Platelet glycoprotein Ibα Kozak polymorphism is associated with an increased risk of ischemic stroke

Ross I. Baker, John Eikelboom, Elizabeth Lofthouse, Nicole Staples, Vahid Alshar-Kharghan, José A. López, Yang Shen, Michael C. Berndt, and Graeme Hankey

Platelets are pivotal to the process of arterial thrombosis resulting in ischemic stroke. Occlusive thrombosis is initiated by the interaction of von Willebrand factor (vWF) and platelet glycoprotein (GP) Ibα. Three polymorphisms have been described in GP Ibα (Kozak T/C polymorphism, variable number of tandem repeats [VNTR], and the human platelet antigen 2a [HPA-2a] [Thr] or HPA-2b [Met] at position 145), each of which may enhance the vWF and GP Ibα interaction. This study investigated whether these polymorphisms are candidate genes for first-ever ischemic stroke. A hospital-based case-control study was conducted of 219 cases of first-ever ischemic stroke and 205 community controls randomly selected from the electoral roll and stratified by age, sex, and postal code. The subtypes of stroke were classified, and blood was collected to perform genotyping analysis for Kozak C or T alleles, VNTR, and HPA-2a/b. It was found that the Kozak T/C genotype was over-represented in the stroke group (32.2%) compared with controls (22.8%) (odds ratio [OR], 1.6; 95% confidence interval [CI], 1.03-2.54; P < .05), and the association was still present even after adjusting for conventional risk factors.

Introduction

Ischemic stroke is a frequent cause of death worldwide and remains a common cause of persistent disability.1,2 A large number of risk factors have been identified and prevention strategies, such as smoking cessation, reducing elevated blood pressure, lowering cholesterol, and maintaining good diabetic control, have been successful in reducing the disease burden. However, it is clear that ongoing platelet activation is still occurring in these patients despite best medical management with the current antithrombotic agents. One possible reason is that these drugs do not prevent shear-induced platelet activation mediated by GP Ibα and vWF.3–8

The vWF and GP Ibα interaction is therefore a target for investigation in patients with ischemic stroke. Recently, 3 relatively frequent polymorphisms in GP Ibα have been described in the general population,9 each of which could increase the potential for shear-induced platelet activation by enhancing the efficiency of the binding of vWF to GP Ibα. The Kozak dimorphism detected by the presence of either thymine (T) or cytosine (C) at position –5 from the initiator ATG start codon, influences messenger RNA translation and the amount of GP Ibα on the platelet surface.9 The C allele proportionally increases the amount of GP Ibα expressed on the platelet surface.10 It is plausible that increasing the density of receptor would predispose to enhanced attachment of vWF, causing platelet activation. Two other polymorphisms affect the structure of GP Ibα. One causes a change in the length of the polypeptide by a variation in the number of 13 amino acid tandem repeats (VNTR)
in the mucinlike macroglycopeptide region.\textsuperscript{13-15} The length varies by multiples of either 1 (D allele), 2 (C allele), 3 (B allele), or 4 (A allele).\textsuperscript{15,16} This polymorphism may have important implications for the function of GP Ib\textalpha{}, as each added repeat would position the ligand-binding region further away from the platelet membrane surface, making it more accessible to ligand binding and more susceptible to shear forces. The other GP Ib polymorphism within the vWF- and thrombin-binding leucine-rich repeat region is based on the presence of threonine or methionine at position 145.\textsuperscript{16-18} This polymorphism is the basis for the human platelet antigen 2 (HPA-2) (Ko) platelet allo-antigen system.\textsuperscript{16-18} It has been shown that methionine 145 (HPA-2b) is in linkage disequilibrium with the VNTR alleles A and B.\textsuperscript{16,18}

The Kozak polymorphism has not been examined in a large number of patients with ischemic stroke and conflicting results have been obtained in 4 reports regarding the importance of the VNTR and HPA-2 polymorphisms in ischemic stroke.\textsuperscript{19-22} To help clarify this issue, the present study examined the prevalence of the VNTR, HPA-2, and Kozak polymorphisms in a large number of patients with confirmed ischemic stroke classified by etiologic subtype and a similar number of people randomly selected from the electoral roll.

**Patients and methods**

**Selection of patients**

Consecutive patients presenting to a university teaching hospital in Western Australia between March 1996 and June 1998 with a first-ever ischemic stroke were approached for consent to participate in our study that was approved by the Ethics Committee of Royal Perth Hospital. Stroke was defined as a clinical syndrome characterized by rapidly developing symptoms lasting more than 24 hours or leading to earlier death and with no apparent cause other than that of vascular origin.\textsuperscript{21} Ischemic stroke was defined as a stroke with either a normal computed tomography (CT) brain scan or evidence of a recent infarct in the clinically relevant area of the brain on a CT or magnetic resonance imaging (MRI) brain scan performed within 3 weeks of the event or at autopsy.\textsuperscript{24} Patients with cerebral hemorrhage or cerebral venous thrombosis were not included. Baseline demographic data (age and sex), history of conventional vascular risk factors (hypertension, diabetes, hyperlipidemia, and current smoker), and history of previous vascular events (myocardial infarction, angina, claudication, and amputation) were obtained. All patients underwent a CT brain scan. Echocardiography and extracranial duplex ultrasound were performed at the discretion of the clinician. An overnight fasting blood sample was obtained for biochemical and genetic analyses within 7 days of the acute stroke event.

On the basis of clinical evaluation and results of imaging studies, the study neurologist (G. J. H.) (who remained blinded to the results of GP Ib\textalpha{} genotyping) classified all strokes into 4 major subtypes according to the following predefined criteria.\textsuperscript{24} (1) Large-artery disease included ischemic stroke with (a) evidence of extracranial or intracranial occlusive large artery disease (eg, Doppler or angiographic), (b) no major cerebral embolic source (atrial fibrillation, recent myocardial infarction [in the past 6 weeks], endocarditis, or prosthetic heart valve), and (c) clinical opinion that the most likely cause of brain infarction was atherothrombosis involving the aortic arch, carotid arteries, or major branches (main stem middle cerebral artery), or vertebral, basilar, and posterior cerebral arteries. (2) Small-artery disease included ischemic stroke with (a) consciousness and higher cerebral function maintained; (b) one of the classical lacunar syndromes (ie, pure motor hemiparesis, pure hemisensory loss, pure hemisensory-motor loss, or ataxic hemiparesis) or nonlacunar small-artery clinical syndromes (eg, basilar branch artery syndromes); and (c) CT or MRI brain scan, performed within 3 weeks of symptom onset, that was either normal or showed a small deep infarct in the basal ganglia, internal capsule, or brainstem. (3) Cardioembolic disease included ischemic stroke with (a) a major cardioembolic source, (b) no definite evidence of occlusive large artery disease, and (c) clinical opinion that the most likely cause of brain infarction was embolism from the heart. (4) Other included ischemic stroke that did not meet the criteria for one of the categories outlined above (eg, perioperative, hypoperfusion, dissection, or procoagulant state), or when there was more than one likely explanation (eg, concurrent large-artery occlusive disease and major cardioembolic source).

Control subjects were randomly selected from the Western Australian electoral roll, stratified by 5-year age group, sex, and postal code. A letter of invitation to participate, together with a stamped and self-addressed envelope, was sent to potential controls. Nonresponders were contacted by telephone. Controls who agreed to participate in the study were given the option of attending the hospital outpatient clinic or being visited at home by the study nurse. Baseline demographic data (age and sex), history of conventional vascular risk factors, and history of previous vascular events were obtained for each control. A blood sample was obtained for genetic analysis.

**DNA amplification**

Genomic DNA was prepared from peripheral blood leukocytes by use of a Triton X-100 (Merck, Melbourne, Australia) salt precipitation method.\textsuperscript{25} Polymerase chain reaction (PCR) was performed by using a Perkin Elmer Cetus DNA thermal cycler (Norwalk, CT). Approximately 100 ng genomic DNA was amplified using 10 \(\mu\)M of each primer, 1-2 U Taq polymerase (Biotech, Perth, Australia), and 20 \(\mu\)M each deoxyribonucleotide triphosphate in buffer containing 67 mM Tris-HCl (pH 8.8), 17 mM ammonium sulfate, 1.5 mM magnesium chloride, 0.45\% Triton X-100, and 0.2 mg/mL gelatin. The DNA fragments were generated from a 35-cycle PCR consisting of 40 seconds at 95\%C (denaturing), 40 seconds at 60\%C (annealing), and 1 minute at 72\%C (extension).

For detection of the Kozak polymorphism, the sequence of the upstream primer was 5'-GAGAGACGGAGCTGAGC-3' and that of the downstream primer was 5'-GGTTGTGCTTCTTGCGAG-3' as previously described.\textsuperscript{12} Samples were restriction digested using 2 \(\mu\)Pfu MI (New England Biolabs, Beverly, MA) at 37\%C for several hours. Digestion of the amplified product from T/T produced 3 bands (125 base pair [bp], 157 bp, and 175 bp), from C/C 2 bands (125 and 332 bp), and from heterozygotes CT 4 bands (125 bp, 157 bp, 175 bp, and 332 bp).

The VNTR polymorphism was detected by using the upstream primer 5'-TCCACTGGCTTCTACAGAC-3' and the downstream primer 5'-GCTGTACAGTCTCCAGGAT-3'.

The HPA-2 polymorphism was detected by allele-specific hybridization, using the common upstream primer 5'-GATGGGACGGCTGCACTG-3' with either the downstream primer for Thr, 5'-CTTCTCCAGCTGGTGTGGGAG-3', or the downstream primer for Met, 5'-CTTCTCCAGCTGGTGTGGGAG-3'.

All DNA fragments were subjected to electrophoresis on 2% agarose gels and visualized under ultraviolet light after staining with ethidium bromide.

**Statistical analysis**

The association of the Kozak, VNTR, and HPA-2 polymorphisms with ischemic stroke was assessed by using a logistic regression model with patient or control as the dependent variable, adjusting for conventional cardiovascular risk factors (smoking, hypertension, diabetes, hyperlipidemia, and previous vascular event). The results were expressed as the odds ratios (ORs) together with their 95% confidence intervals (CIs). Baseline differences between cases and controls were examined by means of the unpaired Student t test for continuous variables and the chi-square test for categorical data. Differences were considered statistically significant for \(P\) values <.05.

**Results**

Clinical characteristics and vascular disease factor data for our patient and control groups are analyzed in Table 1. Age and sex were similar for
of the T/C genotype in the predominantly Caucasian Australian population was similar to a report of the French Caucasian population (23.1%) and within the frequency of 15% to 30% of 4 other different ethnic populations. We found that the T/C genotype was elevated in the stroke group (32.2%) with an unadjusted OR of 1.6 (95% CI, 1.03-2.54; \( P < .05 \)). This increased relative risk was still apparent even when conventional cardiovascular risk factors such as hypertension, diabetes, hyperlipidemia, smoking, and previous vascular event were adjusted with an OR of 1.6 (95% CI, 1.0-2.59; \( P = .05 \)). The CC homozygotes were uncommon, and the study was not powered to examine the role of the CC genotype alone. There appeared to be no overrepresentation of the Kozak polymorphism with the particular subtype of stroke.

The reason why the Kozak polymorphism may be important in the pathogenesis of ischemic stroke could be because the presence of the C allele increases the level of GP Ib\( \alpha \) on the platelet surface. With the use of flow cytometry in washed platelets, the amount of GP Ib\( \alpha \) on the surface of the platelet has been shown to be proportional to the amount of the C allele. Although this finding was not supported by another report, when the C allele was transfected into Chinese hamster ovary cells, it resulted in a proportional increase in surface GP Ib\( \alpha \) expression. Compared with the common T/T genotype (100%), the C/C homozygotes expressed the most (157% ± 26%) and the T/C heterozygotes an intermediate amount (128% ± 16%) of GP Ib\( \alpha \). Because the GP Ib\( \alpha \) molecule is important for platelet adhesion to the vessel wall and binds vWF under conditions of high shear stress, it is likely that increasing the relative density of GP Ib\( \alpha \) on the surface of the platelet would increase platelet adhesion and aggregation.

### Discussion

This report identifies that the Kozak GP Ib\( \alpha \) polymorphism is an independent risk factor for ischemic stroke. The frequency (22.8%) of the T/C genotype in the predominantly Caucasian Australian population was similar to a report of the French Caucasian population (23.1%) and within the frequency of 15% to 30% of 4 other different ethnic populations. We found that the T/C genotype was elevated in the stroke group (32.2%) with an unadjusted OR of 1.6 (95% CI, 1.03-2.54; \( P < .05 \)). This increased relative risk was still apparent even when conventional cardiovascular risk factors such as hypertension, diabetes, hyperlipidemia, smoking, and previous vascular event were adjusted with an OR of 1.6 (95% CI, 1.0-2.59; \( P = .05 \)). The CC homozygotes were uncommon, and the study was not powered to examine the role of the CC genotype alone. There appeared to be no overrepresentation of the Kozak polymorphism with the particular subtype of stroke.

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### Table 1. Distribution of baseline demographics and conventional cardiovascular risk factors in cases and controls

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Patients (n = 219)</th>
<th>Controls (n = 205)</th>
<th>Unadjusted OR</th>
<th>95% CI</th>
<th>( P )*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, y (SD)</td>
<td>66.1 (12.4)</td>
<td>67.0 (11.8)</td>
<td>—</td>
<td>—</td>
<td>.44</td>
</tr>
<tr>
<td>Men</td>
<td>140 (64%)</td>
<td>131 (60%)</td>
<td>—</td>
<td>—</td>
<td>.99</td>
</tr>
<tr>
<td>History of hypertension</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>118 (54%)</td>
<td>68 (33%)</td>
<td>2.35</td>
<td>1.59-3.49</td>
<td>.001</td>
</tr>
<tr>
<td>Diabetes</td>
<td>55 (25%)</td>
<td>22 (11%)</td>
<td>2.79</td>
<td>1.63-4.77</td>
<td>.001</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>52 (24%)</td>
<td>45 (22%)</td>
<td>1.11</td>
<td>0.70-1.74</td>
<td>.66</td>
</tr>
<tr>
<td>Smoking</td>
<td>72 (33%)</td>
<td>36 (18%)</td>
<td>2.30</td>
<td>1.46-3.63</td>
<td>.001</td>
</tr>
<tr>
<td>Previous vascular event</td>
<td>59 (27%)</td>
<td>26 (13%)</td>
<td>2.54</td>
<td>1.53-4.22</td>
<td>.001</td>
</tr>
</tbody>
</table>

OR indicates odds ratio; CI, confidence interval.

*Chi-square test for categorical data, unpaired Student t test for continuous data.

### Table 2. Distributions of Kozak, variable number of tandem repeats, and human platelet antigen–2 mutations in cases and controls

<table>
<thead>
<tr>
<th>Platelet GP Ib( \alpha ) genotype</th>
<th>Controls (n = 205)</th>
<th>Patients (n = 219)</th>
<th>Unadjusted OR</th>
<th>95% CI</th>
<th>( P )*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kozak</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>14 (75.6%)</td>
<td>135 (65.8%)</td>
<td>1 (reference)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>TC</td>
<td>44 (22.8%)</td>
<td>66 (32.2%)</td>
<td>1.62</td>
<td>1.03-2.54</td>
<td>.03*</td>
</tr>
<tr>
<td>CC</td>
<td>3 (1.6%)</td>
<td>4 (2.0%)</td>
<td>1.44</td>
<td>0.32-6.56</td>
<td>.64</td>
</tr>
<tr>
<td>VNTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>4 (4.1%)</td>
<td>5 (4.3%)</td>
<td>1 (reference)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DC</td>
<td>17 (8.8%)</td>
<td>26 (12.6%)</td>
<td>1.36</td>
<td>0.44-4.22</td>
<td>.59</td>
</tr>
<tr>
<td>CC</td>
<td>162 (79.2%)</td>
<td>164 (79.2%)</td>
<td>0.90</td>
<td>0.34-2.39</td>
<td>.83</td>
</tr>
<tr>
<td>BC</td>
<td>8 (2.1%)</td>
<td>9 (2.4%)</td>
<td>1.11</td>
<td>0.22-5.63</td>
<td>.90</td>
</tr>
<tr>
<td>BB</td>
<td>2 (1.0%)</td>
<td>3 (1.4%)</td>
<td>1.33</td>
<td>0.18-10.12</td>
<td>.78</td>
</tr>
<tr>
<td>HPA-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a( ^{\text{b}} )</td>
<td>169 (88.5%)</td>
<td>175 (85.0%)</td>
<td>1 (reference)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>a( ^{\text{b}} ) ( ^{\text{c}} )</td>
<td>19 (9.9%)</td>
<td>31 (15.0%)</td>
<td>1.58</td>
<td>0.85-2.8</td>
<td>.14</td>
</tr>
<tr>
<td>a( ^{\text{b}} ) ( ^{\text{c}} )</td>
<td>3 (1.6%)</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Results are presented as frequencies (%) with unadjusted odd ratio (OR) and 95% confidence intervals (CI). GP indicates glycoprotein; VNTR, variable number of tandem repeats; HPA, human platelet antigen.

*Significant.
platelet would make platelets more adhesive and possibly also to be more readily activated by shear stress, causing thrombosis and vessel occlusion. It may be one mechanism to explain why patients with ischemic stroke have evidence of ongoing platelet activation that is seen even in the chronic phase\textsuperscript{10,21,22} and is associated with poststroke mortality.\textsuperscript{21}

The strengths of our study are that we assembled an inception cohort of more than 200 patients with ischemic stroke and a similar number of community-based controls selected at random from the electoral roll. The diagnosis and etiologic subtype of stroke was determined by an experienced neurologist (G. J. H.) on predefined and accepted objective criteria.\textsuperscript{23,24} He remained blinded to the GP Ib\textsubscript{a} genotypes according to subtypes of ischemic stroke

<table>
<thead>
<tr>
<th>Stroke subtype (n)</th>
<th>Kozak T/C (n = 110)</th>
<th>VNTR CC (n = 326)</th>
<th>CD (n = 43)</th>
<th>DD (n = 17)</th>
<th>BC (n = 9)</th>
<th>BB (n = 5)</th>
<th>HPA-2b (n = 53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large artery (63)</td>
<td>18 (30%)</td>
<td>49 (83%)</td>
<td>5 (8%)</td>
<td>3 (5%)</td>
<td>1 (1%)</td>
<td>1 (2%)</td>
<td>9 (16%)</td>
</tr>
<tr>
<td>Small artery (68)</td>
<td>18 (29%)</td>
<td>46 (72%)</td>
<td>14 (22%)</td>
<td>2 (3%)</td>
<td>2 (3%)</td>
<td>—</td>
<td>12 (19%)</td>
</tr>
<tr>
<td>Cardioembolic (44)</td>
<td>13 (30%)</td>
<td>35 (81%)</td>
<td>4 (9%)</td>
<td>2 (5%)</td>
<td>2 (5%)</td>
<td>—</td>
<td>7 (16%)</td>
</tr>
<tr>
<td>Other (43)</td>
<td>17 (42%)</td>
<td>34 (83%)</td>
<td>3 (7%)</td>
<td>2 (5%)</td>
<td>—</td>
<td>2 (5%)</td>
<td>3 (7%)</td>
</tr>
<tr>
<td>Control (219)</td>
<td>44 (23%)</td>
<td>162 (84%)</td>
<td>17 (9%)</td>
<td>8 (4%)</td>
<td>4 (2%)</td>
<td>2 (1%)</td>
<td>22 (10%)</td>
</tr>
</tbody>
</table>

See Table 2 for abbreviations.

Kozak polymorphism was still a significant independent risk factor for ischemic stroke. In addition there was no significant difference between the frequency of the T/C polymorphism between those with (23.5%) or without (28.7%) vascular disease. It makes any positive finding in our series more significant and generally applicable to all patients with ischemic stroke.

Our study has several potential limitations. First, although cases were classified prospectively and recruited consecutively and controls were randomly selected from the community, potential confounding can never be entirely eliminated in an observational study. Second, the small number of cases among the etiologic subtypes of ischemic stroke may have limited the power of our study to detect potentially important differences in the prevalence of the GP Ib\textsubscript{a} polymorphisms between these groups.

Other gene polymorphisms of GP Ib\textsubscript{a} have been described. One is the VNTR that is in linkage disequilibrium with the HPA-2 polymorphism at position 145.\textsuperscript{16,18} The A and B VNTR variants are associated with methionine 145 (HPA-2b).\textsuperscript{16,18} We found a trend toward the association between the HPA-2a/b genotype and ischemic stroke that was more apparent when conventional risk factors were considered (adjusted OR, 1.8; 95% CI, 0.94-3.4; P = .07). Previous studies concerning the HPA-2 polymorphism are conflicting, but all show a trend of increased risk with the HPA2a/b polymorphism. The significance of the VNTR polymorphism data is also different in several reported studies. The results are summarized in Table 4. Like the present study, one study of 609 stroke patients found no association between the genotype distribution of VNTR in patients and in controls.\textsuperscript{21} This is further supported by the finding that, although the levels of plasma markers of platelet activation (PF4 and β-thromboglobulin) were generally elevated in the stroke group, no differences could be demonstrated according to VNTR genotype.\textsuperscript{21} In a small study, there was a link between the B allele and the BC genotype (OR, 2.83) for cerebrovascular disease.\textsuperscript{19} Like another report\textsuperscript{21} the BC genotype was uncommon in our stroke population (2.1% control versus 2.4% patient). These differences in the apparent significance of the GP Ib\textsubscript{a} polymorphisms in stroke are therefore probably due to variations in the background genotype frequencies of these mutations among normal populations of different ethnic backgrounds, to different patient and control recruitment, to variation in the inclusion criteria, or to differences in attributable risks to other cardiovascular factors in the various cohorts.

We found little variation in the prevalence of the GP Ib\textsubscript{a} polymorphisms and the classification of ischemic stroke according to large-, small-artery, and cardioembolic stroke. One recent study suggests the HPA-2b allele may be more important in those patients with transient ischemic attack (OR, 4.3) followed by lacunar infarction (OR, 2.2) then by atherothrombotic stroke (OR, 1.5).\textsuperscript{22} However, in that study the distribution of type of stroke was different and the cohort was younger when compared with our...
group of patients. These differences in case demographics may account for the lack of association in our study. It suggests that larger studies are required to clarify the effect of the GP Ib polymorphisms in subtypes of ischemic stroke or transient ischemic attack.

Our findings show that the Kozak GP Ibo polymorphism is an independent risk factor for ischemic stroke regardless of etiology. However, further studies are needed to define the role of the other platelet polymorphisms and the interaction of known cardiovascular risk factors with the GP Ib polymorphisms.

References

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