathological conditions.[20,21] We then excluded female subjects because (1) only 15 smokers were found in the females and (2) a significant difference of CBF was observed between males and females (58.9±12.6 and 66.9±14.7 mL/min per 100 g tissue for male and female nonsmokers, respectively; $P<0.001$ by Student *t* test). From the 557 selected male subjects, we extracted 2 groups according to smoking status, as in our previous study[17]: (1) the nonsmokers with a smoking index (measured as cigarettes per day times years) of 0 and (2) the definite

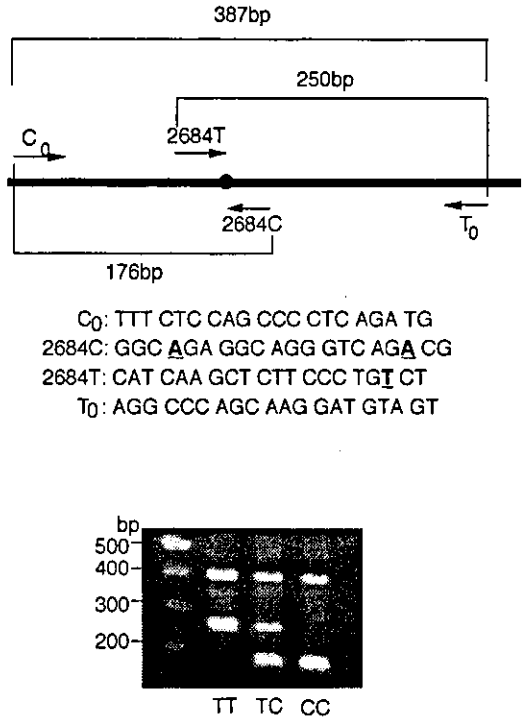

Figure 1. Allele-specific PCR for T-786C polymorphism in the eNOS gene. Top panel shows the primers used in the reaction and the expected PCR products. Underlining in the primer sequences indicates artificially introduced mismatches. Bottom panel shows a typical result of genotyping.

smokers with a smoking index >200. This categorization was reasonable because the oxidative stress evaluated with the urinary IsoP was significantly different between the 2 groups (see Table 1). As a result, 510 male subjects (166 nonsmokers and 344 definite smokers) were included in further analysis. Forty-seven subjects were categorized as having a smoking index between 1 and 199. We excluded this population from the analysis because (1) only 1 CC homozygote of T-786C was found in this population, and (2) the effect of smoking was not clear in this population with a urinary IsoP of 1.49±0.68 ng/mg creatinine, which was not significantly different from that of the nonsmokers. Moreover, when we included these 47 subjects in either the definite smokers or nonsmokers, the results did not change (data not shown).

In addition to these subjects, 96 students (60 male and 36 female students) of Shimane Medical University who voluntarily participated in the study were genotyped as a reference population. Participants gave informed consent, and the study protocol was approved by the ethics committee of Shimane Medical University.

eNOS Genotyping

DNA was extracted from peripheral blood samples. Genotyping of ecNOS4 a/b was performed as described previously.[22] For genotyping of T-786C, a newly developed allele-specific polymerase chain reaction (PCR) was used (Figure 1).[23] The oligonucleotide primers used in the reaction are listed in Figure 1. Artificial mismatches were included in the 2684T and 2684C primers as indicated in Figure 1. Amplification was performed in a total volume of 20 μL containing 50 ng genomic DNA, 0.25 μmol/L 2684T and 2684C primers, 0.063 μmol/L T_0 and C_0 primers, 62.5 μmol/L dNTPs, 45 mmol/L Tris-HCl (pH 8.8), 11 mmol/L ammonium sulfate, 6.7 μmol/L β-mercaptoethanol, 4.5 μmol/L EDTA, 1.5 mmol/L $MgCl_2$, and 0.4 U Taq polymerase (Promega). After a hot start at 96°C, amplification was achieved by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 20 seconds. The C and T alleles gave a 176-

TABLE 2. T-786C Genotype Frequencies in the Studied Populations

	Nonsmokers (N=166)	Smokers (N=344)	Reference (N=96)
TT, n (%)	129 (77.7)	273 (79.4)	78 (81.3)
TC, n (%)	34 (20.5)	64 (18.6)	18 (18.8)
CC, n (%)	3 (1.8)	7 (2.0)	0 (0)
C	0.12	0.11	0.09

C indicates C allele frequency.

and a 250-bp product, respectively, with a 387-bp common product (see Figure 1).

Statistical Analysis

Results are represented as the mean±SD. All statistical analyses were performed by using Statview (version 5.0, SAS Institute Inc). A difference was considered statistically significant at $P<0.05$.

Results

The demographic data of the studied populations are shown in Table 1. A slight difference in body mass index was observed between the definite smokers and the nonsmokers. Triglyceride levels were higher in the smokers. Urinary IsoP in the smokers was nearly double that in the nonsmokers, as reported previously.[16] This observation indicated that the criterion for the definite smokers used in the present study was reasonable as far the level of oxidative stress was concerned. In spite of this, smoking status affected neither CBF nor CVR significantly, as indicated in Table 1.

In the subsequent analysis, we evaluated the effect of the T-786C genotype on smoking. In Table 2, genotype frequencies of T-786C are compared among the nonsmokers, the definite smokers, and the reference population. The 3 cohorts were in Hardy-Weinberg equilibrium, and their genotype frequencies did not differ significantly from one another (χ^2 2.23, df 4, $P=0.69$). This result suggested no apparent genetic stratification in the studied subjects. We used a mixed male and female population as a reference, although the studied populations consisted of males. Because the eNOS gene is located on chromosome 7, the eNOS genotype should be independent of sex. Actually, when the 502 female participants of the health examination were studied, the genotype frequencies were 1.2%, 22.1%, and 76.7% for CC, TC, and TT, respectively, which were not significantly different from those in the male population. In addition, ecNOS4 a/b was genotyped in 257 randomly selected subjects, showing complete linkage disequilibrium between ecNOS4 and T-786C; only 2 haplotypes, C-786/ecNOS4a and T-786/ecNOS4b, were identified in this population (data not shown). Because of this complete linkage disequilibrium, ecNOS4 a/b was not analyzed further.

When the effect of the T-786C genotype on MBP, CBF, and CVR was analyzed, CC homozygotes showed significantly lower CBF (56.6±13.3, 57.6±11.5, and 44.0±7.2 mL/min per 100 g tissue for TT, TC, and CC, respectively; $P=0.03$ by ANOVA) as well as higher CVR (1.76±0.44, 1.72±0.42, and 2.24±0.41 mm Hg · min · mL^{-1} for TT, TC, and CC, respectively; $P=0.01$ by ANOVA) than did heterozygotes and TT homozygotes in the definite smokers

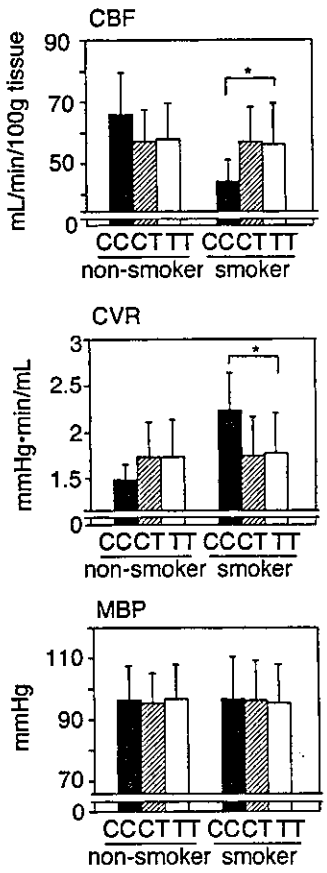

Figure 2. Effects of T-786C genotype on cerebral circulation in smokers and nonsmokers. Effects of T-786C genotype of the eNOS gene on CBF, CVR, and MBP were examined in smokers and in nonsmokers. Each column and line indicates a mean and SD, respectively. *$P<0.05$ vs TC and TT by ANOVA.

(Figure 2). In contrast, there was no apparent difference of CVR or CBF among the 3 genotypes in the nonsmokers (Figure 2). MBP was not different among the 3 genotypes either in the smokers or in the nonsmokers.

Interaction between smoking and T-786C genotype was further supported when the correlation between CBF and the smoking index was analyzed for each of the 3 genotypes separately. The inverse correlation was most evident for CC homozygotes among the 3 genotypes ($r=-0.55$ and $P=0.11$, $r=-0.10$ and $P=0.30$, and $r=-0.05$ and $P=0.32$ for CC, TC, and TT, respectively).

Although age was thought to be inversely correlated with CBF, we found no significant correlation between age and CBF in the nonsmokers ($r=-0.26$, $P=0.75$; data not shown), indicating that age, per se, did not have a major effect on CBF in the studied population. In the smokers, the mean age of CC homozygotes tended to be higher than that of the other 2 genotypes (63.6±10.8 years for CC, 56.8±6.7 years for TC, and 57.5±7.0 years for TT; $P=0.06$ by ANOVA). Even though age itself was not likely to influence CBF significantly, as indicated above, this difference of age among the 3 genotypes might affect the results. To address this possibility, CBF of subjects with the CC genotype was compared with CBF of TT and

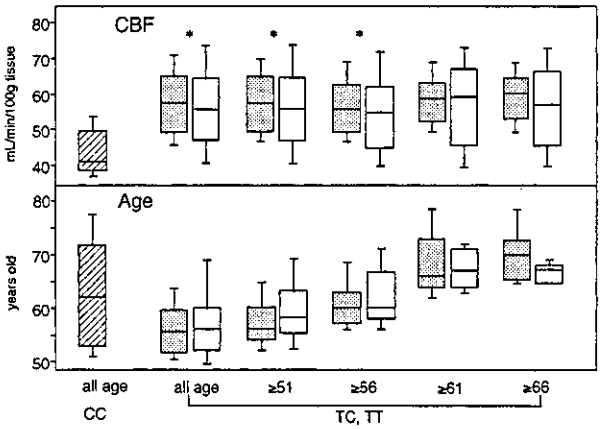

Figure 3. Effects of age on CBF in the smokers with TC and TT genotypes. A stepwise exclusion of younger subjects was performed in TC (shaded bars) and TT (open bars) genotypes. Although the distribution of age went up with the stepwise exclusion (bottom panel), CBF did not change (top panel). *$P<0.05$ among CC (of all ages, hatched boxes), TC, and TT by ANOVA.

TC genotypes extracted according to age (Figure 3). Although the range of age increased with a stepwise exclusion of younger subjects, CBF did not decrease to the level of the CC subjects. This result as well as the fact that no significant correlation was found between age and CBF in the nonsmokers supported the notion that the lower CBF in the CC homozygotes was not due to their higher age.

Recently, some medications for hypertension, diabetes mellitus, and hypercholesterolemia were reported to reduce oxidative stress.[24–28] To evaluate the potential effects of such medications, we performed an analysis after excluding the subjects receiving them. The result was essentially unchanged (for CBF, 57.8±14.5, 59.1±12.8, and 45.2±7.1 mL/min per 100 g tissue for TT, TC, and CC, respectively [$P=0.08$]; for CVR, 1.67±0.42, 1.64±0.43, and 2.25±0.45 mm Hg · min · mL^{-1} for TT, TC, and CC, respectively [$P=0.005$]), implying that the effects of such medications were negligible in the studied population.

Because this polymorphism seemed to influence the cerebral circulation, NOx, stable metabolites of NO, were measured in the subjects. Although NOx tended to be low in CC homozygotes in smokers and also in nonsmokers, no significant difference was obtained among the 3 genotypes (Figure 4).

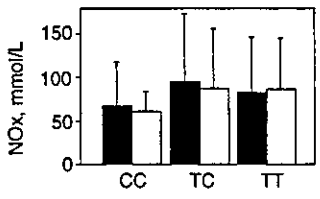

Figure 4. Effects of smoking and T-786C eNOS genotype on plasma NOx. Plasma NOx was measured as described in Methods. Each column and line indicate a mean and SD, respectively. Solid and open bars represent nonsmokers and smokers, respectively.

Discussion

The major finding of the present study is that an interaction between smoking and the eNOS genotype could play an important role in the regulation of cerebral circulation. It should be emphasized that these observations were obtained under a setting of genetic epidemiology, which suggested that the effect of the eNOS genotype could actually influence the cerebral circulation in a general population.

We showed that the effect of T-786C on CVR and CBF was apparent only in smokers. This finding suggested that interaction between smoking and the eNOS genotype increased CVR. T-786C has recently been shown to affect the promoter activity of the eNOS gene; a luciferase promoter assay indicated that a construct with C-786 decreased the promoter activity by 50%.[13] Meanwhile, smoking is known to induce oxidative stress, which is a potent suppressor of eNOS activity.[14,29] Alternatively, such oxidative stress might promote the degradation of NO.[30,31] Inasmuch as smokers showed greater urinary secretion of a biochemical marker for oxidative stress (see Table 1), smoking may cause a further decrease in the NO reservoir in CC homozygotes, resulting in the changes in cerebral circulation. This view was supported by the observation that the correlation between the smoking index and CBF was most evident in the CC homozygotes (see Results).

A similar interaction between smoking and the eNOS gene was observed in coronary artery disease; Wang et al[22] demonstrated that the ecNOS4 a allele, which was in strong linkage disequilibrium with the C-786 allele, was associated with coronary artery disease only in smokers. Nakayama et al[13] showed that the risk of coronary spasm in those with the C-786 allele was greater in smokers than in nonsmokers. Furthermore, measuring the eNOS mRNA and protein levels in placentas, Wang et al showed that T-786C and ecNOS a/b polymorphisms influenced the eNOS expression only in smokers.[14] These observations together with the present study strongly suggest that the eNOS gene polymorphism is a genetic factor potentiating the adverse effect of smoking.

To obtain further evidence for the effects of smoking and eNOS genotype, we measured plasma NOx in our population. The results indicated that although NOx tended to be lower in CC homozygotes, there was no significant difference in levels between smokers and nonsmokers or among the 3 genotypes of eNOS T-786C. We found large variances in plasma NOx levels in our population. Plasma NOx originated from dietary intake as well as from endogenous synthesis.[32] Therefore, overnight fasting has been used to reduce the influence of dietary NOx in most clinical and population-based studies[33–35] because plasma NOx levels after 12 hours of fasting were indicated to be mainly reflective of endogenous NO production.[33,34] However, the results of the present study implied that the noise from dietary intake might still perturb the NOx measurement after overnight fasting in our population. Consistent with this, several studies pointed out that at least 48 hours on a low nitrate diet was necessary to exclude the influence of dietary NOx.[36,37] Accordingly, stricter study designs may be required to clarify the genetic effects of the eNOS gene on plasma NOx levels accurately in our population. In spite of such limitations, the trend of lower NOx

observed in CC homozygotes seems consistent with a previous study showing a significant effect of ecNOS4 on plasma NOx levels in another Japanese population.[35]

White matter changes (or leukoaraiosis) are thought to be closely related to vascular dementia.[38] In this pathological condition, decreased CBF and hyalinized thickening of cerebral arterioles were constantly observed.[20,21,38] It is thought that diffuse arteriopathy based on long-lasting hypertension interacting with other unknown environmental and genetic factors resulted in increased CVR, hypoperfusion, and, finally, demyelination in the white matter to establish such pathological conditions.[38,39] In the present study, we indicated that the combination of smoking and the eNOS genotype could influence CBF and CVR even in the subjects without any pathological white matter changes. Given the pathophysiological roles of NO in the cerebral circulation,[8-11] this observation implies that eNOS is a logical candidate for a genetic risk factor that accelerates vascular dementia, especially in smokers. Because the importance of genetic factors in the development of white matter changes has been pointed out,[38,40] it is of interest to study the effect of eNOS genotype on the progression of leukoaraiosis or vascular dementia under a case-control and a prospective study design.

There are some limitations of the present study. CBF and CVR are likely affected by multiple genetic and environmental factors. Therefore, one should be aware of confounding factors influencing the interpretation of the results. It may be best to apply multiple regression analysis to these parameters to take account of confounding factors. However, this is difficult to do in the present study because (1) the smoking index did have a normal distribution because of the large number of the nonsmokers (ie, smoking index 0), (2) the genotype was a categorical datum that was not easy to include in a regression analysis, and (3) the small number of CC homozygotes did not allow for multivariate analysis, even though we genotyped >1000 subjects. Because of this, we applied a univariate analysis with careful consideration of possible confounding factors. The largest possible confounding factors were sex, white matter changes, and age. We excluded the former 2 and indicated that age, per se, was not likely to have large effects on CBF in our population (see Results).

In the interview, we did not take down information about being a current smoker or exsmoker. Because an in vivo study has suggested that the cessation of smoking results in a rapid decrease of oxidative stress estimated with IsoP,[41] current smoking status, not past smoking status, might have a major effect on the cerebral circulation. However, we could not exclude the possibility of chronic effects of smoking on the cerebral circulation being due to secondary pathological changes in the cerebral arteries caused by a long-lasting decrease of NO activity.[11,42] In this context, it is necessary to examine whether the cessation of smoking can restore CBF in CC homozygotes.

We focused on the 2 polymorphisms, T-786C and ecNOS a/b, in the present study because they have been repeatedly shown to alter eNOS function or NO metabolism.[13,14,35] We did not include Glu298Asp in the study because this SNP was not likely to have a functional role in eNOS activity.[14,43] However, because several studies have suggested an association of this SNP with cerebrovascular disorders,[44] it may be interesting to determine whether it has significant effects on the cerebral circulation in a separate study.

Acknowledgments

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□ORIGINAL ARTICLE□

Frequency and Pathogenesis of Silent Subcortical Brain Infarction in Acute First-ever Ischemic Stroke

Tomohide ADACHI*, Shotai KOBAYASHI and Shuhei YAMAGUCHI

Abstract

Objectives **We have often observed silent subcortical brain lesions on CT or MRI in first-ever ischemic stroke, but there is little published information on the relationship of these lesions to stroke subtypes. Here, we describe the incidence of MRI-detected silent subcortical brain lesions, including infarctions and white matter lesions, in a series of patients with first-ever ischemic stroke classified according to stroke subtypes. We also discuss the pathogenesis of these silent subcortical lesions.**

Patients **We evaluated 171 patients with acute first-ever ischemic stroke.**

Methods **The subjects were divided into three groups: lacunar, atherothrombotic and cardioembolic infarction groups. We evaluated silent subcortical brain infarction (SSBI), enlargement of perivascular space (EPS), and other white-matter lesions using MRI.**

Results **Hypertension was observed in 67.6% of lacunar infarction, 57.1% of atherosclerotic infarction, and 54.1% of cardioembolic infarction. SSBI was more frequently observed in lacunar infarction than the others (lacunar vs. atherothrombotic vs. cardiogenic infarction, 81.5% vs. 44.4% vs. 42.1%, p=0.006). High-grade EPS (grade 2 or higher) was also observed more frequently in lacunar infarction than in the others (lacunar vs. atherothrombotic vs. cardiogenic infarction, 63.3% vs. 24.2% vs. 0%, p<0.001). Scheltens' score of silent subcortical lesions was significantly higher in lacunar infarction than in the others.**

Conclusions **The frequency of silent subcortical ischemic brain lesions was significantly higher in lacunar infarction than in atherosclerotic or cardioembolic infarction. We suggest that the pathogenesis of silent subcortical ischemic brain lesions is common to that of lacunar infarction, that is, small-vessel vasculopathy.**
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Key words: acute ischemic stroke, silent infarct, magnetic resonance imaging, small-vessel disease

Introduction

We have often observed silent subcortical brain lesions on brain CT or MRI in the elderly. Several previous studies have reported the incidence of silent brain infarction, i.e. infarction not associated with any apparent symptoms. In an apparently healthy population, the frequency of silent brain infarction was 7.9%–47% (1–8). In ischemic stroke patients, the frequency was reported to be 11%–68% on the basis of CT and MRI findings (4, 7, 9–11). We have previously reported that 10.6%–13% of apparently healthy adults had silent subcortical brain infarction as revealed by MRI (1, 5). Shinkawa et al found that 12.9% of autopsy cases had silent cerebral infarction in the Hisayama study (6). Okada et al reported that 89% of patients with initial hypertensive brain hemorrhage had silent lacunes manifested on MRI (12). They indicated that hypertensive brain hemorrhage exhibits pathological changes similar to those associated with lacunar brain infarction. However, few reports have described the frequency of silent brain infarction in first-ever ischemic stroke classified according to the subtypes of ischemic stroke, such as lacunar infarction, atherothrombotic infarction, and cardioembolic infarction (10, 11), with only one report being based on MRI findings (11). There has been no report of the frequency of silent lesions among stroke subtypes in Japanese. The pathogenesis of silent brain infarction is not obvious, and the frequencies of other subcortical lesions, such as periventricular hyperintensity, white-matter hyperintensity, and enlargement of perivascular space, among the subtypes of ischemic stroke are also unclear. The relation between the subtypes of ischemic stroke and the pathogenesis of these silent subcortical lesions has not yet been clarified. In the present study, we investigated the frequency of MRI-detected silent subcortical brain infarction (SSBI) and other silent brain lesions in patients with first-ever ischemic stroke according to subtype. The results provide a clue to the possible pathogenesis of silent subcortical brain lesions.

Patients and Methods

We evaluated 171 patients (103 men and 68 women; ages

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ranging from 26 to 93 years; mean age 69 years) with acute first-ever ischemic stroke who had been admitted sequentially to Shimane Medical University Hospital from 1994 to 1997. The information regarding the subjects was gathered by means of a retrospective review of the patients' medical records. These subjects were divided into three groups: atherothrombotic infarction (53 patients, mean age 68 years), lacunar infarction (74 patients, mean age 67 years), and cardioembolic infarction (37 patients, mean age 73 years). Seven cases were unclassified and thus were excluded from this study. This classification followed the criteria of NINDS 90 (13). We used information regarding the onset of stroke, neurological findings, risk factors of ischemic stroke, findings of brain MRI, and intra- and extracranial vascular lesions seen on MR angiography, conventional angiography and duplex carotid sonography, as well as the results of electrocardiography, and ultrasound echocardiography. Atherothrombotic infarction was defined as cases with severe stenosis (>50%) or occlusion of the intracranial or extracranial large vessel on the affected side. Such patients showed cortical symptoms and/or cortical infarction or large subcortical infarction. Lacunar infarction was defined as small subcortical infarction less than 2 cm in diameter, without cortical symptoms and large vessel lesions. Cardioembolic infarction was diagnosed in patients who showed sudden-onset neurological deficit with arrhythmia, e.g., atrial fibrillation, cardiomyopathy, or an intracardiac embolic source on an ultrasound echocardiogram. Lacunar-sized small infarction resulting from large vessel disease was classified as atherothrombotic infarction, and that resulting from cardioembolism was classified as cardioembolic infarction, following the pathogenesis in each case.

We assessed the risk factors for ischemic stroke, i.e., age, sex, hypertension, diabetes mellitus, hypercholesterolemia (history or total cholesterol greater than 220 mg/dl at admission), current smoking and atrial fibrillation. Laboratory analysis involved complete blood cell count and blood coagulation including fibrinogen, prothrombin time, active partial thromboplastin time, and biochemistry studies including fasting blood glucose, total cholesterol, triglyceride, and high-density lipoprotein cholesterol.

Brain MRI was performed using a 1.5T superconducting unit (Signa Advantage, GE; Gyroscan ACS-NT, Philips). We obtained T2-weighted images (TR: 3,800–2,000 msec, TE: 140–100 msec), T1-weighted images (TR: 400–350 msec, TE: 14–12 msec), and proton density-weighted images (TR: 3,800–3,500 msec, TE: 26–20 msec). Transaxial images were obtained with 6 mm thickness without a gap. Two-dimensional Fourier transformation of the images and a 256×256 data acquisition matrix were used. We determined the high signal intensity area responsible for neurological symptoms from the T2-weighted images.

We defined SSBI as high intensity lesion having a size of ≥3 mm on T2-weighted images and low intensity on T1-weighted images, following the criteria of the Workshop Study on "Diagnostic Criteria for Asymptomatic Cerebrovascular Diseases" (14). We measured the size of the high-intensity lesion using axial views. Enlargement of perivascular space (EPS) was defined as a lesion with a maximum diameter of <3 mm, which was seen as a spotty high signal intensity on the T2-weighted image and the same intensity as cerebrospinal fluid on the proton density-weighted image (14, 15). EPS was classified into 4 grades, based on lesions at the basal ganglia: Grade 0: no lesion, Grade 1: 1–5 lesions, Grade 2: 6–10 lesions, Grade 3: >10 lesions. We defined lesions of grade 2 or higher as high-grade EPS. We semiquantitatively evaluated silent subcortical lesions, including SSBI and EPS, on the T2-weighted images, as well as periventricular hyperintensity (PVH), white matter hyperintensity (WMH), basal ganglia hyperintensity (BGH), and infratentorial hyperintensity (ITH), using the modified method reported by Scheltens et al (16, 27). High intensity lesions were scored as either five or six points according to the number and diameter of lesions in each area, such as the frontal, parietal, temporal, and occipital regions. The total score was calculated using these values.

Three observers evaluated the MRIs in a blind fashion. One observer evaluated all the MRIs, while the two other observers evaluated 25 MRIs independently, in order to estimate the reliability of the interpretation of the radiological findings. These observers were unaware of the nature of this study, and had no clinical information regarding the patients, except for that shown on the MRIs.

Lesions in the extracranial carotid artery were evaluated using conventional angiography, and/or carotid duplex sonography (DCS) by means of a 7.5 MHz probe (Ultramark 9, ATL; SSD-2000, Aloka). We classified vascular lesions caused by atherosclerosis into categories of mild stenosis (<50%), severe stenosis (≥50%), and occlusion at the origin of internal carotid artery (ICA). We referred to the example of the European Carotid Surgery Trial and determined the ratio of the diameter of the internal lumen to the whole vessel on angiography and DCS (17). The intracranial arteries were evaluated by 3D-time-of-flight MR angiography and/or conventional cerebral angiography. We defined mild stenosis as a partial narrowing of the caliber of the artery, severe stenosis as partial disappearance of the flow signal, and occlusion as complete interruption of the flow signal (18, 19).

The nominal variables (risk factors for ischemic stroke, frequency of SSBI and grades of EPS) were analyzed using the chi-square test. The relation between age and stroke subtypes was analyzed using the analysis of variance. Differences of PVH, WMH, BGH, ITH, and total scores among stroke subtypes were analyzed using the Kruskal-Wallis test. The relation between SSBI and cerebrovascular risk factors was analyzed using uni- and multivariate logistic regressions. All variables with $p<0.10$ in the univariate analysis were included in multivariate analysis. A value of $p<0.05$ was used as the criterion of statistical significance. The inter-observer reliability in scoring the silent brain lesions was assessed using Pearson's correlation coefficient.

Results

Inter-observer reliability was good for all scores relating to silent brain lesions (R=0.763 to 0.895, average 0.845).

The frequencies of risk factors for ischemic stroke are shown in Table 1. There was no significant difference in age among the three groups. Hypertension was more frequent in the lacunar infarction group, but no statistically significant difference was seen among the three groups; lacunar infarction vs. atherothrombotic infarction vs. cardioembolic infarction was 67.6% vs. 60.4% vs. 56.8%. Atrial fibrillation was significantly more frequent in the cardioembolic infarction group (56.8%) than in the others. Laboratory values showed no significant differences among the three groups.

In the atherothrombotic infarction group, 34.3% of the subjects showed severe stenosis or occlusion of the homolateral internal carotid artery; severe stenosis and occlusion were seen in 25% and 75%, while 57.1% of subjects had stenosis or occlusion of the homolateral middle cerebral artery of the affected side (severe stenosis and occlusion in 20% and 80%, respectively). Other patients with atherothrombotic infarction showed infratentorial infarction and stenosis of the anterior cerebral artery. There was no case with large vessel lesions in the lacunar infarction group. In the cardioembolic infarction group, the frequency of occlusive lesion of the homolateral internal carotid artery was 9.5%, while the frequency of occlusion of the middle cerebral artery was 19.0%.

The frequency of SSBI was 56.7% in all groups (Table 2). The incidence of SSBI was higher in the lacunar infarction group than in the other two groups (lacunar infarction vs. atherothrombotic infarction vs. cardioembolic infarction, 81.1% vs. 45.3% vs. 43.2%, p=0.006). The frequency of high-grade EPS (grade 2 or higher) was 31.6% all groups. The grade of EPS was also higher in the lacunar infarction group than in the other two groups (p<0.001). High-grade EPS amounted to 63.5% in the lacunar infarction group, 24.5% in the atherothrombotic infarction group, and 0% in the cardioembolic infarction group.

Scheltens' score for PVH, BGH, and the total score of silent subcortical lesions were significantly higher in lacunar infarction than in the others (PVH, BGH, and total score; p=0.001,

Table 1. Frequencies of Risk Factors and Atherosclerotic Complications Among Subtypes of Cerebral Infarction

Risk factor	Atherothrombotic infarction (n=53)	Lacunar infarction (n=74)	Cardioembolic infarction (n=37)	P-value
Age	71.6±10.3	71.2±9.3	73.8±10.0	0.417
Sex (male), (%)	36 (67.9)	50 (67.6)	23 (62.1)	0.774
Hypertension, (%)	32 (60.4)	50 (67.6)	20 (54.1)	0.441
Diabetes mellitus, (%)	21 (39.6)	22 (29.7)	7 (18.9)	0.120
Hyperlipidemia, (%)	6 (11.3)	16 (21.6)	3 (8.1)	0.124
Atrial fibrillation, (%)	1 (1.9)	2 (2.7)	21 (56.8)	<0.001
Current smoking, (%)	15 (28.3)	26 (35.1)	6 (16.2)	0.447
Ischemic heart disease, (%)	5 (9.4)	10 (13.5)	5 (13.5)	0.687
ASO, (%)	2 (3.1)	2 (2.7)	2 (5.4)	0.948

ASO: arteriosclerosis obliterans.

Table 2. Frequency of Silent Subcortical Lesions

Silent subcortical lesions	Total (n=171)	Atherothrombotic infarction (n=53)	Lacunar infarction (n=74)	Cardioembolic infarction (n=37)	P-value
SSBI, (%)	97 (56.7)	24 (45.3)	60 (81.1)	16 (43.2)	0.006
EPS, (%)*	54 (31.6)	13 (24.5)	47 (63.5)	0 (0.0)	<0.001
PVH score	5.4	5.0	7.6	2.9	0.001
WMH score	10.9	10.4	14.6	6.6	<0.001
BGH score	11.3	8.1	19.7	4.8	<0.001
Total score	28.8	24.6	43.7	15.1	<0.001

*Grade 2 or greater enlargement of perivascular space. SSBI: silent subcortical brain infarction, EPS: enlargement of perivascular space. Score is average of Scheltens' score of silent subcortical lesions.

Table 3. Univariate Analysis of Risk Factors for SSBI

	Odds ratio (95% confidence interval)	P-value
Age	1.01 (0.99–1.04)	0.323
Sex, female	0.40 (0.19–0.83)	0.015
Hypertension	2.89 (1.50–5.56)	0.001
Diabetes mellitus	1.86 (0.89–3.88)	0.095
Hyperlipidemia	1.12 (0.43–2.92)	0.809
Atrial fibrillation	0.60 (0.27–1.34)	0.218
Current smoking	1.18 (0.59–2.33)	0.639
Alcohol consumption	2.37 (1.21–4.62)	0.011

Table 4. Multivariate Analysis of Risk Factors for SSBI

	Odds ratio			
	Whole infarction	Atherothrombotic infarction	Lacunar infarction	Cardioembolic infarction
Age	–	1.18	–	–
Sex, female	0.46	–	0.15	–
Hypertension	2.97*	12.03*	13.91*	0.27
Diabetes mellitus	1.96	–	–	3.05
Current smoking	–	0.56	–	–
Alcohol consumption	1.63	–	–	6.85

*$p<0.05$. Blanks indicate data not applicable.

<0.001, and <0.001). WMH score was significantly higher in the lacunar infarction group than in the other two groups ($p<0.001$) (Table 2).

The results of univariate analysis of the cerebrovascular risk factors for SSBI are shown in Table 3. Hypertension and alcohol consumption were significant predictors for SSBI. Hypertension, sex, diabetes mellitus, and alcohol consumption were entered into the multivariate analysis (Table 4). Hypertension was the only significant independent predictor for SSBI. Similar univariate analyses for SSBI in the lacunar infarction group, atherothrombotic infarction group, and cardioembolic infarction group were performed (data not shown). Only hypertension was a significant predictor for SSBI in the lacunar infarction and atherothrombotic infarction groups.

Discussion

We present here the frequencies of SSBI according to the subtypes of first-ever ischemic stroke as revealed by MRI. The frequency of SSBI was higher in the lacunar infarction group than in the atherothrombotic infarction and cardioembolic infarction groups. In previous reports, the frequency of SSBI in lacunar infarction was also higher than that in other categories of infarction (10, 12). Boon et al reported that the incidence of silent small infarcts was 29.6% in small deep infarctions examined by CT (10). Chamorro et al also reported that the incidence of silent infarction was 41% in lacunar infarction examined by MRI (11). Lacunar infarction resulted mainly from lipohyalinosis of a small penetrating artery (20–22). We have reported that persons with SSBI should be considered at high risk for clinical subcortical brain infarction or brain hemorrhage, and indicated that the most important mechanism for SSBI was hypertensive small-vessel vasculopathy (5). Laloux et al reported that in patients with silent cerebral infarcts, small-vessel disease may in most cases be the cause of their recent symptomatic cerebral ischemia (23). Boon et al also suggested that small-vessel vasculopathy was a common underlying mechanism of generation of silent small deep subcortical lesions and first symptomatic small deep infarct (10). These reports and the present study suggest that the pathogenesis of SSBI is common to that of lacunar infarction. We suggest that SSBI and lacunar infarction are caused mainly by arteriosclerosis of small penetrating arteries. In our patients, the frequency of SSBI was higher than that described in previous reports. This might reflect a racial difference affecting vascular lesions between Japanese and western populations. For Japanese in Japan, small intracerebral artery disease is more common than in persons of Japanese ancestry in Hawaii (24, 25). Inzitari et al also reported that orientals had intracranial arterial lesions more frequently than extracranial arterial lesions (26). The association between blood pressure and stroke in an eastern Asian population seemed stronger than in a western population, and

the cholesterol concentration was a less important factor in the eastern Asian population (27). Therefore, our results may represent characteristics specific to Japanese and other orientals.

In this study, we examined other silent subcortical brain lesions on MRI. These lesions were also frequently observed in lacunar infarction. The EPS grade was higher in lacunar infarction than in the others. The Scheltens' scores of PVH, BGH, and total score were higher in the lacunar infarction group than in the others, and WMH score was higher in the lacunar infarction group than in the cardioembolic infarction group. Based on these results, we considered that the pathogenesis of these lesions was small-vessel vasculopathy being similar to that of lacunar infarction. EPS is closely related to arteriosclerosis of small penetrating arteries, aging, and hypertension (28). PVH is considered to be an ischemic lesion resulting from small-vessel disease (29, 30). Although the pathogenesis of white matter high intensity lesions on MRI has not been elucidated yet, such lesions may be caused by ischemic processes and seem to be associated with hypertension, aging (8, 31, 32), and arteriosclerosis of small penetrating arteries (33). Thus, WMH could be associated primarily with arteriosclerosis of small vessels, but also partly with atherosclerosis of large vessels. A previous study also suggested that WMH is related to atherosclerosis of large vessels (34). We have previously suggested that the most important and common underlying mechanism for SSBI, focal white matter lesions, and PVH is hypertensive small-vessel vasculopathy (5). Thus, we considered that SSBI and other silent subcortical lesions were related to small-vessel disease.

Hypertension is the strongest risk factor for ischemic stroke, especially in lacunar infarction (20–22). We found that hypertension was frequent in lacunar infarction, but there was no statistically significant difference among the subtypes of ischemic stroke. Hypertension was the most potent risk factor for SSBI as demonstrated by multivariate logistic regression. Several studies have shown that hypertension is a significant risk factor for silent cerebral infarction (3, 5, 6, 10, 12). We have also reported that 28% of asymptomatic adults with SSBI have hypertension, and hypertension is a significant risk factor for SSBI (5). Mast et al reported that the prevalence of hypertension is 73% in lacunar infarction and 64% in non-lacunar infarction (35). These results indicated that hypertension is a common risk factor for both SSBI and lacunar infarction. Millikan and Futrell reviewed several reports on lacunar infarction, and suggested that hypertension is not a specific risk factor, as the incidences of hypertension in patients with lacunes and large infarcts are similar (36). Hypertension may be an essential risk factor for lacunar infarction, as well as for other types of infarction.

Conclusion

In conclusion, the frequencies of SSBI and EPS were higher in lacunar infarction patients than in patients with other types of infarction. Other silent subcortical brain lesions, such as periventricular hyperintensity, white matter hyperintensity, and basal ganglia hyperintensity, were also more commonly observed in lacunar infarction patients than in patients with other types of infarction. The existence of SSBI and EPS in patients who developed symptomatic ischemic stroke suggests the involvement of small-vessel vasculopathy. Hypertension was an important risk factor for SSBI and other silent subcortical lesions, as well as for lacunar infarction.

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□EDITORIAL□

Is Type 2 Diabetes a Risk Factor for Silent Ischemic Brain Lesion?

Key words: MRI, periventricular hyperintensity, white matter hyperintensity, cohort study

Type 2 diabetes (DM) has been known to be a significant risk factor for symptomatic brain infarction not only in the western countries but also in the Hisayama study in Japan (1, 2). In the Hisayama population, lacunar infarction was the most common subtype of cerebral infarction and had a greater variety of risk factors, including not only hypertension but also ECG abnormalities, diabetes, obesity, and smoking, than did atherothrombotic infarction or cardioembolic infarction (2). The mechanism is considered to be acceleration of atheromatous plaque formation in the major intra / extra cranial arteries and microatheroma in branch atheromatous disease. Moreover, it is known that blood coagulability and platelet aggregation are increased in DM patients. In the report based on 1,237 ischemic stroke cases of NINCDS databank including 184 cases with lacunar infarction, DM was a significant risk factor for multiple lacunar infarction (Odds Ratio [OR]: 2.3, Confidential Interval: 1.1–4.5) as well as hypertension (OR: 2.5) (1). Wannamethee (3) emphasized the importance of established type 2 diabetes as an independent risk factor for stroke from a 16.8-year follow-up observation study. There were 347 stroke cases in the 7,649 men. Men who developed diabetes during follow-up (n=320) and men with established type 2 diabetes at screening (n=98) both showed a significantly increased risk of stroke (adjusted relative risk, 2.27). In addition, a J-shaped relationship was seen between nonfasting insulin and risk of stroke in the 5,567 men without diagnosis of diabetes at screening. DeFronzo (4) suggested that insulin resistance is a multifaceted syndrome for DM, obesity, hypertension, dyslipidemia and atherosclerosis.

Concerning the prognosis of brain infarction, Kameyama (5) reported that elderly brain infarction patients with DM showed a significantly poor prognosis 30 years ago. We recently examined the prognosis of the ischemic stroke patients with or without DM using the databank made by our Japanese Standard Stroke Registry Study (JSSRS). The change in Japan Stroke Scale (JSS) of DM group was significantly worsened during admission, and modified Rankin Scale at discharge was also significantly poor in the DM group compared with the non-DM group (6). Tuomilehto et al (7) also reported that diabetic subjects have a very high risk of death from stroke, particularly women.

On the other hand, the role of DM as a risk factor for silent brain infarction or white matter lesion in non-stroke subjects is controversial. Lechner et al (8) reported an increased number of risk factors including hypertension, diabetes, smoking, hyperlipidemia and cardiac disease correlated to the grade of silent white matter lesions.

Our study showed that hypertension (OR: 4.07), diabetes (OR: 2.41), alcohol intake 58 g/day (OR: 2.58), retinal artery sclerosis (OR: 2.14), and age (OR: 1.77) were significant and independent risk factors for subcortical silent brain infarction (cystic lacuna). For white matter lesion except lacuna, and periventricular hyperintensity (PVH) only hypertension and age were independent risk factors (9).

In this issue of the journal, Saitoh et al (10) describe that DM is not a significant risk factor for silent ischemic brain lesion in the Funagata study.

See also p 351.

They studied brain MRI in 187 normal subjects who examined DM using 75 g OGTT. Ischemic rating score and PVH grade were not related to impaired glucose tolerance or DM. Ischemic rating score involved etat crible, lacuna and white matter hyperintensity without T1 low intensity. It would be of interest if they analyze the groups divided into cystic lacuna and T2 hyperintensity without T1 low intensity. Because our previous study which investigated silent brain lesion involving etat crible, cystic lacuna, T2 hyperintensity and PVH showed that the most significant risk factors were age and hypertension, but DM also was mildly related to cystic lacuna (11).

Kertesz et al (12) also demonstrated that the most important risk factors for PVH and "MRI unidentified bright objects" (UBO) were age and hypertension in stroke patients. Liao et al (13) studied the severity of white matter lesions (mainly PVH) associated with hypertension, and found that treated uncontrolled hypertensive subjects have greater odds of white matter lesions than those with treated controlled hypertension in the ARIC study. A postmortem pathological study of incidental subcortical lesions identified on MRI in elderly showed that these histological changes are characteristic of etat crible which, like subcortical MRI lesions, is associated with age and hypertension (14).

Furthermore, a large intervention study for 3,867 DM patients (United Kingdom Prospective Diabetes Study: UKPDS) demonstrated that strict control of DM was significantly reduced the complication rate of microangiopathy but did not

reduce complications of macroangiopathy such as ischemic stroke (15). However, strict control of hypertension with drugs demonstrated a significant risk reduction for stroke (–44%) in DM patients (16).

In conclusion, DM may not be the primary risk factor for silent ischemic brain lesion, especially for white matter lesions including PVH. But silent brain lesion is considered to be a significant risk for symptomatic subcortical infarction. Therefore, it is necessary to elucidate the mechanism causing ischemic stroke in DM.

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Evaluation of the atrial natriuretic peptide gene in stroke

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Abstract

The atrial natriuretic peptide (ANP) gene was, though inconclusive, implied to be etiologically related to stroke in rats and recently in humans. The present study tested the candidacy of ANP for stroke susceptibility by a combination of molecular genetic approaches. First, we undertook an association study using a reported ANP variant, G664A, in two case-control panels independently collected, which involved 970 Japanese subjects. Second, we compared the rat ANP gene sequences and neighboring marker alleles among stroke-prone SHR (SHRSP), normal SHR and WKY of an original inbred colony and we also compared brain ANP expression between SHRSP and normal SHR. In humans, we found no significant association between the 664A variant and stroke in the studied population. In rats, 21 polymorphic sites were identified by direct sequencing of 2170-bp ANP fragments, from which two distinct alleles, SHRSP- and WKY-types, were inferred. From a genealogical point of view, our data indicated that an SHRSP-type allele could not play a determinant role in stroke-proneness. Overall results did not support the disease relevance of ANP, disagreeing with previous reports. Thus, considerable caution should be taken when one attempts to transfer findings in the animal model to humans.

Keywords: Atrial natriuretic factor; Stroke; Genetics; Single nucleotide polymorphism; Association

1. Introduction

Several lines of evidence have supported the substantial involvement of genetic factors in the pathogenesis of stroke [1]. However, studies of the molecular genetics of stroke are not necessarily facile, especially in humans, due to a number of complex features of the disease, e.g. coexistent multifactorial disorders, such as hypertension, diabetes and dyslipidemia. An alternative strategy would be to first explore the predisposition to stroke in the animal model and thereafter, test the corresponding findings in humans, instead of investigating certain candidate genes without any clue. Among animal models thus developed to date, the stroke-prone spontaneously hypertensive rat (SHRSP) is considered of particular interest, since it promotes severe hypertension from the early age of life and also manifests the propensity for stroke on a high salt and low potassium diet [2–4]. Accordingly, this animal has been widely used as a model organism to investigate the etiological relationship between hypertension and stroke.

Recently, two study groups have undertaken genome-wide searches of quantitative trait loci (QTLs) for 'stroke-associated' phenotypes in F2 progeny derived from SHRSP [5,6] and have suggested that predisposition to stroke is at least, in part, under genetic controls independent of hypertension. One group explored genetic susceptibility for latency until the manifestation

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of stroke in F2 progeny between SHRSP and normal (or stroke-resistant) SHR [5], while the other did so for infarct volume after the middle cerebral artery (MCA) occlusion in F2 progeny between SHRSP and Wistar Kyoto (WKY) rats [6]. These genome screens successfully identified four QTLs on three separate rat chromosomes, among which a region around the atrial natriuretic peptide (ANP) gene has drawn substantial attention because markers in the vicinity of ANP on rat chromosome 5 were significantly linked to both phenotypic traits.

Then, the following questions may be asked: (1) whether the ANP gene itself can underlie either (or both) of the 'stroke-associated' phenotypes? (2) whether findings in rats are directly extendable to human stroke? Remarkably, one of the study groups above-mentioned has further proposed ANP as a candidate susceptibility gene based on two lines of evidence. First, SHRSP of a Heidelberg colony (SHRSP/Heidelberg) showed alterations in structure, expression and in vitro function of ANP compared to normal SHR [7]. Second, a case-control study involving 696 white subjects indicated that a single nucleotide polymorphism, G664A, located in exon 1 of the human ANP gene was associated with the risk for stroke [8]. On the contrary, SHRSP of a Glasgow colony (SHRSP/Glasgow) showed no significant differences in either structure or expression of ANP compared to WKY [9]. Rat ANP data from two study groups thus appear discordant and need further confirmation.

Under these circumstances, the present study was designed to evaluate the candidacy of ANP in human and rat stroke. Initially the ANP association was tested in two case-control panels involving 970 Japanese subjects; 270 brain infarction patients and 359 sex- and age-matched controls were included in one panel and 178 patients and 163 controls were included in another panel. This association was primarily tested because ANP could constitute a potential, though not strong, candidate gene for cerebrovascular disorders, whether rat ANP is the susceptibility gene in question or not. Moreover, from a genealogical point of view, disease susceptibility was investigated among SHRSP, normal (or stroke-resistant) SHR and WKY of an original inbred colony, all of which originated from a Wistar rat colony in Japan [4].

2. Methods

2.1. Human subjects

This study was approved by an institutional review committee. Informed consent for participation was obtained from all subjects. Two study panels were independently collected according to classification criteria previously described [10,11]. Briefly, participants in the first panel comprised 270 cases, who were enrolled at the Kitamura Neurosurgery Clinic, Tokyo from September 1996 to May 1997, more than 2 months after the incident of stroke and 359 controls frequency matched by age and sex, who were selected from outpatients at the cardiovascular clinic of the Institute for Adult Diseases, Asahi Life Foundation, Tokyo [10]. Both institutes are in the same area of the megalopolis. The second panel involved three participant groups; (1) 104 subjects with silent brain infarction (SBI) were consecutively enrolled from people undergoing a health screening examination between January 1995 and December 1997 at the Shimane Institute of Health Science; (2) 163 subjects without evidence of SBI on MRI were selected from the same population as controls; and also (3) 74 subjects with symptomatic subcortical infarction were enrolled from outpatients at Shimane Medical University in the corresponding period [11]. Clinical characteristics were defined as a dichotomous phenotype except for age, as depicted in Table 1.

2.2. Genotyping of the G664A polymorphism and statistical analysis

The G664A polymorphism of human ANP was genotyped by the mutagenically separated PCR (MS-PCR) method (Fig. 1) as described in our previous report [12], in which this polymorphism was designated as G191A according to the nucleotide position relative to the transcription start site. Two other single nucleotide polymorphisms (SNPs), C-664G and T1766C, were also genotyped as described previously [12]. The three SNPs had been shown to represent principal ANP polymorphisms in Japanese subjects.

The χ^2-test statistic was calculated between the genotype distribution (or allele frequencies) and stroke status. Because pathological findings of cerebral lesions in SHRSP are similar to those of subcortical (or lacunar) infarction in humans [13], patients with subcortical infarction were analyzed separately in the first panel. Also, because the rat ANP locus is assumed to confer stroke susceptibility independently of hypertension [5,6], each group of cases and controls was stratified by hypertension status to remove its effects on association analysis. Furthermore, confounding influences of six variables listed in Table 1 were assessed in a multiple logistic regression model. The power of a case-control study of the available sample size was determined by calculating the smallest detectable relative risk with 80% power at a 5% Type I error probability [14].

2.3. Experimental animals

We use the name SHR for rats with a low incidence of spontaneous stroke (stroke-resistant SHR or 'SHRSR')

Table 1
Characteristics of the study populations and association analysis of the G664A polymorphism in relation to cerebral infarction

	First panel			Second panel		
	Cases		Controls	Symptomatic subcortical infarction	Silent brain infarction	Controls
No. of subjects (M/F)	270 (127/143)		359 (170/189)	74 (51/23)	104 (62/42)	163 (97/66)
Age (years)	69.5±7.8		68.6±6.4	65.8±10.7*	69.4±9.8*	57.0±7.9
Hypertension (%)	60		57	82*	69*	18
Diabetes (%)	22*		13	28*	12	11
Hypercholesterolemia (%)	57		59	34	52*	37
Smoking (%)	38		34	41	39	34
Genotype distribution for the G664A polymorphism	Total cases	(Subjects with subcortical infarction)				
G/G	230 (85%)	(104 (85%))	298 (83%)	64 (86%)	88 (85%)	127 (78%)
G/A	37 (14%)	(16 (13%))	58 (16%)	10 (14%)	14 (13%)	35 (21%)
A/A	3 (1%)	(3 (2%))	3 (1%)	0 (0%)	2 (2%)	1 (1%)
Frequency of 664A allele	8.0%	(8.9%)	8.9%	6.8%	8.7%	11.3%

In the original report by Rubattu et al. [8], the 664A allele was assumed to confer an increased risk for stroke. First and second panels were collected independently, according to classification criteria previously described in detail [10,11]. Cases in the first panel comprised patients with focal neurological symptoms due to cerebral infarction and excluded those with intracerebral or subarachnoid hemorrhage or those with cardiovascular complications, such as atrial fibrillation, vasculitis and a history of cardiac surgery. Controls in the first panel were ascertained from the same area with the frequencies of age and sex being matched. In the second panel, all subjects were evaluated by MRI; 104 subjects with silent brain infarction and 163 with cerebral infarctions (control group) were selected from people undergoing a health screening examination, while 74 patients with symptomatic subcortical infarction were also included in the study. Classification criteria for confounding factors were as follows: Hypertension was defined by either (1) BP measurements exceeded systolic BP ≧ 160 mmHg and/or diastolic BP ≧ 95 mmHg on two consecutive visits for untreated subjects or (2) chronic antihypertensive treatment of patients. Diabetes mellitus was defined by either (1) fasting plasma glucose concentration ≧ 7.7 mmol/l and/or a diabetic pattern was observed after an oral glucose challenge or (2) those under chronic treatment with oral hypoglycemic agents or insulin. Hypercholesterolemia was defined by either (1) serum cholesterol levels ≧ 5.70mmol/l for those untreated or (2) patients receiving cholesterol-lowering drugs.

* $P < 0.05$ versus controls by t-test of χ^2-test.

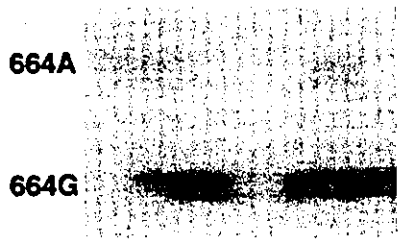

Fig. 1. A G664A polymorphism used for genotyping in the case-control analysis. After MS-PCR, the products were electrophoresed in 6% polyacrylamide/7 M urea gels and transferred to nylon membranes. The membranes were then hybridized with a ^{32}P-labeled primer and subjected to autoradiography.

and SHRSP as a whole hereafter. Ten SHR substrains derived from three colonies were used for DNA analysis in the present study. These included: three SHRSP (A_{1-sb}, A_3 and A_4) and four SHRSR (B_1, B_2, CH and CL) substrains from a colony kept at Kyoto University, Kyoto, Japan (SHRSP/Izm and SHRSR/Izm), SHRSP and SHRSR from the Max Delbruck Center for Molecular Medicine in Berlin-Buch, Germany (SHRSP/Heidelberg and SHRSR/Heidelberg) and SHRSR from the Genetic Resource Section, the National Institute of Health, Bethesda (SHRSR/NIH). The WKY rat from a colony kept at our institution (WKY/Izm) was used as a normotensive control strain. The genealogy of SHR substrains was described elsewhere [2,3,15,16]. Briefly, selective breeding was originally made for stroke-proneness to separate SHRSP from the *A* subline of SHR during F_{24-25} generations (in 1971), by which time three distinct sublines of SHR (the *A–C* sublines) had been maintained in Kyoto. Stroke-resistant SHR, SHRSR, was thereafter derived from the *B* and *C* sublines and seven substrains of SHR (three SHRSP and four SHRSR substrains) have been kept at our institution, all direct descendants of the original colony. SHRSR/NIH was separated from a Japanese colony as early as F_{13} generation and established as an inbred strain at the NIH. SHRSP/Heidelberg was separated from the A_3 substrain of SHRSP/Izm at F_{36} generation (in 1975), but we do not know the details of the origin of SHRSR/Heidelberg. Two SHR substrains of a Japanese colony, descendants of SHRSP (A_3) and SHRSR (B_1), were further investigated in the gene expression study. For this purpose, animals were fed on a regular rat chow diet and sacrificed under pentobarbital anesthesia at 13 weeks of age. All procedures were in accordance with institutional guidelines.

2.4. Sequence comparison of rat ANP among substrains of SHRSP and SHRSR and WKY/Izm

Genomic ANP fragments, 2170-bp in size, were sequenced and compared among seven SHR substrains of a Japanese colony and WKY/Izm. Additionally, a 408-bp fragment spanning from intron 1 to intron 2 was sequenced for SHRSP/Heidelberg, SHRSR/Heidelberg, and SHRSR/NIH. Five overlapping sets of PCR primers were designed to cover a 625-bp 5′-untranslated region (UTR), three exons and two introns and a 591-bp 3′UTR in the rat ANP gene (Fig. 2). After PCR amplification, the products were gel-purified and subjected to cycle-sequencing according to the manufacturer's protocol (Dye-Terminator Cycle sequencing kit) on an ABI 377 DNA sequencer (Applied Biosystems). Information of the PCR primers can be obtained from the authors upon request.

2.5. Substrain comparison of marker alleles on rat chromosome 5

In the region which was assumed to encompass QTLs for 'stroke-associated' phenotypes on rat chromosome 5 [5,6], we examined allele distribution patterns of microsatellite markers among ten substrains of SHR and WKY/Izm. Substrain comparison was made by scoring 42 markers, where the allele size of PCR products was determined in base pair on an ABI 377 DNA Sequencer (Applied Biosystems).

A genetic linkage map of the relevant chromosomal region was constructed by genotyping all informative markers on 110 male F_2 rats involving the A_3 substrain of SHRSP/Izm and WKY/Izm to complete our consensus map. Genotyping and linkage mapping were performed as previously described [17].

As for the ANP locus, to differentiate base substitutions identified in intron 2 of the gene, a set of MS-PCR primers were newly designed as follows:

FP, 5′-AGGATCTGAGCCACGAGCAC-3′
RP-WKY, 5′-TCCCACCAGCCACAGTCTG-3′
RP-SP, 5′-CCAGTGACCAAGTCTTAGCCACCAGC CACAGTCCA-3′
where deliberate differences and base substitutions are underlined [18].

2.6. Gene expression studies

Total RNA was extracted from the whole brain of SHRSP/Izm and SHRSR/Izm for Northern blotting. A semi-quantitative PCR assay was also performed using the full-length cDNAs synthesized from reverse-transcribed mRNA of the brain. The PCR primers were: 5′TCTGATGGATTTCAAGAACCTG3′ (forward) and 5′TCAATCCTACCCC-CGAAGCAG3′ (reverse), where a forward primer was designed to be located on separate exons (exons 1 and 2) in order to eliminate PCR amplification from contaminating genomic DNA. PCR was terminated during the exponential phase and the products were electrophoresed on a 2% agarose gel. Quantitative analysis was performed by densitometric scanning on an AlphaImager 2000 (Alpha Innotech) and normalized by GAPDH levels.

3. Results

3.1. Association of G664A with human stroke

Table 1 shows baseline characteristics in our study panels. Apart from the status of diabetes mellitus, five variables—sex, age, and the status of hypertension, hypercholesterolemia and smoking—were comparable between case and control groups in the first panel. On the other hand, some variables were not comparable among three groups in the second panel, which was inevitable due to the consecutive enrollment scheme. Genotype characterization of the G664A polymorphism was performed by MS-PCR (Fig. 1) and no significant association was seen between case and control groups in either of panels. The results were almost unchanged when each study group was stratified by the presence or absence of hypertension and when confounding factors

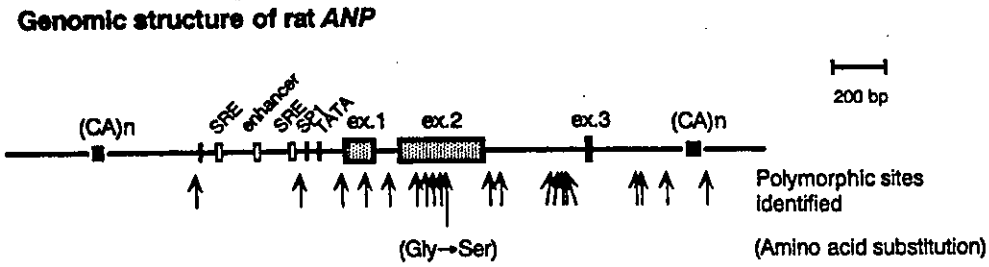

Fig. 2. Sequence differences identified between SP/Izm-type and WKY/Izm-type alleles of the rat ANP gene. Two distinct alleles were inferred from the 21 polymorphic sites identified in the sequenced region. Only a G-to-A substitution at position 485 from the CAP site resulted in an amino acid change, Gly-to-Ser, at residue 100 of prepro-ANP. SRE, serum response element; SP-1, promoter-specific transcription factor.

were adjusted by a multivariate analysis (data not shown). Restricting to patients with subcortical infarction did not influence the results for association. While no significant association was observed between C-664G and T1766C and stroke status, rarer allele frequencies did not exceed 3% in the Japanese population for either of two polymorphisms. This precluded haplotype analysis (data not shown). The prevalence of tested polymorphisms in each study group was consistent with Hardy–Weinberg equilibrium.

3.2. Sequence analysis of rat ANP

A total of 21 base-substitution polymorphisms were identified by direct sequencing of the rat ANP gene (Fig. 2). While six polymorphisms were located in exons, only a G-to-A substitution at position 485 from the CAP site (which was previously numbered as 1125 according to the deposited sequences, K02062 and K02063) [7] resulted in an amino acid change, Gly-to-Ser, at residue 100 of prepro-ANP. In a region of 625-bp upstream of the CAP site, we found three polymorphisms: G-to-T at position −463, C-to-T at position −88, and C-to-T at position 86. Here, a G-to-T substitution at position −463 was localized on the putative PEA-2 polyoma enhancer binding site [19] and a C-to-T substitution at position 86 was 1-bp upstream of the ATG starting codon [20]; these are considered functionally important. Two of the polymorphisms (a G-to-A substitution at position 485 and a G-to-T substitution at position −463) had been shown in a Heidelberg colony [7].

Two distinct types of ANP alleles were inferred from 21 polymorphic sites in the sequenced region. In a Japanese colony, all three SHRSP substrains possessed an identical allele (SP/Izm-type), while four SHRSR substrains and WKY/Izm shared the other allele (WKY/Izm-type). In addition, sequencing of a 408-bp genomic fragment, where six polymorphisms had been detected, revealed that SHRSP/Heidelberg possessed a SP/Izm-type allele, while SHRSR/Heidelberg and SHRSR/NIH possessed a WKY/Izm-type allele (Fig. 3). Judging from four exon 2 polymorphisms reported by Brosnan et al. [9], SHRSP/Glasgow and WKY of a Glasgow colony (WKY/Glasgow) were thought to possess a SP/Izm-type allele.

3.3. Allele distribution patterns on rat chromosome 5

To explore the relevance of substrain differences to 'stroke-associated' QTLs on rat chromosome 5, we determined allele distribution patterns of 42 microsatellite markers among ten substrains of SHR and WKY/Izm (Fig. 3). A small interval (< 10 cM in size) flanked by D5Mit11 and D5Rat24 differed between three SHRSP and four SHRSR substrains of a Japanese colony, whereas allele distribution patterns in the corresponding region were identical among two substrains of a Heidelberg colony and SHRSR/NIH. By contrast, the type of alleles at two adjacent loci, D5Mgh15 and ANP, differed between SHRSP and SHRSR substrains regardless of the origin of colonies.

3.4. Rat ANP gene expression

We found no detectable ANP mRNA expression of the rat brain by Northern blot analysis (data not shown), which appeared in agreement with previous observations in Heidelberg and Glasgow colonies [5,6]. A semi-quantitative assay by reverse-transcription PCR resulted in no significant differences in brain ANP expression between SHRSP/Izm and SHRSR/Izm. In two independent experiments, densitometric units for SHRSP were 91 and 112% of those for SHRSR, respectively, after normalization by GAPDH levels. An example of the reverse-transcription PCR assay is shown in Fig. 4.

4. Discussion

In the present study, we evaluated the ANP gene in relation to stroke susceptibility through a combination of genetic approaches. Our data failed to support the candidacy at large, but brought up several important issues that require careful interpretation. The issues include: (1) how much statistical value can be set on our association analysis in the Japanese subjects; (2) whether the type of rat ANP alleles accounts for part of phenotypic differences between SHRSP and SHRSR (or WKY) at all; and (3) what are the plausible explanations for the observed discrepancies between two previous studies concerning the rat ANP gene [5,6].

Extending findings in rats to humans is certainly tempting, but results should be critically evaluated as far as the rat data remain undefined. While there was no standard method to etiologically stratify stroke patients according to clinical manifestations, we chose to enroll patients with radiographical evidence of cerebral infarction (i.e. abnormal areas on brain CT or MRI) in order to decrease a genetic diversity in overall stroke. Diagnostic criteria for case subjects were no less stringent than those in the original report [8], where cases were sampled in a population-based setting according to individual's medical records or autopsy results. Since our study was performed in a hospital-based setting, one may argue that the results of association could be affected by insufficient matching of controls or other uncontrolled factors. Several findings can refute this criticism. First, despite the use of different enrollment scheme, comparable results were provided for lack of disease association with the ANP locus in two independent populations. Second, the 664A allele frequencies

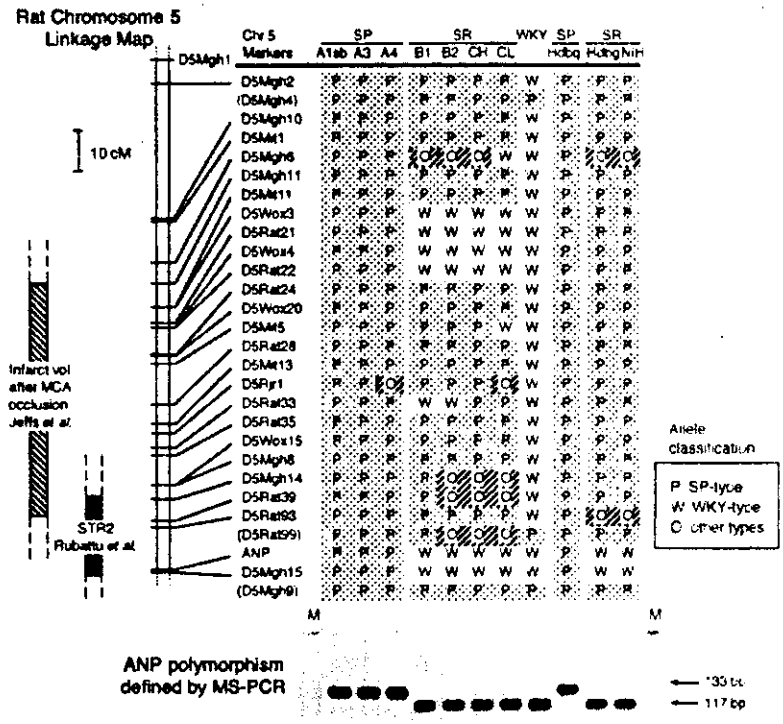

Fig. 3. Substrain comparison in the selected region on rat chromosome 5 (top) and results for an ANP polymorphism defined by MS-PCR (bottom). Marker alleles were categorized into three groups: SP/Izm-type allele (P), WKY/Izm-type allele (W) and alleles different from both types (O). Results for 28 selected markers are depicted in the figure: they were 25 markers informative between A_3 and WKY/Izm and three other markers shown in parentheses. For reference, positions of QTLs for 'stroke-associated' phenotypes are arbitrarily placed to the left of the linkage map, where a likely interval of the QTL for infarct volume after MCA occlusion cannot be precisely defined because Jeffs et al. [6] assigned the ANP locus to the middle of chromosome 5. M, ϕX174 DNA/*Hae*III marker.

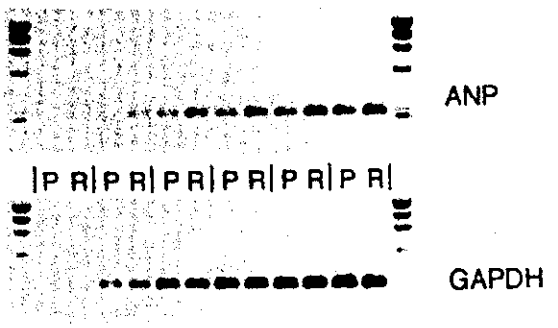

Fig. 4. A quantitative assay by reverse transcription-PCR of brain ANP expression. PCR products were shown with incremental cycles (three additional cycles) from left to right by two consecutive lanes, where products of SHRSP/Izm and SHRSR/Izm were placed in the left and right lanes, respectively. P, SHRSP/Izm; R, SHRSR/Izm.

observed in the present study proved to be concordant with the population frequency (8.9–9.2%) that we previously reported in the Japanese [12]. Furthermore, according to our calculation, a relative risk of >1.9 could have been detected in the available sample of 270 cases and 359 controls in the first panel alone with 80% power at a 5% type I error probability. This allowed us to test the odds ratio of 2.0, which was shown to be indicative of the risk increase in a white population [8]. Also, it has to be noted that the three tested SNPs could represent principal ANP polymorphisms in the Japanese, as demonstrated by our extensive screening [12].

Then we looked into the candidacy of ANP for rat stroke by comparing substrains of SHR. This strategy is based upon the hypothesis that a gene predisposing rats to stroke should have some functional differences between stroke-prone and stroke-resistant strains. Our results showed that two types of ANP alleles differentiated SHRSP from SHRSR substrains at a structural level. To our surprise, however, WKY/Glasgow appeared to possess a SP/Izm-type allele and, more importantly, apart from four substrains tested in the present study, there was a substrain of SHRSR with a SP/Izm-type allele, which had been extinct and only DNA was available from the frozen liver tissue kept at our institution (data not shown). The presence of 21 polymorphisms between two ANP alleles and the presence of a marker (D15Mgh15) showing the same allele distribution pattern as ANP implied that a certain chromosomal fragment rather than a single de novo mutation of the gene had been inherited during the inbreeding process. Taken together, the following situation is probable. There had been at least two distinct types of ANP alleles among ancestral laboratory rats in Kyoto, Japan, from which SHR and WKY were

developed. A few substrains of SHRSR and WKY should have inherited a SP/Izm-type allele, whereas four SHRSR substrains currently kept in our institution happen to have inherited a WKY/Izm-type allele of ANP.

Before we discuss a functional significance of a SP/Izm-type allele, it seems helpful to review 'stroke-associated' phenotypes in the literature. As for latency until the manifestation of stroke, SHRSP/Heidelberg is assumed to have a 'protective' allele of a QTL postulated on rat chromosome 5 compared with SHRSR/Heidelberg [5,21]. In theory, relating a 'protective' allele to SHRSP ironically counterbalances the propensity for stroke. Because such a QTL is unlikely to constitute a principal determinant for selective breeding of stroke-proneness, a 'protective' allele might as well be found in some substrains of SHRSR and WKY. As for infarct volume after the MCA occlusion (or ischemic vulnerability), on the other hand, significant linkage was identified in F2 progeny between SHRSP/Glasgow and WKY/Glasgow [6]. Even so, it remains unclear whether this trait by itself represents a phenotypic difference between stroke-prone and stroke-resistant SHR strains. Some studies have already shown that ischemic vulnerability is observable in SHRSR and may not account for substantial part of strain differences [22,23]. Thus, considerable care should be exercised when we extend the results for 'stroke-associated' phenotypes to stroke-proneness in SHRSP. Nevertheless, it must be stressed that these arguments do not undermine the original findings of linkage to the individual phenotypes because the existence of QTLs is not questionable under given circumstances, i.e. study design and statistical significance levels presented.

Results of the gene expression study fueled further confusion. We found no significant differences in the brain expression of ANP between SHRSP/Izm and SHRSR/Izm, whereas Rubattu et al. reported 3-fold lower expression in the brain of SHRSP/Heidelberg compared with SHRSR/Heidelberg [7]. This discrepancy may be partly attributed to technical problems, as different quantification methods were used to measure relatively low levels of ANP expression. We should also pay attention to differences in blood pressure (BP) profile between Japanese and Heidelberg colonies: SHRSP promotes a severer degree of hypertension from earlier age of life than SHRSR in an original colony [4], whereas both SHRSP and SHRSR are reported to develop a similar degree of hypertension in a Heidelberg colony [5]. It is therefore possible that BP differences in a Japanese colony (a maximal BP difference exceeds 30–40 mmHg) modify the brain ANP expression towards the reduction of strain differences shown in a Heidelberg colony [7]. In fact, elevated BP was supposed to induce higher mRNA expression of brain ANP in SHR when compared to WKY [24]. Alternatively, despite compelling evidence in experiments in vitro [7], functional alterations of a SP/Izm-type allele may not manifest themselves in vivo dependent on the genetic background, i.e. in SHRSP of a Japanese colony. Further investigations are currently in progress to resolve the uncertainties using a more sensitive quantification technique.

Although human and rat parts of our study do not appear complementary to each other, our data collectively lead to the argument that the ANP gene is unlikely to play a major role in the propensity for stroke. In view of a presumably complex interplay between causative genes and confounding phenotypes, such as hypertension, integration of various genetic strategies is required to clarify the pathophysiological role of the ANP gene in stroke. Findings in the current study are indispensable in this regard.

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