VEGF Receptor-2 Variants Are Associated With Susceptibility to Stroke and Recurrence

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Background and Purpose—Dysregulation of vessel wall formation, growth, and maintenance may confer susceptibility of stroke.

Methods—We tested the hypothesis that variants in 2 genes encoding vascular endothelial growth factor and vascular endothelial growth factor receptor-2 are associated with susceptibility to stroke and its recurrence in a Chinese case–control study comprising 1849 patients with stroke and 1798 control subjects and replicated the investigation in an independent study comprising 327 cases and 327 control subjects. The correlation of variants with carotid artery intima media thickness was examined in 1123 healthy individuals.

Results—Compared with their corresponding wild-type genotypes, one coding variant, rs2305948 (Val297Ile), in the vascular endothelial growth factor receptor-2 gene was associated with increased susceptibility to intracerebral hemorrhage (additive model: OR, 2.06; 95% CI, 1.64 to 2.59; \( P = 7.6 \times 10^{-10} \); dominant model: OR, 2.20; 95% CI, 1.70 to 2.84; \( P = 1.5 \times 10^{-8} \)), a promoter variant rs2071559 (−604T>C) in the gene was associated with reduced susceptibility to atherothrombotic stroke (additive model: OR, 0.82; 95% CI, 0.71 to 0.93; \( P = 0.003 \); dominant model: OR, 0.78; 95% CI, 0.65 to 0.92; \( P = 0.004 \)) and was reversely correlated with carotid artery intima media thickness (\( P = 2.8 \times 10^{-13} \)). Replication in the second study yielded similar results. During a median 4.5 years of follow-up for the first stroke population, 355 recurrent strokes were documented. Subjects carrying 297Ile had a higher risk for stroke recurrence (relative risk, 1.40; 95% CI, 1.12 to 1.75; \( P = 0.003 \)), and those with −604C had a lower risk for recurrence (relative risk, 0.71; 95% CI, 0.58 to 0.89; \( P = 0.002 \)) than their wild-type carriers.

Conclusions—The vascular endothelial growth factor receptor-2 gene variants may serve as novel genetic markers for the risk of stroke and its recurrence. (Stroke. 2009;40:2720-2726.)

Key Words: follow-up studies ■ genetics ■ risk factors ■ stroke ■ vascular endothelial growth factor
studies have examined these 2 genes in relation to stroke, especially given that the pathogenic features for ischemic and hemorrhagic strokes are known to differ.

Therefore, we tested the hypothesis that the functional variants altering expression levels or protein structure of VEGF and VEGF receptor-2 are associated with susceptibility to stroke in an initial large case–control stroke study in China, and the findings were then replicated in a second independent case–control study. We also assessed the relationship between genetic variants and stroke recurrence by prospectively following up the first stroke population.

Methods

Stroke Sample Population

The Multicenter Chinese Stroke Study has been described previously. Briefly, 2000 consecutive patients with stroke (age 35 to 74 years) and age-, gender-, and resident area-matched control subjects were recruited from November 2000 to November 2001 from 7 clinical centers in northern China. Stroke, defined as the sudden onset of a nonconvulsive and focal neurological deficit persisting for >24 hours, was confirmed by strict neurological examination, CT, or MRI according to the International Classification of Diseases, 9th Revision. We recruited 3 subtypes of stroke: cerebral thrombosis (thrombosis), lacunar infarction (lacunar), and ICH. The enrolled patients were survivors of acute stroke events. Other types of stroke (embolic stroke and subarachnoid hemorrhage) and severe systemic diseases (collagenosis, inflammation, liver, neoplastic, or renal diseases; endocrine and metabolic diseases but not diabetes) were in the range of exclusion.

This stroke population was followed up at a 2-year period until May 31, 2006, by a standard questionnaire and telephone contact by physician investigators to assess the recurrence of stroke. Before data assessment, we excluded 353 subjects because of lack of definite diagnosis (24 cases), absence of plasma (76 cases, 93 control subjects), insufficient DNA (51 cases, 104 control subjects), and unqualified control subjects who developed stroke during the follow-up. Complete data were available for analysis in 1849 cases and 1798 control subjects.

The second independent case–control study comprised 327 strokes and 327 control subjects that were recruited from October 2004 to August 2005 in Henan Province, China. The diagnostic criteria for stroke were identical to those used by the first case–control stroke study. Because no significant association was found between genetic variants and lacunar stroke in the initial case–control study, only patients with cerebral thrombosis or intracerebral hemorrhage were enrolled in this replication stroke sample population.

The study was approved by the local ethics committees of collaborating hospitals. All participants reported themselves as Han nationality and provided written informed consent.

Biochemical Variable Determination and Clinical Data Collection

Blood samples were collected after a 12-hour overnight fast. In acute medical events, blood collection was delayed for at least 6 weeks. Blood samples were collected after a 12-hour overnight fast. In acute medical events, blood collection was delayed for at least 6 weeks. Plasma and cell buffy coat were separated by centrifuge and kept at −70°C. Biochemical variables, including blood glucose, total cholesterol, triglycerides, and high-density lipoprotein cholesterol, were determined by using an automatic analyzer (Hitachi 7060, Tokyo, Japan). A complete clinical and family history and the conventional vascular risk factors were recorded: cigarette smoking, alcohol consumption, body mass index, and systolic and diastolic blood pressure. Hypertension was defined as a mean of 3 independent measures of blood pressure ≥140/90 mm Hg or the use of antihypertensive drugs. Diabetes mellitus (DM) was diagnosed when the subject had a fasting blood glucose ≥7.0 mmol/L or ≥11.1 mmol/L at 2 hours after oral glucose challenge or treatment with insulin or oral hypoglycemic medication. Stroke severity was measured using the Chinese Scale of Clinical Neurological Deficit of Stroke Patients, which was adapted from the National Institutes of Health Stroke Scale.

Assessment of Stroke Recurrence

The recurrent stroke was defined using the following criteria: there was clinical evidence of the sudden onset of a new focal neurological deficit with no apparent cause other than that of vascular origin occurring at any time after the index stroke or there was clinical evidence of the sudden onset of an exacerbation of a previous focal neurological deficit with no apparent cause other than that of vascular origin occurring >21 days after the index stroke. All reports of stroke events were confirmed by a local independent neurologist based on direct review of patient medical records and brain imaging. In those who had more than one recurrent stroke, only the first one was considered. In cases of death, the medical records and the death certificates were reviewed, and a close relative or a caregiver was interviewed to screen for recurrences.

Gene Variants Selection and Genotyping

The VEGF gene is located on chromosome 6q12. According to the HapMap genotype data and potential biological functions, 3 tagging variants in the promoter region, affecting VEGF expression, were selected: rs833061 (−460T>C), rs1570360 (−116G>A), and rs2010963 (405G>C) with positions defined by the transcription initiation site as +1.

VEGF receptor-2 gene kinase domain-containing receptor is located on chromosome 4q11. We screened the entire coding region and the 2-Kb 5′-promoter region in 96 chromosomes by sequencing. Three common variants were found in the promoter region, rs2071559, rs9994560, and rs7667298, which were in strong linkage disequilibrium (r²>0.9). Two common coding-region variants, rs2305948 (exon_7) and rs1870377 (exon_11), were identified. We selected 2 variants for association studies based on their potential biological functions. By searching putative nuclear factor-binding sites (www.genomatix.de/), we found that the single-base substitution at rs2071559 (−604T>C, defined by the transcriptional initiation site as +1; Genbank Refseq X89776), could affect transcriptional factor E2F binding to the region, which may alter VEGF receptor-2 expression. Exonic variant rs2305948 results in nonsynonymous amino acid change at Val297Ile (Genbank Refseq NM_002253). The amino acid is located at the third extracellular Ig-like domain that is important for ligand receptor binding.

All selected variants were genotyped using the polymerase chain reaction–restriction fragment length polymorphism approach without knowledge of case or control status (primer sequences available on request). Reproducibility of genotyping was confirmed by sequencing in 500 randomly selected samples with 100% concordance.

Correlation Between Variants and Carotid Intima Media Thickness

The correlation between genetic variants and intima media thickness (IMT) was tested in 1123 subjects aged 45 to 83 years (871 men and 252 women) who were randomly selected from a community-based population without a history of stroke. Internal carotid arteries and the common carotid arteries on both sides were measured with a 10-MHz imaging probe. IMT was quantified as described previously.22 Intraobserver coefficient of variation in the reproducibility was 7.9%.

Statistical Analysis

The χ² test was used to examine the Hardy-Weinberg equilibrium for each variant and to compare the distribution of allele and genotype frequencies between cases and control subjects. Unconditional logistic regression models were performed to estimate the ORs and 95% CIs for the association between genotypes and stroke in an additive genetic model (mm versus Mm versus MM) as well as a dominant genetic model (Mm+mm versus MM), in which M denotes the major allele and m denotes the minor allele. Multivariate analyses were adjusted for age, gender, body mass index, fasting triglycerides, total cholesterol, high-density lipoprotein cholesterol,
Table 1. No Association Between VEGF Gene Variants and Stroke in the First Case–Control Study

<table>
<thead>
<tr>
<th>VEGF Gene Variants</th>
<th>MAF, %</th>
<th>P*</th>
<th>MM</th>
<th>Mm</th>
<th>mm</th>
<th>Additive Model (mm vs Mm vs MM)</th>
<th>Dominant Model (Mm + mm vs MM)</th>
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<td>Crude OR† (95% CI)</td>
<td>Adjusted OR‡ (95% CI)</td>
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P* values and †crude ORs (95% CI) were determined by χ² tests, cases vs control subjects.
‡Adjusted ORs (95% CI) and adjusted P value were obtained with multivariate unconditional logistic regression analysis by adjusting for age, gender, clinical centers, body mass index, triglycerides, total cholesterol, high-density lipoprotein cholesterol, blood glucose, blood pressure, smoking, alcohol intake, history of hypertension, and DM.

MAF indicates minor allele frequency; M, major allele; m, minor allele. The MM genotype was the reference.

Results

Clinical Characteristics
The clinical characteristics of patients with stroke and control subjects are shown in Supplemental Table I, available online at http://stroke.ahajournals.org. In the first case–control study, the mean±SD age was 60.4±9.2 years in cases and 59.6±8.5 years in control subjects, and men accounting for 63.4% of patients and 59.2% of control subjects. As expected, patients with stroke had a higher prevalence of conventional vascular risk factors, including cigarette smoking, history of hypertension and DM; higher levels of blood pressure, fasting blood glucose, and triglycerides; and lower level of high-density lipoprotein cholesterol.

Association Between Variants in the VEGF and VEGF Receptor-2 Genes and Stroke
The frequencies distribution of all variants tested in the VEGF and VEGF receptor-2 genes did not deviate significantly from Hardy-Weinberg equilibrium in both the initial case–control stroke population and the replication samples. All tested VEGF variants were not associated with stroke and any subtype of stroke after adjustment for conventional risk factors in the first stroke study (Table 1).

In the VEGF receptor-2 gene, the variant 297Ile was associated with increased susceptibility to overall stroke compared with the wild-type genotype (Table 2). Subgroup analysis showed that the association was stronger for ICH (additive model: OR, 2.06; 95% CI, 1.64 to 2.59; P=7.6×10⁻¹⁰; dominant model: OR, 2.20; 95% CI, 1.70 to 2.84; P=1.5×10⁻⁷). The estimated population-attributable fraction for ICH was 20% for the 297Ile. The C allele of the −604 locus in the promoter region showed significantly

fasting blood glucose, blood pressure, smoking status (never, past, current), alcohol intake (current drinker, yes/no), history of hypertension (yes/no), and DM (yes/no). A 2-stage strategy was used in the present study: (1) test for association in all subgroups in the first stroke population without corrections for multiple testing and the associations with probability value <0.05 was declared as interesting; and (2) then test only the interesting findings in the second replication case–control sample with correction for multiple testing by Simes’ method, a modified Bonferroni procedure.¹²

Person-years of follow-up started from the date of diagnosis of the first-ever stroke until the date of a first recurrence of stroke, death, or the end of the follow-up period (May 31, 2006), whichever came first. For those who were lost to follow-up (89 of 1849 [4.8%]), it ended with the date last known to be alive. Cox proportional hazards models were used to examine the association between variants and the risk of stroke recurrence after adjustment for age (5-year categories), gender, and conventional risk factors, stroke subtypes, neurological deficit, and antihypertensive medication. The correlation between variants and carotid IMT (dependent variable) was analyzed in an additive model by multiple linear regression models. Differences in carotid IMT across the genotypes was examined by one-way analysis of variance. The population-attributable fraction was estimated for variants with the following equation: population-attributable fraction % = 100 × [p(OR−1)−1] × [p(OR−1)+1]; p is the frequency of the at-risk genotypes among control subjects. All probability values are 2-sided, and P<0.05 is considered significant. Analyses were performed with SPSS software, Version 11.0 (SPSS Inc, Chicago, Ill.).
Table 2. Associations Between VEGF Receptor-2 Variants and Stroke in the First Case–Control Study

<table>
<thead>
<tr>
<th>VEGF Receptor-2 Variants</th>
<th>MAF, %</th>
<th>P*</th>
<th>Genotype, n (%)</th>
<th>Crude ORs†</th>
<th>Adjusted ORs†</th>
<th>Adjusted Corrected‡</th>
<th>Crude ORs†</th>
<th>Adjusted ORs†</th>
<th>Adjusted Corrected‡</th>
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<tbody>
<tr>
<td>ns2701559 (−604T&gt;C)</td>
<td>C</td>
<td></td>
<td>TT</td>
<td>1.00</td>
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<td>1.00</td>
<td>1.00</td>
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<td>Control subjects</td>
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<td>TT</td>
<td>0.90</td>
<td>0.86–1.04</td>
<td>1.00</td>
<td>0.90</td>
<td>0.85–1.05</td>
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<tr>
<td>(n=1798)</td>
<td></td>
<td></td>
<td>TC</td>
<td>0.94</td>
<td>0.85–1.05</td>
<td>1.00</td>
<td>0.94</td>
<td>0.81–1.10</td>
<td>0.94</td>
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<tr>
<td>Cases (n=1849)</td>
<td>30.0</td>
<td>0.26</td>
<td>920 (48.9)</td>
<td>0.95</td>
<td>0.86–1.04</td>
<td>1.00</td>
<td>0.93</td>
<td>0.82–1.06</td>
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<td>446 (54.9)</td>
<td>0.81</td>
<td>0.71–0.92</td>
<td>0.003</td>
<td>0.76</td>
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<td>0.004</td>
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<td>Lacunar (n=530)</td>
<td>32.0</td>
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<td>247 (46.6)</td>
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<td>0.90–1.20</td>
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<td>33.0</td>
<td>0.26</td>
<td>227 (44.4)</td>
<td>1.09</td>
<td>0.94–1.26</td>
<td>0.59</td>
<td>1.13</td>
<td>0.89–1.40</td>
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Table 3. Association of VEGF Receptor-2 Variants With Stroke in the Replication Stroke Study

<table>
<thead>
<tr>
<th>VEGF Receptor-2 Variants</th>
<th>Genotype, n (%)</th>
<th>Crude ORs* (95% CI)</th>
<th>Adjusted ORs† (95% CI)</th>
<th>Adjusted P Value</th>
<th>Corrected† P Value</th>
<th>Crude ORs* (95% CI)</th>
<th>Adjusted ORs† (95% CI)</th>
<th>Adjusted P Value</th>
<th>Corrected† P Value</th>
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<td>ns2701559 (−604T&gt;C)</td>
<td>TT</td>
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<td>1.00</td>
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<td>Control subjects</td>
<td>140 (42.9)</td>
<td>143 (43.6)</td>
<td>144 (43.5)</td>
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<tr>
<td>(n=327)</td>
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<tr>
<td>Cases (N=327)</td>
<td>176 (53.8)</td>
<td>128 (39.1)</td>
<td>23 (7.0)</td>
<td>0.67</td>
<td>0.53–0.85</td>
<td>0.004</td>
<td>0.64</td>
<td>0.47–0.87</td>
<td>0.001</td>
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<tr>
<td>Thrombosis (n=209)</td>
<td>118 (56.5)</td>
<td>78 (37.3)</td>
<td>13 (6.2)</td>
<td>0.62</td>
<td>0.47–0.81</td>
<td>0.002</td>
<td>0.58</td>
<td>0.41–0.92</td>
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<tr>
<td>ICH (n=118)</td>
<td>58 (49.1)</td>
<td>50 (42.4)</td>
<td>10 (8.5)</td>
<td>0.78</td>
<td>0.57–1.07</td>
<td>0.17</td>
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<tr>
<td>rs2305948 (Val287Ile)</td>
<td>Val/Val</td>
<td>239 (73.1)</td>
<td>82 (25.1)</td>
<td>1.00</td>
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<tr>
<td>Control subjects</td>
<td>206 (63.0)</td>
<td>108 (33.0)</td>
<td>13 (4.0)</td>
<td>1.55</td>
<td>1.15–2.07</td>
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<td>2.01</td>
<td>1.38–2.94</td>
<td>0.0002</td>
<td>2.14</td>
<td>1.38–3.31</td>
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*Crude ORs (95% CI) were determined by χ² test, cases vs control subjects.  
†Adjusted ORs (95% CI) and adjusted P value were obtained using multivariable conditional logistic regression analysis by adjusting for the same covariates as in Table 1.  
‡Corrected P value was obtained by the Simes’ procedure, a modified Bonferroni correction for multiple testing.  
M indicates minor allele frequency; M, major allele; m, minor allele. The MM genotype was the reference.

Reduced susceptibility to atherothrombotic stroke (additive model: OR, 0.82; 95% CI, 0.71 to 0.93; P = 0.003; dominant model: OR, 0.78; 95% CI, 0.65 to 0.92; P = 0.004; Table 2). The estimated population-attributable fraction was 10% for the −604C.

The associations between the VEGF receptor-2 gene variants and atherothrombotic stroke or ICH were then replicated in the second independent case–control samples (Table 3). After correction for multiple testing by Simes’ procedure, the associations remained statistically significant. An additional analysis stratified by the presence or absence of history of hypertension showed that these positive associations were independent of the status of hypertension in both the initial and replication studies (Supplemental Table II).

Variants in the VEGF Receptor-2 Gene Predicted the Risk of Stroke Recurrence

During a median 4.5 years (range, 0.1 to 6.0 years) of follow-up for 1849 patients with stroke, 89 were lost due to emigration. There was no substantial difference in the frequencies of genotypes and clinical characteristics between the follow-up and lost-to-follow-up subjects. A total of 355 recurrent strokes and 323 deaths from all causes (183 from...
stroke or coronary heart disease, 140 from other causes) were documented.

After adjustment for age, gender, and other conventional vascular risk factors, subjects carrying the 297Ile had an increased risk for stroke recurrence (relative risk, 1.40; 95% CI, 1.12 to 1.75; \( P = 0.003 \); Table 4). Further analyses showed that the 297Ile was associated with almost twice risk for recurrence (relative risk, 1.91; 95% CI, 1.30 to 2.80; \( P = 0.001 \)) in patients with ICH. Subjects carrying the C allele of the −604 locus had a reduced risk for stroke recurrence (relative risk, 0.65; 95% CI, 0.46 to 0.91; \( P = 0.001 \)). The protective role of −604C allele against recurrence was supported that the observed associations are most likely to be real. Because stroke is a quite common disorder in China, the population-attributable fraction may be high even for genetic susceptibility to stroke, but also served as independent predictors of stroke recurrence.

Population stratification may lead to a spurious association. However, in the present study, all subjects were of Han ethnicity. The frequencies distribution of all tested variants also did not deviate from Hardy-Weinberg equilibrium in studied populations. To date, the best strategy to guard against multiple testing is replication of the genetic associations. Thus, in this study, we used multiple replication approaches, including a second independent stroke case-control set and a study of an intermediate phenotype (IMT). In our prospective follow-up, the stroke risk alleles identified in the VEGF receptor-2 gene are not only associated with susceptibility to stroke, but also served as independent predictors of stroke recurrence.

**Discussion**

In this Chinese population, we found that 2 functional variants rs2071559 (−604T>C) and rs2305948 (Val297Ile), in the VEGF receptor-2 gene are not only associated with susceptibility to stroke, but also served as independent predictors of stroke recurrence.

Population stratification may lead to a spurious association. However, in the present study, all subjects were of Han ethnicity. The frequencies distribution of all tested variants also did not deviate from Hardy-Weinberg equilibrium in studied populations. To date, the best strategy to guard against multiple testing is replication of the genetic associations. Thus, in this study, we used multiple replication approaches, including a second independent stroke case-control set and a study of an intermediate phenotype (IMT). In our prospective follow-up, the stroke risk alleles identified in the VEGF receptor-2 gene are not only associated with susceptibility to stroke, but also served as independent predictors of stroke recurrence. The consistency of these results supports that the observed associations are most likely to be real. Because stroke is a quite common disorder in China, the population-attributable fraction may be high even for genetic variants conferring a modestly increased relative risk.

The transition (T>C) at the −604 locus has been found to suppress transcriptional activity and downregulates the expression of VEGF receptor-2; the substitution (Val>Ile) at
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VEGF Receptor-2 Variants and Risk of Stroke

The 297 residue in the VEGF receptor-2 has been shown to reduce the efficiency of binding to VEGF by 50%. It appears that these genetic variants can inhibit VEGF signaling but have different effects on stroke risk. No clear explanation is available for this discrepancy, but it could be due to the differences in pathogenesis between hemorrhagic and atherothrombotic stroke as well as the complex roles of VEGF signaling in vascular biology.

Atherothrombotic stroke results from the narrowing and occlusion of large cerebral arteries most because of atherosclerotic plaque growth and rupture. Recent studies show that increased density of microvessels within the plaque area contributes to the growth and destabilization of the plaque, whereas in stable plaques, there is a marked decrease in the density of immature "leaky" microvessels. Upregulation of VEGF signaling has been linked to neovascularization within the plaque. Therefore, quenching VEGF signaling such as downregulated expression of VEGF receptor-2 would retard the plaque growth and reduce the risk of atherothrombotic stroke in persons with plaque lesions. Our study also provides consistent evidence that the variant -604C was associated with reduced risks of stroke and reversely correlated with IMT, an intermediate phenotype of preclinical atherosclerosis.

On the other hand, ICH results from the spontaneous rupture and bleeding of vessels due to microaneurysms from hypertension, cerebral amyloid angiopathy in the elderly, or unidentified risk factors. VEGF signals are closely associated with development and maintenance of brain vasculature. Animal studies have shown that deficiency of the VEGF receptor-2 gene results in defective endothelial cell development and abnormal blood vessels. VEGF can also prevent oxidized low-density lipoprotein-induced endothelial cell damage and maintain its integrity through VEGF receptor-2 and downstream PI3-kinase/Akt signal pathways. The lack of sufficient VEGF signaling could result in endothelial dysfunction, vascular degeneration, and formation of weak, thin-walled vasculature, which can reduce vessel compliance and increase the risk of spontaneous vessel wall rupture and ICH under some stresses such as hypertension and increased shear stress.

We did not find evidence that the variants in the VEGF gene promoter region contributed to the risk of stroke or its recurrence. The present study had >80% power to detect an association with ORs of 1.20 for alleles at 10% to 40% frequency. However, considering the linkage disequilibrium structure of the VEGF gene, a more comprehensive evaluation across the gene is still required. In addition, although VEGF receptor-1 has weak stimulatory activity for angiogenesis because of much weaker tyrosine kinase activity compared with VEGF receptor-2, recent evidence has shown that it is also expressed in the monocytes and may play a role in the inflammatory process. Studies of VEGF receptor-1 and atherosclerosis will be helpful for clarifying the etiology of stroke.

In summary, our data suggest that VEGF receptor-2 variants are novel genetic risk markers for atherothrombotic and hemorrhagic strokes as well as independent predictors for the recurrence of stroke. Although the present study is limited to the lack of information on pathological subtypes of recurrent strokes, these findings support the contention that genetic variants influencing atherosclerosis progression and vascular structure can modify stroke risk and give impetus to further evaluate the role of angiogenesis in stroke etiology, which may provide a new target for stroke prevention and treatment.

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Disclosures

None.

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