Polymorphisms in Genes of the Endothelin System and Cerebral Small-Vessel Disease

Kelly Gormley, BSc; Steve Bevan, PhD; Ahamad Hassan, MRCP; Hugh S. Markus, FRCP

Background and Purpose—Endothelial dysfunction has been implicated in the pathogenesis of cerebral small-vessel disease (SVD). Endothelin (ET), released by the endothelium, plays a crucial role in vasoconstriction in the cerebral circulation and could contribute to the pathogenesis of cerebral SVD. Circulating ET levels may not reflect vascular production of endothelin-1 (ET-1), most of which is abluminal. Studying genetic associations, particularly of functional polymorphisms that alter activity of the ET system, is an attractive method of determining whether ET plays a role in SVD pathogenesis. We determined whether genetic variants in components of the ET system are a risk factor for cerebral SVD.

Methods—Three hundred SVD patients and 600 community controls were genotyped. Polymorphisms in the ET-1 gene (K198N), the ET receptor type A (ETA), (∼231G>A and +1222C>T), and the ET type B (ETB) receptor (G57S and L277L) were genotyped. Polymorphisms were studied both individually and as haplotypes. With brain imaging, cases were subtyped into those with lacunar infarct without leukoaraiosis and those with leukoaraiosis.

Results—No significant differences were observed between SVD cases and controls for any individual single-nucleotide polymorphism or the ETA haplotype. There were no differences between cases with isolated lacunar infarct or with lacunar infarct and leukoaraiosis.

Conclusions—This study, in a well-phenotyped population, does not support a role for genetic variation in the ET system as a risk factor for cerebral SVD. (Stroke. 2005;36:1656-1660.)

Key Words: endothelins • genetics • small-vessel disease • stroke

Lacunar infarction (LI), resulting from disease of the cerebral small vessels, can occur in isolation or may be accompanied by patchy or confluent changes in the periventricular white matter on CT or MRI. This appearance is referred to as leukoaraiosis (LA) and is thought to be related to small-vessel disease (SVD). The pathogenesis of this condition is incompletely understood. Hypertension is the major risk factor but fails to account for all of the risk. Recent evidence suggests genetic factors are important. A twin study that investigated high-signal lesions on MRI, which are believed to reflect asymptomatic SVD, suggested that the heritability was 71%. Analysis of family history data suggests that a family history of stroke at a young age is an independent risk factor for lacunar stroke.

Considerable evidence suggests that endothelial dysfunction plays a crucial role in the pathogenesis of SVD. Neuroimaging studies demonstrate endothelial disruption. Neuropathologic studies demonstrate reduced white-matter blood flow and autoregulatory capacity. Nitric oxide, derived from the endothelium, is responsible for the maintenance of cerebral blood flow and autoregulation. Soluble markers of endothelial activation are elevated independent of conventional risk factors in patients with lacunar stroke and LA. Knockout mice lacking the endothelial nitric oxide synthase (eNOS) gene develop vascular lesions resembling those seen in cerebral SVD. Functional polymorphisms in eNOS have also been associated with lacunar infarction in humans.

In addition to vasodilatory substances such as nitric oxide, the cerebral endothelium secretes vasoconstrictive substances known as endothelins (ETs). The balance between these substances is thought to regulate basal tone and may be altered in SVD. As yet, no studies have addressed the contribution of disturbances in the ET system in cerebral SVD. One problem with investigating this association is that ET levels may not reflect vascular production of endothelin-1 (ET-1), most of which is abluminal. Studying genetic associations, particularly of functional polymorphisms that alter activity of the ET system, is an attractive method of determining whether ET does play a role in SVD pathogenesis. This is an area of clinical importance, because drugs that modulate ET function are becoming available and could offer a novel avenue of treatment in the disease.

ET-1 is derived from a precursor, proendothelin (PPET), the gene for which is located on chromosome 6 and consists of 5 exons. The nucleotide sequence encoding ET-1 is found within exon 2 and corresponds to amino acids 53 to 231.
TABLE 1. Polymorphisms and Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Location</th>
<th>Genotyping Technique</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>K198N</td>
<td>6p24</td>
<td>Allele-specific PCR</td>
<td></td>
</tr>
<tr>
<td>ET&lt;sub&gt;a&lt;/sub&gt; receptor</td>
<td>-231G&gt;A</td>
<td>4q31</td>
<td>Pyrosequencing</td>
<td></td>
</tr>
<tr>
<td>ET&lt;sub&gt;a&lt;/sub&gt; receptor</td>
<td>+1222C&gt;T</td>
<td>4q31</td>
<td>Pyrosequencing</td>
<td></td>
</tr>
<tr>
<td>ET&lt;sub&gt;b&lt;/sub&gt; receptor</td>
<td>L277L</td>
<td>13q22</td>
<td>Pyrosequencing</td>
<td></td>
</tr>
<tr>
<td>ET&lt;sub&gt;b&lt;/sub&gt; receptor</td>
<td>G57S</td>
<td>13q22</td>
<td>Restriction fragment length polymorphism</td>
<td></td>
</tr>
</tbody>
</table>

PCR indicates polymerase chain reaction; Gen, generic prime; F, forward; R, reverse; Seq, sequencing prime.

Subjects and Methods

Study Population

Three hundred consecutive white patients presenting with SVD at participating stroke services were recruited. SVD was defined as a clinical lacunar syndrome with accompanying lesion on MRI or CT. All patients underwent brain imaging and imaging of the carotid arteries with duplex or MR angiography. Patients with subcortical lesion ≥1.5 cm in diameter, cortical infarct of any size, a potential cardioembolic source, and large-vessel disease defined as carotid, vertebral, or basilar intracranial artery stenosis ≥50% were excluded. Six hundred age- and sex-matched controls free of symptomatic cerebrovascular disease were recruited by sampling family physician lists from the same geographic locations as the patients. The study protocol was approved by local research ethics committees, and informed consent was obtained from all participants.

Genotyping

Genomic DNA was extracted from blood with the use of Nucleon Bacc3 kits (Tepnel Life Sciences). For the K198N polymorphism in ET-1, genotyping was performed by allele-specific polymerase chain reaction. For the 2 polymorphisms in the ET<sub>a</sub> receptor, −231G>A and +1222C>T, and the single nucleotide polymorphism (SNP) in the ET<sub>b</sub> receptor, L277L, pyrosequencing on the PSQ HS96A platform was used. This technique uses an enzyme cascade system to allow real-time detection of SNPs and a reference sequence immediately following the polymorphic site. An additional polymorphism within the ET<sub>b</sub> receptor, G57S, was genotyped by restriction fragment length polymorphism. A 218-bp fragment was amplified and digested with BsaI. In the presence of the G allele, the enzyme cuts twice, resulting in fragments of 46, 106, and 66 bp. The presence of the A allele disrupts a recognition site, so the enzyme cuts only once to give fragments of 152 and 66 bp. These differences were resolved on a 3% agarose gel. Primer sequences were constructed with the aid of Primer3. Accession numbers refer to sequences reported on the Human Gene Mutation Database or by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

SVD Subtyping

Of the 300 cases, 222 (74%) underwent MRI and 71 (24%) underwent CT. Of the remaining 7 cases, 4 were graded on the basis of formal radiologic reports and 3 were unable to be subtyped. Patients were then divided into two groups: LI (≥1 focal lesion and absent/mild LA) or LA (≥1 focal lesion and moderate/severe LA) according to a previously validated method.

Statistical Analysis

Univariate analysis was performed with χ<sup>2</sup> statistics. Binary logistic regression was used to assess the effects of dominant and recessive models at each locus. To do this, genotypes at each locus were coded as follows: 1 = wild-type homozygotes, 2 = heterozygotes, and 3 = mutant homozygotes. A dominant model compared genotypes 3 and 1 (i.e., heterozygotes) versus 2 and 3 (i.e., wild-type and mutant homozygotes). A recessive model compared genotypes 1 and 2 (i.e., heterozygotes) versus 3 (i.e., mutant homozygotes).
TABLE 2. Demographics of Study Groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n=600)</th>
<th>All Cerebral SVD (n=300)</th>
<th>Cerebral SVD Subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LI (n=137)</td>
<td>LA (n=160)</td>
</tr>
<tr>
<td>Age, y</td>
<td>66.85 (8.15)</td>
<td>67.10 (10.26)</td>
<td>63.52 (10.31)*</td>
</tr>
<tr>
<td>Male sex</td>
<td>387 (64.5)</td>
<td>198 (66.0)</td>
<td>93 (67.9)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>254 (42.6)</td>
<td>223 (74.3)*</td>
<td>93 (67.9)*</td>
</tr>
<tr>
<td>Ever smoked</td>
<td>361 (60.3)</td>
<td>213 (71.5)†</td>
<td>100 (73.5)†</td>
</tr>
<tr>
<td>Current smoker</td>
<td>93 (15.5)</td>
<td>174 (19.4)*</td>
<td>39 (28.7)*</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>37 (6.2)</td>
<td>17 (5.7)</td>
<td>7 (5.1)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>23 (3.8)</td>
<td>29 (9.7)*</td>
<td>17 (12.4)</td>
</tr>
</tbody>
</table>

Values in parentheses denote SD for continuous data and percent for categorical data.

*P<0.0005, †P<0.005, †P<0.05 vs controls; §P<0.0005, †P<0.05 vs LI.

Results

Subject Characteristics

There were no differences in age and sex between cases and controls (Table 2). Hypertension, smoking, and diabetes mellitus were all more common in cases than controls (Table 2). One hundred thirty-seven cases (45.6%) were subtyped as LI and 160 (53.3%) as LA. The remaining 3 cases could not be subtyped because of unavailability of original scans and therefore were excluded from the subtype analysis.

Genotype Distributions

All genotype frequencies were in Hardy-Weinberg equilibrium in the control population (−231G>A, P=0.9801; +1222C>T, P=0.9303; L277L, P=0.3962; G57S, P=0.77; and K198N, P=0.5708).

The genes were considered under both recessive and dominant models. The results from the recessive model, which is more likely for genes involved in metabolic pathways, are presented in Table 3. The only exception to this was the case of the G57S polymorphism in the ETα receptor. No mutant homozygotes were found in this instance. There were only 16 heterozygotes in the entire population, so the results given are for possession of 1 copy of the SNP versus the wild type. No significant differences were observed in genotype distributions between cerebral SVD cases and controls for any individual SNP (K198N, P=0.25; −231G>A, P=0.29; +1222C>T, P=0.32; G57S, P=0.95; and L277L, P=0.66 for SVD compared with controls). Neither were there any significant differences between the 2 SVD phenotypes: LI versus controls: K198N, P=0.99; −231G>A, P=0.57; and L277L, P=0.77; LA versus controls: K198N, P=0.17; −231G>A, P=0.49; and L277L, P=0.98. No differences were observed when a dominant model was used for the K198N, −231G>A, +1222C>T, and L277L polymorphisms.

Haplotype Analysis

The 2 genotypes for the ETα receptor resulted in 4 possible haplotypes, the frequencies of which are given in Table 4. Haplotypes were examined for those individuals who carried 1 or more copies only, owing to the small numbers who were homozygous for the individual haplotypes. The haplotype distributions did not differ between cases and controls (data not shown).

Haplotype analysis was repeated with the SNPs in the ETβ receptor, G57S and L277L. However, given the rarity of the minor allele of G57S, the genotype GA at this locus occurred only on the background of the wild type at the L277L locus. Further haplotype analysis therefore did not give any additional information to the analysis of the individual SNPs.

TABLE 3. Odds Ratios for Associations Between ET Gene System Polymorphisms and All Cerebral SVD, Isolated LI, and LA Phenotype

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>All SVD</th>
<th>LI</th>
<th>LA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>K198N</td>
<td>1.150 (0.864–1.531)</td>
<td>1.084 (0.401–2.928)</td>
<td>1.898 (0.875–4.119)</td>
</tr>
<tr>
<td>ETα receptor</td>
<td>−231G&gt;A</td>
<td>0.908 (0.543–1.520)</td>
<td>0.959 (0.485–1.895)</td>
<td>0.890 (0.462–1.714)</td>
</tr>
<tr>
<td>ETα receptor</td>
<td>+1222C&gt;T</td>
<td>1.110 (0.705–1.756)</td>
<td>0.589 (0.274–1.267)</td>
<td>1.550 (0.923–2.605)</td>
</tr>
<tr>
<td>ETβ receptor</td>
<td>L277L</td>
<td>1.205 (0.794–1.829)</td>
<td>1.354 (0.791–2.319)</td>
<td>1.054 (0.613–1.810)</td>
</tr>
<tr>
<td>ETβ receptor</td>
<td>G57S</td>
<td>1.036 (0.357–3.012)</td>
<td>2.769 (0.355–21.616)</td>
<td>0.589 (0.185–1.881)</td>
</tr>
</tbody>
</table>

CI indicates confidence interval. These were calculated by recessive models. In the case of the G57S SNP, possession of 1 copy of the SNP was compared with wild-type homozygotes.
TABLE 4. Associations Between Different ETA Receptor Haplotypes and Cerebral SVD

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Overall Frequency (N=1800), No. (%)</th>
<th>All SVD Cases (n=600), No. (%)</th>
<th>Controls (n=1200), No. (%)</th>
<th>P</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-C</td>
<td>263 (14.6)</td>
<td>91 (15.2)</td>
<td>172 (14.3)</td>
<td>0.67</td>
<td>1.099 (0.803–1.503)</td>
</tr>
<tr>
<td>≥1 copy</td>
<td></td>
<td></td>
<td></td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>A-T</td>
<td>266 (14.8)</td>
<td>94 (15.7)</td>
<td>172 (14.3)</td>
<td>0.43</td>
<td>1.166 (0.661–1.580)</td>
</tr>
<tr>
<td>≥1 copy</td>
<td></td>
<td></td>
<td></td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>G-C</td>
<td>969 (53.8)</td>
<td>316 (52.7)</td>
<td>653 (54.4)</td>
<td>0.48</td>
<td>0.955 (0.662–1.375)</td>
</tr>
<tr>
<td>≥1 copy</td>
<td></td>
<td>247 (41.2)</td>
<td>498 (41.5)</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>G-T</td>
<td>302 (16.8)</td>
<td>99 (16.5)</td>
<td>203 (16.9)</td>
<td>0.84</td>
<td>0.757 (−1.387–1.024)</td>
</tr>
<tr>
<td>≥1 copy</td>
<td></td>
<td>90 (15.0)</td>
<td>177 (14.8)</td>
<td>0.88</td>
<td></td>
</tr>
</tbody>
</table>

Results are shown for 1 or more of each copy of the haplotype.

Discussion

Considerable evidence suggests that endothelial dysfunction may play a role in cerebral SVD. This makes genes controlling endothelial function attractive candidate genes in this disease. Previous studies have implicated polymorphisms in genes involved in nitric oxide metabolism and vasodilation in disease pathogenesis. The ETs play a crucial role in vasoconstriction. However, this study, in a well-phenotyped group of patients with cerebral SVD, does not support a role for genetic variation in the ET system as a risk factor for cerebral SVD.

It has been suggested that the pathogenesis in cerebral SVD may be heterogeneous, differing between patients with larger, isolated, lacunar infarcts and those with LA and multiple, small lacunar infarcts. It is particularly in this latter subtype that endothelial dysfunction has been implicated. However, we also found no differences in the distribution of genotypes between the 2 types of cerebral SVD.

We looked at genes encoding a number of components in the ET system. Wherever possible, we focused on polymorphisms that have been shown to be functional and associated with other cardiovascular disease. The ET-1 polymorphism studied herein has been associated with elevated ET levels and implicated in blood pressure control. The 2 ETA polymorphisms that we examined have both been associated with disease or cardiovascular function, the first with migraine and the second with pulse pressure. We also examined 2 polymorphisms in ETB, 1 of which, the L277L polymorphism, is not functional and therefore would only be associated via linkage disequilibrium with a nearby variant.

In this study, we focused on a well-characterized group of patients with a specific stroke phenotype. Previous association studies in stroke have given conflicting results. This may reflect a number of issues, including sample size and adequate phenotyping. Recent family studies have suggested that the genetic component may vary for different stroke subtypes, being particularly strong for both SVD and large-vessel disease. It has been shown that the sample size required to detect associations with a specific stroke subtype is very much smaller if a well-characterized group with a specific phenotype is studied, rather than unselected patients with ischemic stroke. Our sample size would detect modest associations on the order of an odds ratio of 1.5 to 2 according to these power calculations.

Although our study is the first to examine ET polymorphisms and cerebral SVD, it provides no evidence for genetic variation in this system as a disease risk factor. We have excluded a role for the polymorphisms studied or any closely linked polymorphisms in disease pathogenesis. However, we cannot completely exclude a role for genetic variation in this system as a risk factor for cerebral SVD. First, further screening of the genes is required to identify other potential functional polymorphisms. Second, there are multiple mechanisms that may modulate the effects of ET-1. The first such control is at the level of mRNA synthesis. PPET mRNA is short lived, which may offer protection against overproduction. Its effect as a vasoconstrictor is also offset by the release of nitric oxide, prostacyclins, and natriuretic peptides. As a consequence, further studies into the ET system are required to fully elucidate the roles of genetic variation in this system on cerebral SVD.

Acknowledgments

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References

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