The COX-2 G/C −765 polymorphism may modulate the occurrence of cerebrovascular ischemia
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In the atherosclerotic plaque, cyclooxygenase-2 (COX-2) catalyzes prostaglandin E formation, which acts as a proatherogenic factor. A polymorphism, G/C −765, within the COX-2 promoter region modulates gene expression and the risk of cerebrovascular disease. We have evaluated the relation between COX-2 G/C −765 genotypes and the occurrence of cerebrovascular ischemia. We evaluated the COX-2 G/C −765 polymorphism in 110 consecutive patients with a documented history of acute ischemic cerebrovascular disease, in 110 age-matched and sex-matched subjects without such history, and in a general population (\(n = 324\)) from the same geographic area. The frequency of the COX-2 −765C allele in patients (0.21; 95\% confidence interval (CI), 0.16–0.26) was similar to those found in controls (0.28; 95\% CI, 0.22–0.34) and in the general population (0.26; 95\% CI, 0.23–0.29). Carriers of the COX-2 CC genotype differed between patients (0.02; 95\% CI, 0.00–0.05) and controls (0.10; 95\% CI, 0.04–0.16), \(P = 0.019\); odds ratio, 0.17 (95\% CI, 0.04–0.79) or the general population (0.08 (95\% CI, 0.05–0.11), \(P = 0.023\); odds ratio, 0.22 (95\% CI, 0.05–0.95)). In a multiple logistic regression analysis adjusted for confounding variables, smoking status (\(P < 0.001\)), atrial fibrillation (\(P = 0.004\)) and COX-2 G/C −765 polymorphism (\(P = 0.016\)) independently contributed to cerebrovascular ischemia, with CC carriers exhibiting a lower risk (odds ratio, 0.07; 95\% CI, 0.01–0.61). Our data show an association between the COX-2 G/C −765 gene polymorphism and cerebrovascular ischemia, suggesting that the COX-2 gene is a susceptibility locus for the risk of cerebrovascular ischemic disease. Blood Coagul Fibrinolysis 17:93–96 © 2006 Lippincott Williams & Wilkins.

Keywords: stroke, thrombosis, cyclooxygenase-2, genetics, risk factors

Introduction
Stroke is a major complication of atherosclerotic cardiovascular disease and a leading cause of morbidity and mortality in western countries [1]. The number of determinants of thrombosis known today has led to the concept of thrombosis as a multigenic and multicausal disease; that is, disease will only develop in the presence of several interacting risk factors [2]. However, such risk factors account for only a portion of the future ischemic episode [3]. Cross-sectional, longitudinal, and twin studies strongly support an inherited component to cerebrovascular risk [4,5]. It is well recognized that a history of parental cerebrovascular disease is associated with an increased risk of stroke [6]. Studies investigating only young and middle-aged patients with stroke found a greater influence of family history of stroke compared with studies including older patients [6].

The arachidonic acid metabolism plays an important role in the pathogenesis of the atherosclerotic plaque and its ischemic complications. Actually, in-vivo and in-vitro studies have reported a relationship between eicosanoid biosynthesis and atherosclerosis [7]. Moreover, clinical studies have shown the efficacy of lowering prothrombotic prostaglandins, such as thromboxane \(A_2\), in reducing arterial thrombosis [8].

A new variant in the cyclooxygenase-2 (COX-2) promoter, a guanine to cytosine substitution at position −765 (−765G>C), has recently been found [9]. Patients carrying the −765C allele had markedly (30\%) lower promoter activity, lower plasma levels of C-reactive protein and, more interestingly, presented a lower risk of myocardial and ischemic stroke [10].

In a setting of patients presenting with acute ischemic cerebrovascular disease, we have investigated the prevalence of COX-2 −765G>C genotypes, and compared the results with those found in an age-matched and sex-matched group of controls and in a large group of healthy subjects from the same geographic area.

Methods
After approval of the local Ethics Committee, the study was carried out according to the Principles of the Declaration of Helsinki; informed consent was obtained from all participants.
Patients

From 8 April 2003 to 13 March 2004, 150 consecutive patients with a clinical diagnosis of acute cerebrovascular disease (79 men, 71 women; mean age, 67.2 years; age range, 19–90 years) were admitted to the Emergency Department Stroke Unit of the University ‘La Sapienza’ of Rome. In order to confirm the clinical diagnosis and define stroke subtypes according to TOAST criteria [11], patients underwent brain computed tomography scan and/or magnetic resonance imaging, Doppler sonography of extra-cranial and intra-cranial vessels and transthoracic/transesophageal echocardiography. The presenting episode was arterial ischemic cerebrovascular event in 112 patients (64 men and 48 women, 25–90 years old), hemorrhagic stroke in 26 patients (10 men and 16 women, 44–90 years old), cerebral vein thrombosis in six patients (one man and five women, 19–49 years old), and non-cerebrovascular disease in six patients (four men and two women, 35–74 years old). Thus, 112 patients were enrolled in the study. Of these, 16 patients (eight men and eight women; mean age, 72.1 years; age range, 49–88 years) had a diagnosis of transient ischemic attack and 96 patients (56 men and 40 women; mean age, 68.7 years; age range, 25–90 years) a diagnosis of ischemic stroke.

A complete clinical summary with emphasis on personal and family history for stroke, angina pectoris, myocardial infarction, peripheral arterial disease, venous thromboembolism and vascular risk factors (high blood pressure, hyperlipidemia, diabetes mellitus, cigarette smoking, alcohol consumption) was obtained from all subjects by specially trained staff, according to a previously described questionnaire [12]. In addition to detailed and specific questions about symptoms of ischemic heart disease, peripheral vascular disease and previous vascular surgery, as defined according to the World Health Organization questionnaire for cardiovascular disease, the questionnaire also contained specific questions concerning stroke and habitual use of drugs. Hypertension was defined as a longstanding use of antihypertensive drugs or as a systolic blood pressure greater than 140 mmHg and/or a diastolic blood pressure greater than 90 mmHg in the sitting position on at least three different occasions at the time of admission. Subjects with fasting blood glucose levels higher than 126 mg/dl or on treatment with diets and drugs that lower plasma glucose levels were classified as diabetics. Smokers were divided into subjects who currently smoke or previously did and those who never did. Subjects with fasting blood cholesterol levels higher than 200 mg/dl were defined as hypercholesteroleemics. Untypability occurred in two patients with ischemic stroke.

Controls

Controls were 112 healthy individuals who attended the same hospital during the same period, matched with cases for sex, age (± 2 years) and area of origin. Their clinical histories excluded the presence of ischemic cerebrovascular disease. Since the assessment of the COX-2 −765G>C polymorphism was unsuccessful in two patients, the relative matched controls were excluded from the analysis of the effect of the polymorphism investigated.

While patients were being recruited, we interviewed 352 volunteers free of personal history of cardiovascular and cerebrovascular disease, either visitors to or employees of the ‘Casa Sollievo della Sofferenza’ Hospital, San Giovanni Rotondo, Southern Italy. Of these, 25 refused and untypability was observed in three blood specimens. Thus, the relative frequency of the COX-2 −765G>C alleles and genotypes was estimated in 324 subjects (167 men and 157 women; mean age, 47.3 years; age range, 31–74 years) from the same ethnic background.

Isolation of DNA and genotype analysis

Blood samples were collected into vacuum tubes containing 0.129 mol/l trisodium citrate and DNA was extracted from peripheral blood leukocytes according to standard protocols [13]. COX-2 −765G>C alleles were investigated as described by Cipollone et al. [10].

Statistical analysis

All the analyses were performed according to the SPSS/PC V10.0 statistical package (SPSS Inc., Chicago, Illinois, USA) and following the recommended procedures. The Kolmogorov–Smirnov test, a non-parametric method, was used to compare the distributions of the variables in patient and control settings. The allele frequencies were estimated by gene counting, and genotypes were scored. The observed numbers of COX-2 genotypes were compared with those expected for a population in Hardy–Weinberg equilibrium using a chi-squared test. The Pearson’s χ² statistics was employed to evaluate the association of the clinical condition with respect to categorical variables. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Adjusted ORs and 95% CIs were calculated by appropriate models, which were set up to evaluate in a logistic analysis the independent contribution of each variable to the ischemic event. An enter method was employed to set up the system, and the log-likelihood and the Wald χ² statistics are presented. For all the tests, significance was established at a P value less than 0.05.

Results

Clinical characteristics

As a whole, 112 patients presenting with acute arterial ischemic cerebrovascular disease were investigated. Based on data from the ethiopathogenic work-up, 64 of them were defined as large artery atherothrombotic events and 23 were defined as cardioembolic events. In two cases a dissection of a cerebral artery was detected,
whereas in one case a lacunar infarct was identified. In six patients, causes of the stroke event were undetermined. A diagnosis of transient ischemic attack was made in 16 patients.

Clinical characteristics of the patients and matched controls are presented in Table 1. At the time of the ischemic cerebrovascular event, 34 patients (30.6%) were taking acetylsalicylic acid whereas none of them was prescribed COX-2 inhibitors.

Patients were comparable with controls with respect to a personal history of myocardial infarction, angina, diabetes mellitus and hypercholesterolemia, whereas they showed a significantly higher percentage of hypertension, atrial fibrillation and smoking (Table 1).

**COX-2 –765GC polymorphism**

The distribution of the COX-2 –765GC genotype frequencies differed significantly between patients and controls, and differed slightly between patients and the general population (Table 2). In the patients’ group, the frequency observed for the COX-2 –765CC genotype was significantly lower than that observed in controls [0.02 (95% CI, 0.00–0.05) versus 0.10 (95% CI, 0.04–0.16); P = 0.019, respectively]. A similar figure was observed when patients’ data were compared with those observed in a group of apparently healthy subjects (Table 2). When compared with controls and individuals from the general population, the OR for having an ischemic cerebrovascular disease was significantly lower in CC carriers (OR, 0.17; 95% CI, 0.04–0.79 and OR, 0.22; 95% CI, 0.05–0.95, respectively).

The genotype frequencies were not different from those predicted from the Hardy–Weinberg equilibrium in patients, controls and in the setting from the general population (chi-squared test; P > 0.1).

No association was found between COX-2 alleles and genotypes and the subtype of ischemic cerebrovascular disease (data not shown). No differences with respect to COX-2 alleles and genotypes were found with respect to sex, age, hypertension, diabetes mellitus, smoking status, personal and family history of myocardial infarction, angina, atrial fibrillation and assumption of acetylsalicylic acid (always P > 0.05). This was true in cases as well as in controls.

The independent nature of the contribution of the COX-2 –765CC genotype to the occurrence of acute ischemic cerebrovascular disease was assessed in a multiple logistic regression model in which, in addition to the COX-2 765GC polymorphism, a series of relevant covariates were included. Under these circumstances, the analysis showed a significant decrease (OR, 0.07; 95% CI, 0.01–0.61) of the occurrence of acute ischemic cerebrovascular disease in CC carriers as compared with non-CC carriers, confirming the strength of the association observed in the univariate analysis (OR, 0.17). In addition, hypertension (OR, 1.82; 95% CI, 1.03–3.23) and atrial fibrillation (OR, 3.64; 95% CI, 1.33–9.93) were significantly related with

### Table 1 Subject characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (n = 112)</th>
<th>Controls (n = 112)</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [mean (range)]</td>
<td>69 (25–90)</td>
<td>68 (23–91)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Men [%]</td>
<td>65 (58%)</td>
<td>65 (58%)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Myocardial infarction [%]</td>
<td>19 (17%)</td>
<td>19 (17%)</td>
<td>1.56</td>
<td>0.73–3.33</td>
</tr>
<tr>
<td>Angina [%]</td>
<td>3 (3%)</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Atrial fibrillation [%]</td>
<td>20 (18%)</td>
<td>6 (5%)</td>
<td>3.84</td>
<td>1.48–6.97</td>
</tr>
<tr>
<td>Hypertension [%]</td>
<td>66 (59%)</td>
<td>50 (45%)</td>
<td>1.78</td>
<td>1.05–3.02</td>
</tr>
<tr>
<td>Diabetes mellitus [%]</td>
<td>28 (25%)</td>
<td>18 (16%)</td>
<td>1.74</td>
<td>0.90–3.37</td>
</tr>
<tr>
<td>Hypercholesterolemia [%]</td>
<td>24 (21%)</td>
<td>20 (18%)</td>
<td>1.26</td>
<td>0.65–2.43</td>
</tr>
<tr>
<td>Smoking [%]</td>
<td>24 (21%)</td>
<td>13 (12%)</td>
<td>2.08</td>
<td>1.00–4.33</td>
</tr>
</tbody>
</table>

*Matched variable.

### Table 2 COX-2 –765GC alleles and genotypes according to the occurrence of acute cerebrovascular disease

<table>
<thead>
<tr>
<th>Allele</th>
<th>General population</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>648</td>
<td>220</td>
</tr>
<tr>
<td>G</td>
<td>0.648</td>
<td>[0.74 (0.71–0.77)]</td>
<td>0.72 (0.66–0.78)</td>
</tr>
<tr>
<td>C</td>
<td>0.26</td>
<td>[0.23–0.29]</td>
<td>0.28 (0.22–0.34)</td>
</tr>
<tr>
<td>Genotype</td>
<td>n</td>
<td>924</td>
<td>110</td>
</tr>
<tr>
<td>GG</td>
<td>0.56 (0.51–0.61)</td>
<td>60 [0.55 (0.46–0.64)]</td>
<td>65 [0.59 (0.50–0.68)]</td>
</tr>
<tr>
<td>GC</td>
<td>0.37 (0.32–0.42)</td>
<td>39 [0.36 (0.31–0.41)]</td>
<td>43 [0.39 (0.34–0.44)]</td>
</tr>
<tr>
<td>CC</td>
<td>0.08 (0.05–0.11)</td>
<td>11 [0.10 (0.04–0.18)]</td>
<td>2 [0.02 (0.00–0.05)]</td>
</tr>
</tbody>
</table>

Allele and genotype numbers presented as observed (95% confidence interval). General population versus controls: genotype distribution, P = NS; CC versus non-CC, P = NS. General population versus patients: genotype distribution, P = 0.008; CC versus non-CC, P = 0.023 (two-sided Fisher’s exact test); odds ratio, 0.22 (95% confidence interval, 0.05–0.95). Controls versus patients: genotype distribution, P = 0.036; CC versus non-CC, P = 0.019 (two-sided Fisher’s exact test); odds ratio, 0.17 (95% confidence interval, 0.04–0.79).
the occurrence of an acute event of cerebrovascular ischemia.

Discussion

COX-2-derived eicosanoids mediate inflammation, a known risk factor for atherosclerosis and its related cerebrovascular and cardiovascular ischemic complications [14].

In a setting of patients presenting with an acute episode of arterial ischemic cerebrovascular disease, we have prospectively evaluated the role of the COX-2 −765G>C polymorphism and we have found a significant and independent inverse relationship between the personal history and the −765CC carrier status. In the attempt to assess whether the relationship between the COX-2 −765G>C gene polymorphism and the occurrence of acute ischemic cerebrovascular disease would be offset by other significant variables, a multiple logistic regression model was performed including, in addition to the COX-2 −765G>C gene polymorphism, the presence of additional known risk factors. Findings from this statistical analysis showed a significant reduction of the occurrence of acute ischemic cerebrovascular disease in carriers of the COX-2 −765CC genotype (adjusted OR, 0.07; 95% CI, 0.01–0.61), confirming the strength of association observed in univariate analyses (crude OR, 0.17). The association was independent of the presence of known classical risk factors for cerebrovascular disease, such as hypertension and atrial fibrillation, suggesting a protective effect of the −765CC genotype. In the present report, the distribution of the COX-2 −765G>C genotypes did not differ between controls and subjects free of personal history of cardiovascular and cerebrovascular disease sampled from the general population. Likewise, allele frequencies were similar between the two groups. We have selected age-matched and sex-matched control subjects among inpatients without ischemic cerebrovascular disease. However, the differences between patients and controls were comparable with those between patients and a setting of subjects free of personal history of cardiovascular and cerebrovascular disease. These findings further strengthen the evidence of the association we have found between the clinical phenotype and the COX-2 −765G>C polymorphism. In addition, control COX-2 −765G>C allele and genotype frequencies comparable with ours have been reported in another Italian series [10].

One major concern of case–control studies such as this is the possibility of a selection bias by chance or sample design influencing the results. To reduce this risk we have selected two different settings of controls. However, the replication of such an association in different populations is warranted. A prospective and not a case–control study would be needed to explore etiologic associations. Because of the lack of prospective studies, our calculated ORs only reflect the possibility of an association between the COX-2 −765G>C polymorphism and the occurrence of arterial cerebrovascular ischemia.

Among subjects carrying the −765GC and −765CC genotypes, the OR for having a myocardial infarction or atherothrombotic ischemic stroke was found to be significantly reduced compared with that for patients carrying the −765GG genotype [10]. At variance with that study, we did not find a higher risk of occurrence of acute cerebrovascular disease in −765 GC carriers. Differences in the study design and in the sample size, informativeness of the marker used, and the statistical power may well explain inconsistencies between the two studies. The inclusion of all patients presenting with acute arterial ischemic cerebrovascular disease might have diluted the protective effect of the −765GG genotype.

In conclusion, we have provided further evidence that the COX-2 −765G>C polymorphism may modulate the ischemic cerebrovascular risk.

References