The Combined Effect of Paraoxonase Promoter and Coding Region Polymorphisms on the Risk of Arterial Ischemic Stroke Among Young Adults

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Background: Serum paraoxonase (PON1) is a high-density lipoprotein–associated esterase with antioxidant and antiatherogenic properties that has recently been implicated in the pathogenesis of cardiovascular disease. Interindividual variability in PON1 levels is determined by the Q192R and L55M coding region polymorphisms and by 2 recently described polymorphisms in the promoter of the PON1 gene, C(−107)T and G(−824)A.

Objective: To determine the association of the PON1 promoter polymorphisms with arterial ischemic stroke (AIS) in the young.

Design, Setting, and Patients: We studied 118 young patients (age, <45 years) with a first nonfatal AIS of undetermined etiology and 118 control subjects, matched simultaneously for age and sex. The PON1 C(−107)T polymorphism was determined by single-stranded conformational polymorphism analysis and the G(−824)A substitution by polymerase chain reaction and restriction enzyme digestion.

Results: The presence of the low-expressor −107T allele was associated with an independent increase in overall risk of AIS (odds ratio [OR], 2.69; 95% confidence interval [CI], 1.06-6.78; P=.04) by conditional multiple logistic regression analysis. Among individuals with the 192RR genotype, the presence of the −107T allele led to a higher but nonsignificant risk, yielding an OR of 4.15 (95% CI, 0.35-48.76; P=.15) when compared with noncarriers of the T allele and 17.01 (95% CI, 1.74-166.35; P=.02) when compared with noncarriers of either variant. No significant difference between groups was found regarding the G(−824)A polymorphism.

Conclusion: These findings suggest that the PON1 −107T allele is independently associated with the risk of AIS, in addition to interacting with and potentiating the risk conferred by the Q192R polymorphism.

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DETECTION OF THE PON1 G(–824)A POLYMORPHISM

We adopted the nucleotide count corresponding to the PON1 promoter sequence accessible in GenBank (accession number AF051133). As the G(–824)A polymorphism does not affect a naturally occurring restriction site, we designed a mutagenic sense oligonucleotide primer containing a single base pair (bp) change 4 positions upstream from nucleotide –824 (sequence 5′-TATCAGTGAACCTCTCTGACACG-3′, with A being the mutated base), which, in combination with the G nucleotide at the position of interest, created a restriction site for PvuII (Figure 1A). With the mutagenic primer and the antisense primer 5′-CTCAAGCTTACGTTCATTCCAA-3′, a fragment of 200 bp was obtained by polymerase chain reaction (PCR) at 54°C annealing temperature and digested with 5 U of PvuII, yielding fragments of 176 and 24 bp in the presence of the –824G nucleotide. The G→A substitution disrupted the target sequence for PvuII, and only 1 fragment of 200 bp was observed (Figure 1B).

DETECTION OF THE PON1 C(–107)T POLYMORPHISM

As no artificial restriction sites could be created around the C(–107)T polymorphism, we used nonradioactive single-stranded conformational polymorphism (SSCP) analysis for detection of the base pair change. Amplification of a PCR fragment of 129 bp containing nucleotide –107 was obtained by using the sense primer 5′-GCGCTTCTGTGCACCTG-3′ and antisense primer 5′-CAGACACCGGGCCTAG-3′, at 68°C annealing temperature.

Nonradioactive SSCP analysis was performed on an automated nucleic acid electrophoresis system (PhastSystem; Amersham Pharmacia, Uppsala, Sweden). The PCR products were diluted at a ratio of 1:2 with a formamide-based denaturing loading buffer, denatured at 95°C for 5 minutes, quenched on ice, and electrophoresed on precast nondenaturing 8% to 25% gradient polyacrylamide gels (PhastGels; Amersham Pharmacia) at 4°C for 450 volt-hours. The DNA bands were visualized by automated silver staining (PhastGel DNA Silver Staining Kit; Amersham Pharmacia).

The SSCP analysis yielded 3 different electrophoretic patterns. The PCR fragments corresponding to each pattern were purified and sequenced with a fluorescence dideoxy termination method using an automated sequencer (Big Dyes, Model ABI 377-96; Applied Biosystems, Foster City, Calif). The DNA sequencing was conducted by the Molecular Genetics Core at Boston University School of Medicine, Boston, Mass. The sequencing results are shown in Figure 2.

STATISTICAL ANALYSIS

Clinical characteristics among patients and controls were compared by 2-tailed t test for age and the χ² test or the Fisher exact test, when appropriate, for categorical variables. Odds ratios (ORs) are given with their 95% confidence intervals (CIs). Hardy Weinberg equilibrium was tested by the χ² test (4 df). The extent of linkage disequilibrium between polymorphism pairs was expressed by the standardized coefficient D'.

A conditional logistic regression model, stratified by a variable that accounted for matching on age (±2 years) and sex, was used to evaluate the association of the PON1 promoter polymorphisms with stroke risk after adjusting for the PON1 coding region polymorphisms, ethnicity, and vascular and prothrombotic risk factors. Carriers of the ~107T allele were analyzed using the ~107CC genotype as the reference group. All statistical analyses were performed using SAS software (SAS version 8.2; SAS Institute Inc, Cary, NC).

![Figure 1. Methodology for detection of the paraoxonase (PON1) G(–824)A polymorphism. A, The normal sequence flanking the polymorphism is shown at the top. Amplification of a 200-base pair (bp) polymerase chain reaction (PCR) fragment with a mutagenic sense primer that inserted a T→A substitution 4 positions upstream from the polymorphic site created an artificial restriction site for PvuII in the presence of the −824G nucleotide. B, Electrophoretic patterns of the digestion products for the G(–824)A polymorphism run on 2.5% agarose gel. Lane 1 shows a 100-bp DNA ladder; lanes 2, 3, 6, 7, and 10, −824GA genotype; and lane 8, −824AA genotype.](https://www.archneurol.com/content/61/3/351.full)
RESULTS

Table 1 shows the demographic characteristics and the prevalence of vascular and inherited prothrombotic risk factors among patients with AIS and controls. Conventional vascular risk factors were more prevalent among patients than controls; however, only hypertension and smoking reached statistically significant differences by univariate analysis. No significant differences between patients and controls were found regarding the prothrombotic polymorphisms.

The genotype distributions and allele frequencies of the PON1 −107 and −824 polymorphisms among patients and controls are shown in Table 2. All genotype distributions were in Hardy-Weinberg equilibrium in both groups. For both polymorphisms, the genotype distributions and allele frequencies did not differ significantly between patients and controls by univariate analysis. The distribution of the PON1 promoter genotypes was further analyzed as a function of the PON1 55 and 192 coding region genotypes, which have been described previously in this population.13 Strong linkage disequilibrium was observed between the promoter polymorphisms and the 55 coding region polymorphism (for −107 × 55, $D' = 0.303 [P < .001]$; for −824 × 55, $D' = -0.935 [P < .001]$). The 192 coding region polymorphism was weakly associated with the −107 polymorphism ($D' = -0.158 [P = .02]$), but not with the −824 polymorphism ($D' = 0.085 [P = .55]$). In addition, the promoter −107 and −824 genotypes were strongly linked to each other ($D' = -0.586 [P < .001]$), the −107T allele rarely being associated with the −824A allele.

The findings from the univariate analyses were further studied in a conditional multiple logistic regression model, including the PON1 promoter and coding region polymorphisms, ethnicity, and the vascular and inherited prothrombotic risk factors (Table 3). The presence of the −107T allele (TT + TC genotypes) was associated with an independent, 2.7-fold increase in susceptibility to AIS (OR, 2.69; 95% CI, 1.06-6.78; $P = .04$). Our group has previously shown a 4-fold increase in risk associated independently with the 192RR genotype in this population.13 After incorporating the promoter polymorphisms in the logistic model in this study, the risk estimate for the 192RR genotype increased to almost 5 (OR, 4.81; 95% CI, 1.23-18.78; $P = .01$), suggesting an influence of the −107 polymorphism on the risk associated with the 192 genotype. Hypertension showed the strongest association with AIS, independently increasing the susceptibility 15-fold, whereas the OR associated with smoking was 2.76. In addition, patients of African descent were at a 4.6-fold higher risk of AIS than white patients, which is in accordance with previously reported ethnic differences in stroke risk among young adults.14 No significant associations between the PON1 −824 and 55 polymorphisms and the risk of stroke were found in the logistic regression analysis.

As some authors have suggested that the PON polymorphisms have a cumulative effect on the risk of cardiovascular disease,15 we analyzed the combined effects of the promoter and coding region polymorphisms on the risk of AIS. Table 4 shows the crude and adjusted risk estimates in subgroups defined by outcome and the 192 and

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**Table 1** shows the demographic characteristics and the prevalence of vascular and inherited prothrombotic risk factors among patients with AIS and controls. Conventional vascular risk factors were more prevalent among patients than controls; however, only hypertension and smoking reached statistically significant differences by univariate analysis. No significant differences between patients and controls were found regarding the prothrombotic polymorphisms.

**Figure 2.** Electrophoretic pattern of the paraoxonase (PON1) C(−107)T polymorphism by single-stranded conformational polymorphism (SSCP) analysis and the corresponding genotypes as determined by sequencing. Three different patterns were detected by SSCP, as seen on the gel. Lanes 1 and 3 correspond to the CC genotype; lanes 2, 4, 6, and 7, to the TT genotype; and lanes 5 and 8, to heterozygosity.

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Table 1. Characteristics of Study Subjects*

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>Patients (n = 118)</th>
<th>Controls (n = 118)</th>
<th>OR (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, y</td>
<td>36.3 ± 6.6 36.8 ± 6.8</td>
<td>35.6 ± 6.6 35.8 ± 6.6</td>
<td>1.00 (0.75-1.34)</td>
<td>.99</td>
</tr>
<tr>
<td>Male</td>
<td>54 (45.8) 54 (45.8)</td>
<td>50 (42.5) 50 (42.5)</td>
<td>1.20 (0.95-1.50)</td>
<td>.12</td>
</tr>
<tr>
<td>Ethnic background</td>
<td>White 88 (74.6) 96 (81.4)</td>
<td>88 (74.6) 96 (81.4)</td>
<td>1.00 (0.37-2.94)</td>
<td>.99</td>
</tr>
<tr>
<td>African American</td>
<td>28 (23.7) 21 (17.8)</td>
<td>28 (23.7) 21 (17.8)</td>
<td>1.00 (0.37-2.94)</td>
<td>.99</td>
</tr>
<tr>
<td>Asian</td>
<td>2 (1.7) 1 (0.8)</td>
<td>2 (1.7) 1 (0.8)</td>
<td>1.00 (0.37-2.94)</td>
<td>.99</td>
</tr>
<tr>
<td>Family history of CVD†</td>
<td>39 (33.1) 30 (25.4)</td>
<td>39 (33.1) 30 (25.4)</td>
<td>1.00 (0.37-2.94)</td>
<td>.99</td>
</tr>
<tr>
<td>Conventional vascular risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>46 (39.0) 10 (8.5)</td>
<td>47 (39.6) 11 (9.2)</td>
<td>1.00 (0.25-4.00)</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>Smoking</td>
<td>65 (55.1) 42 (35.6)</td>
<td>66 (54.8) 43 (36.4)</td>
<td>1.00 (0.25-4.00)</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>39 (33.1) 31 (26.3)</td>
<td>39 (33.1) 31 (26.3)</td>
<td>1.00 (0.25-4.00)</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>7 (5.9) 3 (2.5)</td>
<td>7 (5.9) 3 (2.5)</td>
<td>1.00 (0.25-4.00)</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>Inherited prothrombotic risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor V Leiden§</td>
<td>4 (3.4) 4 (3.4)</td>
<td>4 (3.4) 4 (3.4)</td>
<td>1.00 (0.25-4.00)</td>
<td>.99</td>
</tr>
<tr>
<td>Prothrombin G20210A</td>
<td>5 (4.2) 3 (2.5)</td>
<td>5 (4.2) 3 (2.5)</td>
<td>1.00 (0.25-4.00)</td>
<td>.99</td>
</tr>
<tr>
<td>MTHFR C677T¶</td>
<td>17 (14.4) 11 (9.3)</td>
<td>17 (14.4) 11 (9.3)</td>
<td>1.00 (0.25-4.00)</td>
<td>.99</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; CVD, cardiovascular disease; MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio.
*Study subjects have been described previously by Voetsch et al.13 Unless otherwise specified, data are expressed as number (percentage) of subjects.
†Calculated from a conditional logistic model accounting for age and sex.
§Indicates heterozygosity.
¶Indicates homozygosity.

−107 genotypes. Patients who did not carry the 192RR genotype (QQ+QR genotypes) or the −107T allele (CC genotype) were used as the reference group and attributed a risk of 1.00. While the presence of the −107T allele alone conferred an only slightly increased adjusted risk of AIS (OR, 1.61; 95% CI, 0.75-3.46; P = .15), carriers of the 192RR genotype had a nonsignificant, 4-fold higher adjusted risk than the reference group (OR, 4.10; 95% CI, 0.84–19.98). Interestingly, the risk of AIS in individuals carrying both the −107T allele and the 192RR genotype was 17-fold higher than in noncarriers (OR, 17.01; 95% CI, 1.74–166.35; P = .02). This finding indicates that the risk conferred by the −107T allele is higher in carriers of the RR genotype (OR, 4.13; 95% CI, 1.35–12.86; P = .01) compared with that in noncarriers (OR, 1.61), suggesting a synergistic interaction between these 2 polymorphisms. No such effect was found between the 192 and –824 polymorphisms.

**COMMENT**

Paraoxonase has emerged as a novel risk factor for cardiovascular disease in the past decade, but the risk associated with the promoter polymorphisms is not well characterized. The data presented herein are, to our knowledge, the first to analyze the role of the PON1 promoter polymorphisms in AIS. In a strictly matched patient and control population younger than 45 years, we found that the presence of the −107T allele was associated with an independent 2.7-fold increase in risk of AIS. This result is in accordance with the 2 studies to date that have analyzed the correlation of the −107 polymorphism and coronary artery disease. James and colleagues12 found that the −107TT genotype independently doubled the susceptibility to coronary artery disease in diabetics (OR, 2.12; 95% CI, 1.19-3.70). A second study by this group determined that the high expressor genotype protected against the risk of coronary artery disease in patients aged 60 years or younger (OR, 0.60; 95% CI, 0.37-0.90), but not in older patients.15 The magni-
The −107 and −824 polymorphisms were found to be in strong linkage disequilibrium with each other and with the PON1 55 coding region polymorphism. Linkage between the 55 and the promoter polymorphisms, in particular at position −107, has previously been identified in several studies.8,10,11,12,16,17,18 The polymorphic −107 site lies within the GGG consensus sequence of an Sp1-binding site, which may explain its effect on promoter activity.8

The −107 and −824 polymorphisms were found to correlate the polymorphisms with the intermediate phenotype.8

Table 4. Combined Effect of PON1 192 and −107 Genotypes on Risk for AIS

<table>
<thead>
<tr>
<th>192 and −107 Genotypes</th>
<th>Patients (n = 118)</th>
<th>Controls (n = 118)</th>
<th>Crude OR (95% CI)†</th>
<th>Adjusted OR (95% CI)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>QQ + OR/CC</td>
<td>34</td>
<td>43</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>QQ + OR/CT + TT</td>
<td>65</td>
<td>69</td>
<td>1.20 (0.66-2.20)</td>
<td>1.61 (0.75-3.46)</td>
</tr>
<tr>
<td>RR/CC</td>
<td>9</td>
<td>4</td>
<td>3.53 (0.92-13.51)</td>
<td>4.10 (0.84-19.98)</td>
</tr>
<tr>
<td>RR/CT + TT</td>
<td>10</td>
<td>2</td>
<td>7.21 (1.41-36.91)</td>
<td>17.01 (1.74-166.35)</td>
</tr>
</tbody>
</table>

Abbreviations: AIS, arterial ischemic stroke; CI, confidence interval; OR, odds ratio; PON1, paraoxonase gene.

*All ORs are relative to the reference category, the QQ + OR/CC genotype.
†Accounts for age and sex.
‡Adjusted for ethnicity and vascular and inherited prothrombotic risk factors.
§P < 0.05
||For the RR/(CT + TT) vs RR/CC genotypes, OR, 4.15; 95% CI, 0.35-48.76.

The analysis of the combined effect of the 192 coding region and the −107 promoter polymorphisms suggested an interesting interaction between the 2 polymorphic alleles. The risk associated with the −107T allele was higher among carriers of the 192RR genotype compared with noncarriers (ORs, 4.13 vs 1.61). Carriers of both the −107T allele and the 192RR genotype had an adjusted risk for AIS of 17.01, which is 2.6-fold higher than would be expected by multiplying the risk conferred by each polymorphism individually. Although the sample size for this analysis is small, yielding wide and occasionally nonsignificant CIs, these results are strengthened by their biological plausibility. These findings are in accordance with the description by James and colleagues8,17 of a complex interaction between the −107 and 192 polymorphisms. However, rather than the modulating effect of the −107 polymorphism found by their group, our results are novel in that they show that the presence of the −107T allele enhances the risk conferred by the coding region 192RR genotype. Evidence from our study and previous studies supports the hypothesis that the promoter and 192 polymorphisms influence PON1 activity and vascular risk by separate mechanisms.12 Furthermore, these findings support the current view that cerebrovascular disease is a complex trait and multifactorial in etiology, frequently due to underlying gene-gene or gene-environment interactions.

In this study, we have described 2 novel methods for the detection of the PON1 promoter −107 and −824 polymorphisms. Previous investigators have genotyped these polymorphisms by hybridization with allele-specific oligonucleotides.11,12 We identified the −107 polymorphism by nonradioactive SSCP, followed by automated silver staining, and the −824 polymorphic site by recognition of a restriction site artificially introduced into the amplified DNA fragment. These methods proved to be rapid, sensitive, and reliable PCR-based procedures, which had the advantage of not requiring the use of radioactive labeling.

Some limitations of our study should be discussed. First, this study includes only patients with nonfatal AIS, and the possibility of survival bias must be taken into account. Second, PON1 concentration and activity measurements were not performed, and we could not correlate the polymorphisms with the intermediate phenotypes. This is an allelic association study and, therefore, cannot provide evidence of the mechanism by which the PON1 polymorphisms influence the development of AIS. Finally, although this is a large cohort of young patients with AIS, we did not have sufficient statistical power to analyze some of the subgroups conclusively. Confirmation of our results requires studies with larger numbers of subjects to assess the combined effect of the PON1 coding region and promoter polymorphisms and the mechanistic implications of these observations.

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Author contributions: Study concept and design (Drs Voetsch, Damasceno, and Loscalzo); acquisition of data (Drs Voetsch and Damasceno); analysis and interpretation of data (Drs Voetsch, Panhuysen, and Loscalzo, and Ms Benke); drafting of the manuscript (Dr Voetsch); critical revision of the manuscript for important intellectual content (Drs Voetsch and Loscalzo); statistical expertise.
(Ms Benke and Dr Panhuysen); obtained funding (Drs Voetsch, Damasceno, and Loscalzo); administrative, technical, and material support (Dr Loscalzo); study supervision (Dr Loscalzo).

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REFERENCES