Association of 5′ Upstream Promoter Region of Prostacyclin Synthase Gene Variant With Cerebral Infarction

Tomohiro Nakayama, Masayoshi Soma, Duolikun Rehemudula, Yukie Takahashi, Hideko Tobe, Mikano Satoh, Jiro Uwabo, Masako Kunimoto, and Katsuo Kanmatsuse

The aim of this study was to investigate whether there is an association between the promoter region of the prostacyclin synthase gene and cerebral infarction (CI). Using the polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) method, we found a variable-number tandem repeat polymorphism in the 5′-upstream promoter region of the prostacyclin synthase gene. This region contains transcriptional factors–binding sites of Sp1 (CCCGCC) and AP-2 (CCGCCACCC). The alleles varied in size from three to seven repeats of nine base pairs (bp). We performed an association study using the polymorphism in 111 patients and 152 control subjects. The transcriptional activity of the abnormal promoter region allele was determined by luciferase assay. The overall distribution of alleles differed significantly between both groups. Logistic linear regression analysis revealed the small number repeat allele to be found more frequently with CI. Transcriptional activity increased with increasing numbers of repeats. This study provides consistent support for the association between CI and the PGIS gene. Am J Hypertens 2000;13:1263–1267 © 2000 American Journal of Hypertension, Ltd.

KEY WORDS: Prostacyclin synthase gene, promoter, cerebral infarction, polymorphism, variable number of tandem repeat.

Cerebral infarction (CI) is a common disorder of multifactorial origin. However, it is not clear whether genetic variants influence the development of this disease. It has been reported that cerebrovascular infarctions resulting from complications of atherosclerosis are associated with disturbances in microcirculatory flow caused by intravascular platelet activation.1

Prostacyclin inhibits platelet aggregation, smooth muscle cell proliferation, and vasoconstriction.2-4 Prostacyclin synthase (EC 5.3.99.4), which catalyzes the formation of prostacyclin from prostaglandin H2, is widely distributed, predominantly in vascular endothelial and smooth muscle cells.5,6 The prostacyclin synthase gene is localized to 1q21 to q227 and thought to be a candidate gene for cardiovascular disease. We recently reported the organization of this gene.8 Previously, we identified a family with a nonsense mutation in exon 2 of the prostacyclin synthase gene, and this family has a history of CI and hypertension.9 These findings suggested that abnormality of the prostacyclin synthase gene may lead to altered vasodilation and platelet aggregation.

In the present study, we searched for mutations or polymorphisms in the 5′-flanking region of the prostacyclin synthase gene in patients with CI and as-
sessed the effect of these genetic variations on transcriptional activity.

MATERIALS AND METHODS

Subjects The study group consisted of 111 patients (mean age, 65.8 ± 9.6 years) with CI diagnosed by computed tomography (CT) or magnetic resonance imaging (MRI). All patients had neurologic deficits that persisted for ≥1 month. A total of 152 subjects without CI (mean age, 66.5 ± 4.6 years) were studied as control subjects. Control subjects had vascular risk factors such as hypertension, hypercholesterolemia, or diabetes mellitus, but no cerebrovascular disease. Individuals with atrial fibrillation were excluded from both the CI and non-CI groups. Informed consent was obtained from each subject according to a protocol approved by the Human Studies Committee of Nihon University.

Single Strand Conformation Polymorphism For single-strand conformation polymorphism (SSCP) analysis, two oligonucleotide primers were designed to (5'-ACATTTTCCCCCCAGGCTGCTGC-3', sense), (5'-CGGCTCAGTAGCAGCAGCAACAG-3', antisense) genomic sequences of the prostacyclin synthase gene.7 DNA was extracted from whole blood according to standard procedures. Polymerase chain reaction (PCR)-SSCP was performed as described previously, on DNAs from 90 patients with CI.9

Genotyping Genomic DNA was extracted from peripheral blood leukocytes by a standard method. Two oligonucleotide primers (PGIS-F, 5'-ACATTTTCCCCCCAGGCTGCTGC-3' and PGIS-R, 5'-CGGCTCAGTAGCAGCAGCAACAG-3') were used to amplify about 200-bp fragment. The PCR was conducted in a volume of 10 μL containing 50 ng of genomic DNA and 0.125 pmol each of 32P-labeled PGIS-F primer and unlabeled PGIS-R primer. The PCR conditions were described previously.10 To establish a control for the number of tandem repeats, DNA sequencing was done by the dideoxynucleotide chain termination procedure after subcloning fragments amplified from genomic DNA into pCR 2.1 (Invitrogen, Carlsbad, CA). Plasmid clones containing defined numbers of tandem repeats were used as standard templates for PCR. The seven tandem repeat allele PCR product has a size of 317 bp. The three tandem repeat allele of PCR product is 281 bp.

Transcriptional Activity To make human prostacyclin synthase reporter gene constructs, the PCR product was reamplified and subcloned into the TA cloning vector for PCR products (Invitrogen). After digestion with Sac I and Xho I, each product was subcloned into the Sac I/Xho I sites of the luciferase reporter gene vector, pGV-B2 (Toyo Inkt Inc, Tokyo, Japan). All constructs were verified by sequencing the inserts and flanking regions of the plasmids.

Human aortic smooth muscle cells (HASMC) were purchased from Kurabo Inc (Osaka, Japan) and cultured in HuMedia-SG2 medium containing 5% heat-inactivated fetal bovine serum, 0.5 ng/mL human endothelial growth factor (hEGF), 2 ng/mL human fibroblast growth factor-B (hFGF), 50 μg/mL gentamycin, and 50 ng/mL amphotericin B (all from Kurabo Inc) in six-well plastic tissue culture plates. A quantity of 2 mL medium was used per well. The HASMC were used between passages 3 and 6 for transfections. For transfection of HASMC with prostacyclin synthase promoter constructs, calcium phosphate (CaPO4) method using a mammalian transfection kit (Stratagene, CA) was used. Prostacyclin synthase promoter plasmid DNA (2 μg) and a plasmid containing the thymidine kinase gene pRL-TK, (0.2 μg, to normalize for transfection efficiency) were mixed with 5 μL of 2.5 mol/L CaPO4 and 50 μL of 2× BBS (50 mmol/L N,N-Bis-2-aminoethanesulfonic acid and buffered saline, 280 mmol/L NaCl, and 1.5 mmol/L Na2HPO4) according to the manufacturer’s protocol. The transfection solution was incubated for 20 min at room temperature with 60% to 70% confluent HASMC. Cells were incubated for 24 h under 3% CO2, 35°C. The next day, the medium was removed and replaced with complete media for an additional 24 h under 5% CO2, 37°C. The HASMC were then lysed (400 μL), and extracts were centrifuged to remove intact cells and debris. Extracts were then used for measurement of luciferase activity (50 μL). Luciferase activity was measured at least three times in duplicate using a double luciferase assay system (PickageneDual, Toyo Inkt) with a Berthold luminometer. All data were normalized as relative light units/pRL-TK activity.

Statistical Analysis Allele frequencies were calculated from the genotypes of all subjects. Hardy-Weinberg equilibrium was assessed by χ² analysis. The overall distribution of alleles was analyzed by 2 × 5 contingency tables, and a P value of <.05 was considered significant. Individual differences of allele frequencies were tested using 2 × 2 contingency tables for each allele by combining the remaining alleles into one category, and a P value of <.01 (ie, .05/5) was considered significant to correct for the number of comparisons made. The association of cerebral infarction (CI) and genotypes was evaluated by multiple logistic regression analysis; CI was regarded as the dependent variable, and the number of the repeats, age, and sex were considered independent variables. The overall distributions of alleles between hypertensive patients and nonhypertensives, and between diabetic patients and nondiabetics, were assessed by χ²
analysis. Differences in transcriptional activities of polymorphic alleles were analyzed with one-way repeated measure analysis, and a \( P \) value of \(<.05\) was considered significant.

**RESULTS**

Using SSCP, we discovered a nine-bp nucleotide repeat polymorphism positioned \(-6\) nucleotides from the start codon. There are Sp1 (CCGCC) and AP-2 (CCGCCAGCCC) binding sites in this region (Fig. 1). Alleles based on variations in the number of nine-bp nucleotide repeats were determined by typing 263 unrelated subjects. The alleles varied in size from three to seven repeats. The estimated allele frequencies are shown in Table 1.

We genotyped 111 CI and 152 non-CI individuals. The observed and expected heterozygosities in the non-CI group were 96.6% and 97.3%, respectively, which were in good agreement with predicted Hardy-Weinberg equilibrium values (\( \chi^2 = 53.5, df = 14, P > .05 \)). The overall distribution of the alleles differed significantly between the CI and non-CI group (\( \chi^2 = 11.6, df = 4, P = .020 \)). Multiple logistic linear regression analysis adjusted by age and sex showed alleles containing small numbers of repeats to be associated with CI (odds ratio, 1.38; 95% confidence interval, 1.11 to 1.71). The logistic linear regression curve between CI and the total number of repeats for both chromosomes is shown in Fig. 2. The overall distributions of alleles were not significantly different between the hypertensive and nonhypertensive groups, and between subjects with diabetes mellitus and nondiabetic groups.

Using the human prostacyclin synthase gene sequence,\(^7\) we designed PCR primers that amplified the region in human genomic DNA from bp \(-1600\) to \(+22\). Previous analysis of the prostacyclin synthase gene revealed that the 5'-upstream region lacks a TATA-box and contains a single transcriptional start site 22 bp upstream of the initiation site. To determine whether our construct was functional for basal transcription in HASMC, a series of constructs that contained approximately 1.6-kb fragments of the polymorphic 5'-flanking sequence in front of the luciferase reporter gene were made. Luciferase reporter gene constructs were then transiently transfected into HASMC for determination of prostacyclin synthase promoter activity. The transcriptional activity increased with increasing numbers of repeats (Fig. 3).

**DISCUSSION**

Numerous factors such as diabetes mellitus, hypertension, smoking, and antiphospholipid antibodies have been reported to influence the pathogenesis of stroke. Cerebral infarction is thought to be a multifactorial disease like essential hypertension and diabetes mellitus. Several candidate genetic variants related to this disease have been studied using polymorphic genetic markers, but these studies have failed to show any association of CI with genes such as apolipoprotein A,\(^11\) apolipoprotein E,\(^12\) or methylenetetrahydrofolate reductase.\(^13\) However, apolipoprotein A-I,\(^14\) and an-

---

**TABLE 1. ALLELE FREQUENCY OF 9 BASE PAIR NUCLEOTIDE REPEAT IN THE COREPROMOTER REGION OF THE PROSTACYCLIN SYNTHASE GENE**

<table>
<thead>
<tr>
<th>Group</th>
<th>3 Repeat</th>
<th>4 Repeat</th>
<th>5 Repeat</th>
<th>6 Repeat</th>
<th>7 Repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral infarction ((n = 111))</td>
<td>5</td>
<td>39</td>
<td>7</td>
<td>167</td>
<td>4</td>
</tr>
<tr>
<td>Non–cerebral infarction ((n = 152))</td>
<td>1</td>
<td>32</td>
<td>9</td>
<td>250</td>
<td>12</td>
</tr>
</tbody>
</table>

---

**FIG. 1.** Nine base pair nucleotide repeat polymorphism in the promoter region of the prostacyclin synthase gene. ATG in bold type indicates the start codon, and the first nucleotide upstream of the ATG start codon is numbered \(-1\). Cis-elements are underlined. The 9 bp nucleotide repeats are indicated by lines above the sequence.
giotensin converting enzyme gene polymorphisms were shown to be associated with the risk for CI in the Japanese population. In association analyses using genetic markers, selection of subjects and verification of positive data are very important. Discrepancies in results reported for the same gene may be attributed to different criteria used in selection of subjects or to racial differences in the populations studied. In this experiment, we strictly defined the non-CI subjects.

After the discovery of prostacyclin, a powerful physiologic antiaggregatory factor, arterial thrombosis caused by platelet aggregates has been considered to be a deficiency of prostacyclin synthesis. Several investigators reported that abnormal prostacyclin synthesis or metabolism may be a risk factor in patients with CI. An accelerated rate of prostacyclin degradation in the blood might also lead to CI. Although prostacyclin reduced CI in animal models, many therapeutic trials of prostacyclin failed to show significant clinical improvement.

Previously we identified a nonsense mutation in exon 2 of the human prostacyclin synthase gene in a family with a history of CI and essential hypertension. This mutation was found in only one woman of 300 subjects comprising 150 essential hypertensive and 150 normotensive individuals. This suggested that prostacyclin synthase gene abnormalities may be associated with CI. We hypothesized that a genetic variant with a functional difference could be linked to CI even if its frequency in the population was very low. The presence of genetic heterogeneity, however, makes prediction of the degrees of overlap between many genes in a population difficult. Our study suggests that the differences of prostacyclin activities are allele-dependent, and may influence the risk for CI.

In this study, we found a novel nine-bp nucleotide repeat polymorphism in the 5'-upstream promoter region of the prostacyclin synthase gene and showed that the overall distributions of allele frequencies differed significantly between CI and non-CI groups. Logistic analysis revealed that the risk of CI increases with smaller numbers of tandem repeats. Furthermore, each allele of the prostacyclin synthase gene has different transcriptional activity. Alleles with smaller numbers of tandem repeats have lower transcriptional activity. The region between −150 and the start codon is GC-rich, and it has been reported that this position contains the basal promoter region. Thus, this polymorphism may influence basic transcriptional activity. It has recently been reported that the alleles of the 3 and 4 repeat of this polymorphism seem to be a risk factor for higher pulse pressure and systolic hypertension. It is consistent with our result, because hypertension is one of the risk factors of CI.

In the present study, the clinical data for the risk of CI are in good agreement with the experimental data from reporter gene analysis of transcriptional activity. Our study suggests that prostacyclin function depends on the different alleles of the prostacyclin synthase gene and may influence the risk for CI.

**ACKNOWLEDGMENTS**

We acknowledge Dr. N. Fukuda, Dr. Y. Watanabe, and Dr. Y. Izumi for collecting samples. This work was financially supported by a grant from the Ministry of Education, Science and Culture of Japan (High-Tech Research Center, Nihon University), a research grant from an alumni association of Nihon University School of Medicine, and Tanabe Biomedical Conference, Japan.
REFERENCES


