Association of estrogen receptor-alpha gene polymorphisms with stroke risk in patients with metabolic syndrome


Objective – The vascular protective effects of estrogens are mediated by their binding to the two known estrogen receptors. In this study, we examine the association of stroke with two common polymorphisms of the ESR1 gene in patients with metabolic syndrome. Materials and methods – DNA from 130 patients hospitalized for ischemic stroke and 240 healthy controls were genotyped for ESR1 PvuII and XbaI polymorphisms. Results – Comparing female and male patients, it was found that CCGG diplotype is more frequent in male patients \((P = 0.03)\). In addition, the AA genotype is associated with the onset of stroke at a younger age in the male patient group \((P < 0.05)\). Conclusions – These findings suggest that PvuII and XbaI polymorphisms may affect the age at onset of the first stroke and the probability of developing cerebrovascular disease.

Introduction

Estrogen receptor-alpha is a ligand-activated transcription factor involved in the regulation of cellular pathways. These pathways play an important role in vascular wall physiology and function, as vascular endothelial and smooth muscle cells contain estrogen receptor-alpha protein (1, 2).

According to recent studies, there is evidence that estrogen receptor-alpha gene variation is responsible for a range of important estrogen-dependent characteristics, including responses to lipid profile (3), atherosclerotic severity to hormone replacement therapy (4), coronary reactivity (5, 6), and coronary heart disease (7).

The roles that reproductive steroids play in cerebrovascular pathophysiology and ischemia are an important area of ongoing investigation. It is suggested that genetic variations in estrogen receptor-alpha gene may influence the risk for cardiovascular disease (8, 9) and myocardial infarction (10–12).

In the present study, we examine the association of estrogen receptor-alpha (ESR1) \(c.454-397T > C\) (PvuII) and \(c.454-351A > G\) (XbaI) polymorphisms with ischemic stroke in patients with signs of metabolic syndrome, in the reference center of northwest Greece, an area with homogeneous population and limited recent immigration.

Materials and methods

Subjects

One hundred and thirty patients with metabolic syndrome, 84 males and 46 females, hospitalized in the Stroke Reference Center of northwest Greece from January 2003 to December 2005 were identified in a prospective way. As controls, 240 healthy individuals, age and sex matched, were enrolled. The 100 male and 140 female controls had neither stroke nor metabolic syndrome. Diagnosis of metabolic syndrome was according to the ATP III guidelines and three or more of the following criteria were fulfilled: obesity, dyslipidemia,
hypertension, and elevated glucose levels. A computer tomography (CT) of the brain at the acute phase was performed. Performing magnetic resonance imaging (MRI), in 15 days from the onset of stroke, revealed ischemic lesions not showed by CT scan at the acute phase. For determination of the subtype of ischemic stroke, TOAST (Trial of ORG 10172 in Acute Stroke Treatment) (13) criteria were used. Subtype definition was based on imaging findings (CT scan, MRI and vascular imaging), electrocardiogram (ECG), echocardiography (trans-thoracic or trans-esophageal when needed), assessment of prothrombotic syndromes, and risk factor profiles. Patients were grouped into two subtypes. The first group included patients with large artery atherosclerosis and the second one included patients with small artery occlusions known as lacunars. Cardioembolic stroke patients and stroke patients of other determined cause were excluded. Stroke patients of undetermined cause despite the extensive evaluation, were classified in large or small vessels groups according to CT and MRI findings.

All patients gave detailed interviews and a physical examination was performed. Demographic features, clinical features, severity of stroke using the modified Rankin scale, biochemical parameters, and established risk factors for stroke were recorded. Body mass index (BMI) of each patient was calculated as weight (kg)/height (m)². Blood samples were obtained after overnight fasting for lipid profile. Whole blood samples from both patients and control groups were used for isolation of peripheral blood leukocytes for the genetic analysis. The Institutional Ethics Committee approved the study protocol in accordance with the Helsinki declaration and all participants gave informed consent.

Biochemical assays

Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured by enzymatic methods (Olympus 600 Clinical Chemistry Analyzer, Olympus, Tokyo, Japan). Low-density lipoprotein (LDL) cholesterol was calculated by the Friedewald equation [LDL = total cholesterol (mg/l) – HDL cholesterol (mg/l) – triglycerides (mg/l)/5] (14).

Genotyping

DNA was extracted from peripheral blood leukocytes using standard procedures. The primers: 5’-CTGCACACCTATCTGTATCTTTTGTACAT-3’ and 5’-TCTTTTCTCTGACACCCCTGG-CGTCGATTTATCTGA-3’ were used to amplify a part of intron 1 and exon 2 of the ESR1 gene, containing both polymorphic sites -397T/C (rs2234693) and -351A/G (rs9340799). The -397T/C and -351A/G single nucleotide polymorphisms (SNP) are located 397 and 351 bp, respectively, upstream from the first nucleotide of exon 2 of ESR1.

Polymerase chain reaction (PCR) amplification was performed in a total volume of 25 µl consisting of 1 µl extracted DNA, 0.2 mM dNTP, 0.4 µM of each primer, 1x Taq DNA polymerase buffer, 4 mM MgCl₂ and 1 U of Taq DNA polymerase. The thermal cycling for the ESR1 PCR was as follows: denaturation at 94°C for 2 min, 33 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 30 min with a final extension at 72°C for 10 min. PCR products were subsequently digested with restriction enzymes PvuII and XbaI. Enzyme digestion products were separated by 2% agarose gel electrophoresis and visualized by exposure to ultraviolet light after ethidium bromide staining. The resulting genotypes for PvuII (-397T/C) and XbaI (-351A/G) polymorphic sites were characterized as TT/TC/CC and AA/AG/GG, respectively, and diplotype were also recorded, matching each genotype of PvuII (-397T/C) polymorphism with each genotype of XbaI (-351A/G) polymorphism.

Statistical analysis

Allele frequencies at the individual loci were estimated by counting. The agreement of genotype frequencies with Hardy-Weinberg equilibrium expectations was tested using the chi-square test. Stepwise multiple regression analysis was performed to identify independent predictors of stroke in patients with metabolic syndrome. Continuous data was expressed as the mean ± SD. P value of <0.05 was set as statistically significant. All analyses used the SPSS (version 12.0; SPSS Inc, Chicago, IL, USA).

Results

The mean age of male patients was 57.8 ± 9.2 and of females was 61.7 ± 8.0 while the mean age at first stroke was 56.1 ± 9.1 for men and 59.6 ± 8.5 for women. The clinical characteristics are presented in Table 1. Both polymorphisms were in Hardy-Weinberg equilibrium in the study population and the control group.

The allele distribution, allele frequencies, and genotypes were not significantly different between control and patient groups. No direct association of PvuII and XbaI polymorphisms with stroke
occurrence was identified in both genders (Table 2). Furthermore, a noteworthy gender-specific difference ($P < 0.05$) was that male patients with ESR1 -351A/A genotype had an earlier onset of stroke (53.3 ± 8.1 years) than male patients with -351 G/A and -351 G/G genotypes (56.9 ± 9.4 years). This association was independent of the risk factors (dyslipidemia, hypertension, and diabetes mellitus) and independent of the BMI.

Comparing male and female patients, female patients had less frequently the CCGG diplotypes than men ($P = 0.03$) while there was no difference comparing healthy men with healthy women (Fig. 1). Additionally, the TTAA diplotype tended to be more frequent in female patients than female controls (36.9% vs 26.4%, $P < 0.1$) (Table 3).

These associations were independent of the BMI, a marker used to represent the other risk factors (dyslipidemia, hypertension, and diabetes mellitus). Moreover, there was no association between the type of stroke, defined by the imaging findings, and the severity of stroke and the genotypes. Total cholesterol, triglyceride, and HDL-cholesterol levels gave no evidence of association with alleles and genotypes.

**Discussion**

Gender-specific differences in the incidence of hypertension, coronary artery disease stroke, and generally in the development of arteriosclerosis are attributable to the indirect effect of estrogen on
risk factor profiles, such as cholesterol levels, glucose metabolism, and insulin levels (9). There is evidence that estrogen receptor can mobilize signals at the plasma membrane and in the cytoplasm of the cell walls of the vessels. Consequently, understanding of the estrogen function and regulation may be the key to the development of specific therapeutics that will mediate the prevention and treatment of vascular diseases. Experiments in ESR1 and ESR2 knockout mice showed that ESR1 is the link, which mediates the ability of estradiol to protect the brain against injury (15). Furthermore, ESR1 expression is up-regulated after brain injury, provided a mechanism by which estradiol exerts its protective role (16).

Our results indicate that there may be an earlier stroke insult in males associated with ESR1 genotypes and in particular ESR1 AA genotype at the -351 polymorphic site. This result is potentially unrelated to risk factors and BMI, as in our study individuals with the ESR1 AA genotype had equal mean BMI, if not lower, compared with the other male patients. This is the first study, to our knowledge, to associate ESR1 AA genotype with young age at stroke insult in males.

According to our study, female patients had less frequently the CCGG diplotype compared with men patients, indicating that CCGG diplotype makes men more sensitive to develop cerebrovascular ischemia compared with women (Fig. 1). These results are in concordance with a study of 2709 male participants (17) associating ESR1 variations with a higher risk of stroke. In particular, when men with CC genotype were compared with those with CT or TT genotype at the -397 polymorphic site, they had a relative risk of 1.92. This result was essentially unchanged at 1.84, after additional adjustment for BMI, cholesterol, hypertension, diabetes, and smoking status.

Similar results were observed in recent studies associating ESR1 polymorphisms with vascular disease. In particular, the prospective study of 1739 unrelated men and women of the Framingham study (8) demonstrated an increase of atherothrombotic cardiovascular disease in men with CC genotype compared with individuals with the CT or TT genotypes at the -397 polymorphic site. Participants with the CC genotypes had 3.0-fold greater odds of myocardial infarction compared with individuals with the CT or TT genotypes. This study provided no informative results for women, as there were an insufficient number of women with cardiovascular events. In addition, a study of more than 7000 white men (11) provided evidence that CC genotype, present in 20% of individuals, was a risk factor for non-fatal acute myocardial infarction, after adjustment for established cardiovascular risk factors.
controls (Table 2), similar to the results of the Rotterdam study (10).

In contrast to the previous studies, Koch et al. (12) examined 3657 patients with myocardial infarction and 1211 control individuals. The genotype and diplotype distributions of the -397T/C and -351A/G polymorphisms were not significantly different between the two groups. Separate analysis in men and women did not reveal sex-related associations of specific genotypes or diplotypes of the polymorphisms with myocardial infarction.

All these findings underscore a potentially important role of ESR1 in influencing the development of atherosclerosis and in accelerating the transition from subclinical atherosclerosis to plaque rupture and acute thrombotic cardiovascular disease events such as myocardial infarction and stroke. The ESR1 gene has been shown to mediate three direct effects of estrogen on the vessel wall: alteration of endothelial nitric oxide production (18), inhibition of the vascular injury response (19) and acceleration of re-endothelialization (20). There is recent evidence that ESR1 c.454-397C allele results in a relative high level of ESR1 transcription (10). The c.454-351A > G polymorphism may be responsible for the observed association with stroke risk because of linkage disequilibrium with the c.454-397T > C SNP or the functional significance of the polymorphism itself.

In summary, although the small number of cases enrolled in this study may limit the value of our findings, is nevertheless indicative of the significance of the ESR1 in stroke. In conclusion, this study has shown for the first time, the association of ESR1 genotypes and diplotypes with the incidence of stroke in men and women and the association of these polymorphisms with the age at onset of the first stroke in men, supporting the significance of ESR1 in cerebrovascular disease.

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References