Association Analysis of Glutathione S-transferase Omega-1 and Omega-2 Genetic Polymorphisms and Ischemic Stroke Risk in a Turkish Population

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Abstract

Objectives: Oxidative stress is a known risk factor for the pathogenesis of atherosclerosis, the main cause of ischemic stroke. Glutathione S-transferase (GST) omega-1 and omega-2, members of phase II enzymes, play a role in the antioxidant system. The single nucleotide polymorphisms (SNPs), C419A and A424G in GST omega genes can cause a decrease in enzyme activity. The aim of this study was to investigate the possible association between these polymorphisms and ischemic stroke risk in a Turkish population.

Methods: The genotypes and allele frequencies for 239 patients and 130 controls were determined by the PCR/RFLP method. No significant differences were found between patients and controls in terms of genotype and allele frequencies.

Results: The frequency of the polymorphic 'A' allele was 0.358 in patients and 0.342 in controls for the C419A polymorphism in the *GSTO1* gene. The frequency of the polymorphic 'G' allele for *GSTO2* A424G SNP was 0.370 in patients and 0.404 in controls. The combined homozygous wild type genotype 'CCAG' was significantly higher in control group than in the patients.

Conclusion: No significant difference was observed between the stroke patients and controls in terms of genotypes and allele distributions. Double combine haplotype CCAA was found to be protective against ischemic stroke when compare to other haplotypes. However, different genotypes of *GSTO1* and *GSTO2* were observed to have effects on stroke risk in subgroups of diabetics and smokers. In conclusion, the current study is the first to report this finding.

Keywords: Genetic polymorphism, Glutathione S transferase omega 1, Glutathione S transferase omega 2, ischemia, stroke, SNPs

Running Head: *GSTO1* and *GSTO2* Genetic Polymorphisms and Ischemic Stroke Risk in Turkish Population

Introduction

Stroke is defined as the acute loss of neurological function due to interruption of blood flow to the brain. The most common stroke type, ischemic stroke, is caused by stenosis, which is an obstruction within a blood vessel supplying blood to the brain. Carotid atherosclerosis is a risk factor for stroke [1]. Atherosclerotic plaque formation starts with the build-up of oxidized LDL in macrophages and other phagocytes, which leads to the formation of foam cells [2]. Oxidized-LDL is formed due to an imbalance between the generation of free radicals and antioxidant defense mechanisms, a situation called oxidative stress. Oxidized-LDL plays the role of a promoter in vasoconstriction and platelet aggregation and adhesion, which are related to atherosclerosis [3].

Glutathione S-transferases (GSTs) are members of an important multifunctional enzyme family that play important roles in phase II reactions. GST omega, a class of GSTs, has two isoforms: GST omega 1 and GST omega 2, which are encoded by the *GSTO1* and *GSTO2* genes, respectively, located on chromosome 10q24.3 [4]. GST omega enzymes function as

thioltransferases and dehydroascorbate reductases; they promote antioxidant activity and can also metabolize drugs and toxins [4]. GST omega 1 and 2 enzymes have cysteine residue in their active sites, unlike other GST enzymes. The cysteine residue plays a role in thiol/disulphide homeostasis, which is related with atherosclerotic disease [5]. In addition, GST omega enzyme expression was found in glial cells of the brain, which may play important antioxidant roles in this tissue. [6]. Therefore, we focused on GST omega enzymes in this study. The activities of these enzymes are, however, affected by missense mutations. *GSTO1* C419A (rs4925) is a single nucleotide polymorphism (SNP) in exon 4 on nucleotide 419 from cytosine to adenine, which leads to a change in the coded 140th amino acid from alanine to aspartic acid. The polymorphic genotype causes reduced enzyme activity [7]. The *GSTO2* A424G (rs156697) polymorphism found in exon 4 is a substitution from adenine to guanine at position 424, which causes 142th amino acid conversion from asparagine to aspartic acid [4,8].

Due to their antioxidant functions, these enzymes may also be important in the pathogenesis of ischemic stroke. C419A SNP of the *GSTO1* gene reduces the enzyme activity, and thus, the oxidative stress reaches higher levels and induces brain-related diseases [9]. According to a previous study, the Asp/Asp and Ala/Asp genotypes of the *GSTO1* C419A SNP are significantly associated with an increased risk of cerebrovascular diseases, such as vascular dementia and stroke [9]. On the other hand, GSTO1 C419A SNP was not found to be related with stroke volume [10].

There is no study in the literature regarding the relationship between the genetic polymorphisms C419A in the *GSTO1* gene and A424G in the *GSTO2* gene, and ischemic stroke in Turkish population. Therefore, this study was aimed to investigate the possible association between the *GSTO1* C419A and *GSTO2* A424G SNPs and ischemic stroke risk in a Turkish population. This

study also determined the genotype and allele frequencies of GST Omega Class SNPs in a Turkish population and compared them with those in other populations.

Materials and Methods

Study Population

The study population is composed of 239 patients with acute hemispheric ischemic stroke and 130 symptom-free controls from the Central Anatolia Region of Turkey. The study was approved by the ethical committee of Gülhane Medical Academy, and was carried out according to the principles of the Declaration of Helsinki. The ischemic stroke patients having an anterior circulation stroke that resulted from carotid artery atherosclerotic disease were included in the study from October 2005 to April 2011. The cerebral infarction was initially diagnosed on the basis of neurological examination and brain computer tomography (CT) scan and then transthoracic echocardiographic examination, Holter study and transcranial Doppler emboli detection procedure to rule out emboli source. The inclusion criteria for patients are having anterior circulation stroke, no other major illnesses, including autoimmune diseases, neoplasms, coagulopathies, hepatic or renal failure, no known embolic source (aortic arch, cardiac or carotid), no family history of hematological, autoimmune or chronic inflammatory diseases, no history of myocardial infarction within 3 weeks or of transient ischemic attack or stroke at any time. Our classification system is similar to that described by Trial of ORG 10172 in Acute Stroke Treatment (TOAST). The present study included the TOAST ‘large-vessel disease’ group, Oxfordshire Community Stroke Project (OCSP) ‘total anterior’, and ‘partial anterior circulation infarcts’ groups. Excluding other TOAST groups with anterior circulation stroke we reduced the sample size further with Oxfordshire Community Stroke Project ‘total anterior’ and ‘‘partial

anterior circulation infarcts'. The control subjects were selected randomly from the neurology outpatient clinics; they did not have a stroke or transient ischemic attack at any time. All the other criteria and tests were applied to control samples; additionally, the following criteria were also taken into consideration: patients not having carotid stenosis (lumen narrowing) > 70% or ulcerated carotid plaque.

Genotype Determination

Blood samples were collected from subjects in the stroke and control groups into Na-EDTA-containing tubes for DNA isolation, which was performed by the salting out method [11]. Standard polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) method was used to determine the genotypes for *GSTO1* C419A and *GSTO2* A424G polymorphisms. The primer pairs used for obtaining PCR products for the *GSTO1* C419A (rs4925) region were F: 5′-GAA CTT GAT GCA CCC TTG GT-3′ and R: 5′-TGA TAG CTA GGA GAA ATA ATT AC-3′, and those for the *GSTO2* A424G (rs156697) region were F: 5′-AGG CAG AAC AGG AAC TGG AA-3′ and R: 5′-GAG GGA CCC CTT TTT GTA CC-3′ [12]. The PCR mixtures were prepared with PCR buffer, dNTP mix, $MgCl_2$, Taq polymerase, DNA samples and appropriate primers. The 254-bp *GSTO1* PCR product was digested with the *Cac8I* restriction enzyme to fragments for C419A and *MboI* restriction enzyme for 424G. The digestion products were observed and analyzed on a 2.5% agarose gel, and the allele and genotype frequencies were obtained by the direct gene counting method.

Statistical Analyses

Statistical analyses were performed using Statistical Package for Social Science 20.0 statistical software (SPSS Chicago, IL, USA). Normality of the sample distribution of each continuous variable was tested with the Kolmogorov-Smirnov test. Continuous variables were expressed as

mean ± SD. Median and interquartile range (IQR) values were also assigned for variables that were not distributed normally. Differences of continuous variables were evaluated by the independent samples T-test or Mann-Whitney U test, depending on the shape of the distribution curves. Categorical variables were expressed as proportions and compared using the $\chi 2$ test or Fisher's exact test. Allele frequencies were determined by the gene counting method, and departures from the Hardy-Weinberg equilibrium were evaluated by the $\chi 2$ test. In order to determine the effects of vascular risk factors, lipid parameters, and the *GSTO1* and *GSTO2* genotypes on the prediction of ischemic stroke, logistic regression analyses with backward selection method were performed. The Hosmer-Lemeshow goodness of fit test was used for calibration. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Clinical Characteristics and Conventional Risk Factors

In this study, two SNPs, C419A in the *GSTO1* gene and A424G in the *GSTO2* gene, were investigated in a sample population comprising 239 ischemic stroke patients and 130 controls. The demographic features, clinical laboratory test results, and the prevalence of conventional risk factors for patients and controls in the study population are summarized in Table 1. The mean ages of stroke patients (65.6±13.5) and subjects in the control group (64.2±12.2) were close and there were no significant differences between them (P=0.067). The percentage of males in the patient group (55.6%) was higher than that in the control group (51.5%). As expected, the prevalence of conventional risk factors for ischemic stroke attack, such as hypertension, diabetes mellitus, smoking, and obesity, were significantly higher in the patient group than in the control group. According to the clinical laboratory test results, HDL-cholesterol levels were significantly lower in patients (1.08±0.31 mmol) than in controls (1.20±1.17 mmol/L, P=0.001).

[Table 1 near here]

GSTO1 and GSTO2 Genotype and Allele Frequencies

Table 2 shows the distributions of genotypes and allele frequencies for C419A of the *GSTO1* gene and A424G (424A>G) of the *GSTO2* gene. No significant difference was observed between the stroke patients and controls in terms of genotype and allele distributions of *GSTO1* C419A and *GSTO2* A424G polymorphisms. For *GSTO1* C419A, the minor allele (A) frequency was 0.358 for patients and 0.342 for controls (P=0.675). For GSTO2 A424G, the frequency of the minor allele (G) was found to be 0.370 for patients and 0.404 for controls (P=0.370).

[Table 2 near here]

Table 3 shows distribution of combined haplotypes for *GSTO1* C419A (rs4925) and *GSTO2* A424G (rs156697) SNPs. Prevalence of combined homozygous wild type genotype for *GSTO1* gene and heterozygous genotype for *GSTO2* gene (CCAG) was significantly higher (15.4%) in control group than in the patients (7.5%).

[Table 3 near here]

Genotype distributions were stratified into diabetic/non-diabetic and smoker/non-smoker groups in order to clarify the effects of conventional risk factors for different *GSTO1* and *GSTO2* genotypes on ischemic stroke attacks, as shown in Table 4. The proportion of stroke patients with diabetes to control subjects with diabetes compared to the proportion of stroke patients without diabetes to control subjects without diabetes was the highest among the *GSTO1* CA+AA genotype group (OR=3.873, P=0.000). Similarly, minor allele carriers of *GSTO2* (AG+GG) had a higher risk of diabetes-related stroke (OR=2.715, P=0.004), compared to the wild-type *GSTO2*

genotype groups. In case of the smoker and non-smoker stratifications, a higher risk of ischemic stroke was observed in carriers of mutant *GSTO1* (OR=3.556, *P*=0.002) and *GSTO2* alleles (OR=3.171, *P*=0.004), compared to the wild-type genotypes.

[Table 4 near here]

Logistic Regression Analysis

The logistic regression analysis was applied with backward selection method to evaluate the effects of conventional risk factors, lipid parameters, and *GSTO1* and *GSTO2* genotypes on ischemic stroke risk. The conventional risk factors such as age, sex, hypertension, diabetes mellitus, obesity, smoking, lipid parameters (total cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol), and the *GSTO1* and *GSTO2* genotypes, were added as covariates, and the results are given in Table 5. Hypertension (OR=3.043, 95% CI: 1.840–5.031, *P*=0.000), smoking (OR=3.258, 95% CI: 1.658–6.50, *P*=0.001), and obesity (OR=2.593, 95% CI: 1.129–5.956, *P*=0.025) were revealed as significant risk factors for ischemic stroke, while HDL-cholesterol played a protective role against stroke by decreasing the risk (OR=0.270, 95% CI: 0.111-0.654, *P*=0.004). The model predicted 70.4% of cases correctly and Hosmer-Lemeshow goodness of fit test pointed out that the calibration of the model was satisfactory (chi-square=5.093; 8 degrees of freedom; *P*=0.658).

[Table 5 near here]

Discussion

The present study was aimed to investigate the possible associations between the genetic polymorphisms of GST Omega 1 and GST Omega 2 and ischemic stroke risk in Turkish

population. The genetic polymorphisms of GSTO1 and GSTO2 cause reduction in enzyme activity and increase the ROS formation [7]. A high degree of ROS in blood causes the peroxidation of the lipids, LDL and HDL, and subsequently, atherothrombotic situations and ischemic stroke attacks [3].

Oxidative stress promotes the formation of atherosclerosis. Members of the Glutathione S-Transferase enzyme family play important roles in detoxification and metabolism, and decrease oxidative stress. In our previous study, we have focused on the relationship between the genetic polymorphisms in GSTT1 and GSTM1 genes, as well GST activity and ischemic stroke risk [13]. We found that GSTT1 and GSTM1 null genotypes, together with hypertension, may play a significant role in the pathogenesis of ischemic stroke. In two recent studies, genetic polymorphisms in GSTP1 in addition to GSTT1 and GSTM1 were investigated in relation to ischemic stroke in Turkish population. While any direct relation between these polymorphisms and ischemic stroke was not found, the risk increased in smoker group [14, 15]. Impaired enzyme activity due to C419A SNP in the GSTO1 gene and A424G SNP in the GSTO2 gene may increase the risk of ischemic stroke. However, no significant difference was found between the two groups directly. Previous studies [9, 10], have also considered a correlation between oxidative stress and stroke; hence, the polymorphisms in genes expressing GST omega enzymes were focused upon. Kölsch and co-researchers investigated the effect of C419A polymorphisms in the GSTO1 gene on Alzheimer disease, vascular dementia, and stroke in a German population. They found that Asp/Asp genotypes or Asp/Ala genotypes may be a genetic risk factor for cerebrovascular disease. Furthermore, the logistic regression analyses showed that the presence of at least one A allele associated with an increased risk of stroke [9]. Moreover, in another study which studied the association of GSTO1 C419A polymorphism and stroke, a major effect could

not be found, but the researchers concluded that if the selected study population was larger in size, the results would be more accurate [10].

In addition, according to double combined haplotype analysis, CCAG genotype significantly higher in control than patients. This haplotype can be considered to have a protective effect for ischemic stroke. The distribution of the other haplotypes was not found to be significant.

In the present study, logistic regression analyses revealed that hypertension, smoking, and obesity are significant risk factors for ischemic stroke; additionally, high-density lipoproteins were found to be significant protective factors against ischemic stroke (OR=0.270, P=0.004). Similar results were found in previous studies [16, 17].

Diabetes mellitus, one of the independent risk factors of ischemic stroke, is associated with high blood pressure, high blood cholesterol, atherosclerosis, and obesity. According to a previous study, it is revealed that the thromboembolic stroke risk was two-fold higher in patients with diabetes than in the controls, which were Japanese men [18]. In another previous study involving a Turkish population, the prevalence of diabetes was found to be significantly higher in stroke patients than in non-diabetic groups [16]. Being a risk factor for stroke, it was concluded that diabetic individuals carrying a mutant GSTO1 allele have a higher risk of ischemic stroke. Smoking can cause changes in the structure of plasma fibrinogen, and this structural damage can trigger the blocking of vessels [19]. According to a study, smoking doubles the risk of ischemic stroke; it is considered that the reason for this may be nicotine and carbon monoxide [20]. In parallel with the results of previous studies, our results have shown that smoking is a risk factor for stroke; additionally, having mutant alleles for the GSTO1 and GSTO2 genes cause a higher risk of this disease in smokers.

The comparison of GSTO1 C419A and GSTO2 A424G allele frequencies in different populations and the control group of the present study is shown in Table 6. The frequency of the minor allele (A) for GSTO1 C419A (0.342) was close to that found in another study carried out on a Turkish population (0.311) [21]. On the other hand, in another study also carried out on a Turkish population, the frequency of this allele was considerably different from our results [22]. The frequency of minor allele 'A' for GSTO1 gene was reported as 0.085, which is closer to that of the Taiwanese, Chinese, and Italian populations; they were lower than the values of our results. This may result from differences in the nature of the study populations used. In our study, the blood sampling area was Ankara, which is a cosmopolitan city. Thus, the study population included individuals from central Anatolia and all of Turkey. However, in the study of Takeshita et al., the blood sampling was done only in Adana, which is not considered as a cosmopolitan city. According to previous studies, while the frequency of the minor allele 'A' was lower in Taiwanese, Chinese, and Italian populations than in Turkish populations [12, 23-26], its frequency in German, American, and Serbian populations was closer to the value obtained in our results [9, 27, 28]. The frequency of the minor type 'G' allele for the GSTO2 A424G SNP was found to be 0.404 in the present study, which is closer to that of Brazilian (0.374) and Iranian populations (0.370) [29, 30]. The American, Japanese, Taiwanese, Serbian, and Thai populations show lower minor allele frequencies for this polymorphism [12, 22, 23, 27, 28, 31, 32].

[Table 6 near here]

To our knowledge, the present work is the first study about the investigation of the relationship between GSTO1 C419A and GSTO2 A424G genetic polymorphisms and ischemic stroke risk in a Turkish population. No direct association was revealed between different genotypes and the alleles of the GSTO1 and GSTO2 genes and the risk of ischemic stroke. On the other hand, some

risk assessments were performed, and a correlation was found between some risk factors and the risk of ischemic stroke in subgroup analyses concerning diabetics and smokers carrying the mutant allele.

Limitations of the present study include number of control subjects. However, it should be noted that the sample was clinically well defined and strictly selected so as to keep mean age of controls and stroke patients similar, as age is the strongest determinant of stroke. It was highly difficult to find elderly subjects who meet the required criteria as controls, i.e. no history of ischemic stroke, transient ischemic attack and ischemic heart disease at any time or myocardial infarction within 3 weeks and no more than 70 % carotid stenosis.

Conclusion

To our knowledge, the present work is the first investigating relationship between *GSTO1* C419A and *GSTO2* A424G genetic polymorphisms and ischemic stroke risk in a Turkish population. No direct association was revealed between different genotypes and the alleles of the *GSTO1* and *GSTO2* genes and the risk of ischemic stroke. Double combine haplotype CCAA was found to be protective against ischemic stroke in overall population. On the other hand, some risk assessments were performed, and a correlation was found between some risk factors and the risk of ischemic stroke in subgroup analyses concerning diabetics and smokers carrying the mutant allele.

Acknowledgments

This work was supported by the Middle East Technical University Research Project Fund. We would also like to thank all the patients and controls who provided blood samples for this study.

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