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ORIGINAL ARTICLE

# The C718T polymorphism in the 3′-untranslated region of glutathione peroxidase-4 gene is a predictor of cerebral stroke in patients with essential hypertension

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**In the present study we have investigated the association of three single nucleotide polymorphisms in glutathione peroxidase (GPx) genes *GPX1* rs1050450 (P198L), *GPX3* rs2070593 (G930A) and *GPX4* rs713041 (T718C) with the risk of cerebral stroke (CS) in patients with essential hypertension (EH). A total of 667 unrelated EH patients of Russian origin, including 306 hypertensives (the EH–CS group) who suffered from CS and 361 people (the EH–CS group) who did not have cerebrovascular accidents, were enrolled in the study. The variant allele 718C of the *GPX4* gene was found to be significantly associated with an increased risk of CS in hypertensive patients (odds ratio (OR) 1.53, 95% confidence interval (CI) 1.23–1.90, $P_{adj}$=0.0003). The prevalence of the 718TC and 718CC genotypes of the *GPX4* gene was higher in the EH–CS group than the EH-alone group (OR=2.12, 95%CI 1.42–3.16, $P_{adj}$=0.0018). The association of the variant *GPX4* genotypes with the increased risk of CS in hypertensives remained statistically significant after adjusting for confounding variables such as sex, body mass index (BMI), blood pressure and antihypertensive medication use (OR=2.18, 95%CI 1.46–3.27, *P*=0.0015). Multiple logistic regression analysis did not reveal any interaction between various combinations of *GPX1*, *GPX3* and *GPX4* genotypes regarding the risk of CS in patients with EH. The study demonstrated for the first time that the C718T polymorphism in the 3′-untranslated region of the *GPX4* gene could be considered as a genetic marker of susceptibility to CS in patients with EH.**
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**Keywords:** cerebral stroke; essential hypertension; genetic susceptibility to disease; glutathione peroxidase genes; single nucleotide polymorphism

## INTRODUCTION

According to JNC 7 (the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure), the relationship between blood pressure and cerebral-stroke risk is strong, continuous, graded, consistent, independent, predictive and etiologically significant.[1] Essential hypertension (EH) remains the most important well-documented, modifiable risk factor for stroke, and the treatment of hypertension is among the most effective strategies for preventing both ischemic and hemorrhagic stroke.[2] Although EH is frequently complicated by cerebral stroke (CS), there are many patients who did not suffer the cerebrovascular disease suggesting genetic susceptibility to stroke in some, but not in all hypertensive individuals. The increase of our understanding of the role of genetic factors in determining the susceptibility to stroke development may provide an insight into the mechanisms of cerebrovascular complications, and help to identify new therapeutic targets and prophylactic strategies to afford neuroprotection promptly.

Growing evidences indicate that the increased production of reactive oxygen species and oxidative stress, a condition occurring when this balance is disrupted by excessive production of reactive oxygen species and/or by inadequate antioxidant defenses, in the brain are among the causative mechanisms of cerebral tissue damage in both ischemic and hemorrhagic strokes.[3–5] It is noteworthy that increased vascular oxidative stress is thought to be involved in the pathogenesis of both EH[6] and CS.[7,8]

One of the antioxidant enzymes protecting against oxidative stress is glutathione peroxidase (GPx), which reduces hydrogen peroxide ($H_2O_2$) and organic hydroperoxides to $H_2O$ and the corresponding alcohols using reduced glutathione as an essential co-substrate. In the genes coding for these enzymes, several single nucleotide polymorphisms have been described. As oxidative stress can be involved in the

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etiology of hypertension and cerebrovascular disease, polymorphic genes encoding for GPxs can be putative candidates for the genetic susceptibility to CS. Several genetic studies have been done so far to search for the association between polymorphisms of glutathione peroxidase 1 (*GPX1*) and 3 (*GPX3*) genes and the risk of CS.[9–12] These association studies were designed for use of normotensive subjects as a control population and did not assess the stroke risk in the hypertensive population. In the present study, we have investigated, for the first time, the prevalence of three single nucleotide polymorphisms in GPx genes (*GPX1* rs1050450, *GPX3* rs2070593 and *GPX4* rs713041), and the association between these genetic polymorphisms and CS risk in patients with EH.

## METHODS

### Study population

A total of 667 unrelated subjects with EH, including 306 hypertensives who suffered from CS (EH–CS group) and 361 hypertensives (EH group) who did not have cerebrovascular accidents, were enrolled in the study. Patients who suffered CS (281 hypertensives were with ischemic stroke, 25 hypertensives suffered from hemorrhagic stroke) were recruited at the Neurology Clinics of Kursk Emergency Medicine Hospital between 2007 and 2010. Hypertensive patients without a clinical history of cerebrovascular disease were enrolled from the Cardiology Clinics of both Kursk Regional Clinical Hospital and Kursk Emergency Medicine Hospital during two study periods; the first was between 2003 and 2006 as described in our recent paper,[13] and the second was between 2007 and 2010. All study participants were of Russian origin from Central Russia. All patients provided their informed consent before the study and the protocol was approved by the Ethical Review Committee of Kursk State Medical University.

### Diagnosis

Demographic data were obtained for each subject from medical records at the time of the enrollment and included current age, sex and family history of hypertension and stroke. Patients were defined as hypertensive according to the World Health Organization criteria and (or) if they were receiving any antihypertensive medication. All the study subjects had a clinical history of hypertension of more than 1 year. Untreated hypertensive patients had established hypertension defined by as seated systolic and/or diastolic blood pressure above 140 and/or 90 mm Hg, respectively, during at least two separate measurements. All hypertensive patients had no clinical signs, symptoms and laboratory findings suggesting secondary hypertension. The hypertensives-comprising EH group (control group) had no clinical history of any cerebrovascular disease. All hypertensives who suffered from CS (EH–CS group) were examined by qualified stroke neurologists. The diagnosis of CS was based on the findings in the medical record and results of physical examination, and was confirmed with magnetic resonance imaging of the brain. We did not recruit hypertensive subjects with major cardiac, renal, hepatic, endocrine diseases, skeletal disorders and cancer.

### DNA analysis

Genomic DNA was isolated from 5 ml of peripheral blood samples collected in K3-EDTA tubes by venipuncture and maintained at −20 °C until processed. DNA purification was carried out using SDS–proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. Genotyping of the polymorphisms of the GPx genes was done using PCR followed by restriction fragment length polymorphism analyses. Primers for genotyping of rs2070593 and rs713041 polymorphisms were designed using GeneFisher2 Interactive PCR Primer analysis software (http://bibiserv.techfak.uni-bielefeld.de/genefisher2).

A 363-bp genomic fragment containing the polymorphism G>A (rs2070593) of the *GPX3* gene was amplified by using the forward primer: 5′- TCTCCAACCACACTATCTACC-3′ and reverse primer: 5′- GAGGTATCAG TTAGAGCAGAAC-3′. PCR conditions were as following: initial denaturation at 95 °C for 5 min, followed by 37 cycles of denaturation (94 °C for 40 s), annealing (57 °C for 30 s) and extension (72 °C for 40 s), with the final extension at 72 °C for 10 min. The PCR product containing the polymorphism rs2070593 was digested with 7 U of *Psp*CI restriction enzyme (Sibenzyme, Novosibirsk, Russia) producing two fragments of 183 and 180 bp (these bands overlap each other at electrophoresis) in case of the G allele and leaving the A allele uncut (one fragment of 363 bp) on 2%-agarose gel.

The C718T polymorphism (rs713041) of the *GPX4* gene was amplified within a 226-bp genomic fragment with forward primer: 5′-TTTCTAGCTCCAC AAGTGTGTG-3′ and reverse primer: 5′-AGATCCAGCAGGCTAATTTGTC-3′. The thermal cycling comprised an initial denaturation step at 95 °C for 4 min, followed by 35 cycles at 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, with the final extension at 72 °C for 7 min. The PCR product was subjected to restriction digestion with 10 U of the *Bss*T1I enzyme (Sibenzyme) at 60 °C for 4 h followed by 2.5%-agarose gel electrophoresis. For the 718C allele, *Bss*T1I cleaves the 226-bp PCR fragment into a 145-bp and an 81-bp fragment, whereas three fragments were generated for the 718T allele: 97, 81 and 48 bp.

The P198L polymorphism (rs1050450) of glutathione peroxidase-1 (*GPX1*) was genotyped as described by Hu and Diamond.[14] PCR amplification was performed in a final volume of 25 µl of the reaction mixture containing 1.5 U of *Taq* DNA polymerase (Sibenzyme), about 1 µg DNA of each patient's DNA, 0.25 µM each primer, 250 µM of dNTPs, 2 mM of $MgCl_2$, 1× PCR buffer of the following composition: 67 mM Tris-HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$ and 0.01% Tween-20. Digested products were resolved through ethidium bromide-stained 2%-agarose gels and visualized under UV light on the GDS-8000 Computer Detection System (UVP Inc, Upland, CA, USA). A 'no template' control (water) was used in each restriction fragment length polymorphism assay. The genotyping results were scored by two independent investigators who did not know whether a sample was from the EH–CS or the EH group. In addition, about 10% of the samples from each study group were randomly selected to perform direct sequencing of the PCR products of each polymorphism. Sequencing was carried out by Sanger methodology on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and 100% concordance with restriction fragment length polymorphism assays was shown providing confidence in using the full dataset in subsequent analysis.

### Statistical data analysis

Categorical variables (sex, family history for hypertension and stroke, and antihypertensive medication use) are expressed as percentage and were compared using Pearson's $\chi^2$ test. Parametric data (age, blood pressure and body mass index (BMI)) are reported as mean ± s.d. and were compared using two-sided Student's *t*-test. Allele frequencies were estimated by the gene counting method. $\chi^2$ test (d.f.=1) was used to identify significant departures from Hardy–Weinberg equilibrium. Frequencies of alleles and genotypes of GPx genes were compared between patients by using Pearson's $\chi^2$ test. The magnitude of the association between the polymorphisms and the stroke risk was expressed as odds ratio (OR) with 95% confidence intervals (CI). An independent association between the *GPX4* gene polymorphism and the risk of CS was assessed using multiple logistic regression analysis after adjusting for potential confounding factors such as sex, BMI, blood pressure and antihypertensive medication use. Multiple logistic regression analysis was also used to test any interaction between various combinations of *GPX1*, *GPX3* and *GPX4* genotypes regarding to the risk of CS in patients with EH. A *P*-value of 0.05 or less was considered to indicate statistical significance after adjusting for multiple comparisons ($P_{adj}$). All statistical analyses were performed using STATISTICA for Windows version 8.0 software (StatSoft Inc, Tulsa, OK, USA).

## RESULTS

Baseline characteristics of the study population are shown in Table 1. The mean ages of the EH–CS and the EH groups were 62.2 ± 10.4 and 62.2 ± 13.8 years, respectively ($P>0.05$). The two groups matched by sex and, therefore, there was no significant difference in this variable. Both mean systolic and diastolic blood pressure were significantly higher in the EH–CS group as compared with the EH group. BMI was higher in hypertensives who suffered from CS than in those who had no cerebrovascular accidents. There was no statistically significant difference in antihypertensive medication use between the groups. Percentages of positive family history of hypertension and stroke were comparable among the groups.

**Table 1 Baseline characteristics of study population**

| *Baseline characteristics* | *EH–CS[a] group (n=306)* | *EH[b] group (n=361)* | P-*values* |
|---|---|---|---|
| Age, mean ± s.d. | 62.2 ± 10.4 | 62.2 ± 13.8 | 0.99 |
| Gender (M, male; F, female) | M 158 (51.6%) | M 171 (47.4%) | 0.27 |
| | F 148 (48.4%) | F 190 (52.6%) | |
| Mean systolic blood pressure (mm Hg) | 175.8 ± 29.1 | 169.4 ± 24.6 | 0.002 |
| Mean diastolic blood pressure (mm Hg) | 98.4 ± 17.2 | 95.6 ± 15.8 | 0.03 |
| Body mass index (kg m$^{-2}$) | 26.6 ± 3.4 | 25.7 ± 4.8 | 0.01 |
| Antihypertensive-medication use | Yes, 264 (86.3%) | Yes, 327 (90.6%) | 0.08 |
| | No, 42 (13.7%) | No, 34 (9.4%) | |
| Family history of hypertension[c] | Yes, 247 (81.0%) | Yes, 262 (78.2%) | 0.38 |
| | No, 58 (19.0%) | No, 73 (21.8%) | |
| Family history of cerebral stroke[d] | Yes, 117 (38.4) | Yes, 77 (36.0) | 0.58 |
| | No, 188 (61.6) | No, 137 (64.0) | |

[a]EH–CS: patients with essential hypertension who suffered cerebral stroke.
[b]EH: patients with essential hypertension who did not have cerebrovascular accident.
[c]Data were obtained from 305 EH–CS patients and 335 EH patients.
[d]Data were obtained from 305 EH–CS patients and 214 EH patients.

**Table 2 Genotype and allele frequencies of glutathione peroxidase genes for hypertensives who suffered cerebral stroke and those with no cerebrovascular accident**

| *Alleles/genotypes* | *EH–CS[a] group (n=306)* n *(%)* | *EH[b] group (n=361)* n *(%)* | *OR[c] (95% CI[d])* | P-*value*[e] |
|---|---|---|---|---|
| *GPX1 P198L (rs1050450)* | | | | |
| Variant allele 198L | 0.310 | 0.263 | 1.26 (0.99–1.60) | 0.06 |
| Genotype P198PP | 145 (47.4) | 195 (54.0) | 1.30 (0.96–1.77) | 0.09 |
| Genotype 198PL | 132 (43.1) | 142 (39.3) | 1.17 (0.86–1.59) | 0.32 |
| Genotype P19LL | 29 (9.5) | 24 (6.6) | 1.47 (0.84–2.58) | 0.18 |
| *GPX3 930G>A (rs2070593)* | | | | |
| Variant allele 930A | 0.299 | 0.277 | 1.11 (0.88–1.41) | 0.38 |
| Genotype 930GG | 149 (48.7) | 184 (51.0) | 1.10 (0.81–1.49) | 0.56 |
| Genotype 930GA | 131 (42.8) | 154 (42.7) | 1.01 (0.74–1.37) | 0.97 |
| Genotype 930AA | 26 (8.5) | 23 (6.4) | 1.36 (0.76–2.44) | 0.29 |
| *GPX4 C718T (rs713041)* | | | | |
| Variant allele 718C | 0.618 | 0.514 | 1.53 (1.23–1.90) | 0.0001 |
| Genotype 718TT | 43 (14.1) | 93 (25.8) | 2.12 (1.42–3.16) | 0.0002 |
| Genotype 718TC | 148 (48.4) | 165 (45.7) | 1.11 (0.82–1.51) | 0.49 |
| Genotype 718CC | 115 (37.6) | 103 (28.5) | 1.51 (1.09–2.09) | 0.01 |

[a]EH–CS: patients with essential hypertension who suffered cerebral stroke.
[b]EH: patients with essential hypertension who did not have cerebrovascular accident.
[c]OR: Odds Ratio not adjusted for the confounding variables.
[d]CI: 95% confidence interval.
[e]*P*-values not adjusted for multiple comparisons.

The genotype distribution and allele frequencies for genes *GPX1*, *GPX3* and *GPX4* are provided in Table 2. Genotype frequencies of GPx genes were in agreement with Hardy–Weinberg equilibrium in all groups ($P>0.05$). There were no significant differences in the genotype distribution for the rs1050450 and rs2070593 polymorphisms between the study groups. However, variant allele 198L of the *GPX1* gene tended to be associated with an increased risk of CS in hypertensive patients (OR=1.26, 95%CI 0.99–1.60, $P$=0.06, $P_{adj}$=0.18). In contrast, variant allele 718C of the *GPX4* gene was found to be significantly associated with an increased risk of CS in hypertensives (OR=1.53, 95%CI 1.23–1.90, $P$=0.0001, $P_{adj}$=0.0003). The prevalence of the 718TC and 718CC genotypes of the *GPX4* gene was higher in the EH–CS group as compared with the EH group (OR=2.12, 95%CI 1.42–3.16, $P$=0.0002, $P_{adj}$=0.0018). The association of the variant *GPX4* genotypes with the increased risk of CS in hypertensives remained statistically significant (OR=2.18, 95%CI 1.46–3.27, $P_{adj}$=0.0015) after adjusting for confounding variables such as sex, BMI, blood pressure and antihypertensive-medication use. Multiple logistic regression analysis did not reveal any interaction between various combinations of *GPX1*, *GPX3* and *GPX4* genotypes regarding to the risk of CS in patients with EH (data not shown).

## DISCUSSION

### Summary of study findings

The study was designed to assess the associations of GPx genes and CS in patients with EH. It has been found for the first time that the C718T polymorphism of the glutathione peroxidase-4 gene can be considered as a risk factor for CS in hypertensive patients. The allele C718, the 718TC and 718CC genotypes of the *GPX4* gene were found to be associated with an increased risk of CS in hypertensive patients.

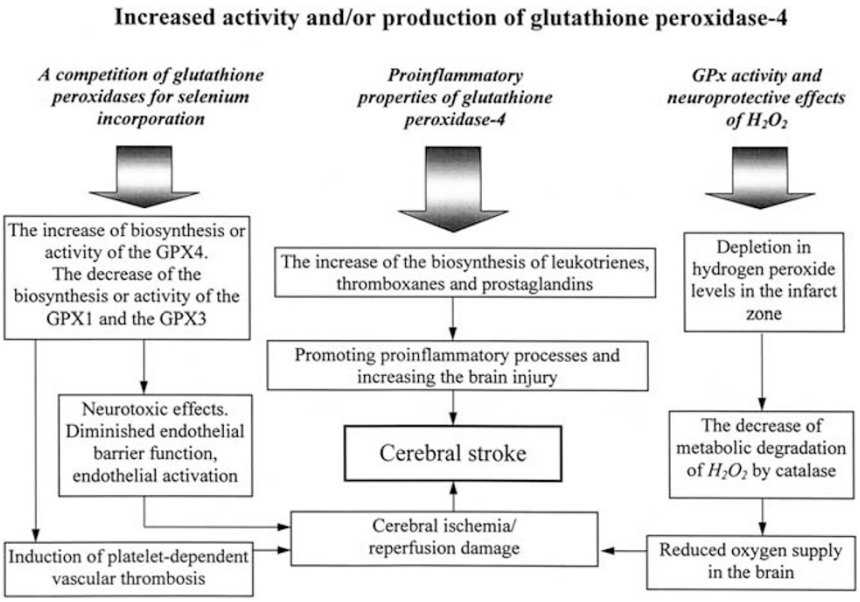


**Figure 1** Possible role of glutathione peroxidase-4 in the pathogenesis of CS.

The association remained statistically significant after adjusting for confounding variables such as sex, BMI, blood pressure and antihypertensive-medication use. We found no statistically significant associations of CS with the *GPX1* and *GPX3* gene polymorphisms either separately or in combination with the *GPX4* gene.

**Biological role and functional studies of the GPX4 gene**

The GPX4 is an intracellular antioxidant selenoprotein catalyzing a decrease of $H_2O_2$, organic hydroperoxide, and lipid peroxides within membranes and lipoproteins by reducing glutathione levels, and it protects the cells against oxidative damage.[15] The gene for glutathione peroxidase-4 is expressed in most tissues, including the brain (Gene Expression Atlas, available at http://www.ebi.ac.uk/gxa/gene/ENSG00000167468). Although the *GPX4* gene is highly polymorphic, a limited number of studies[16–19] have been done to investigate genetic variations within the *GPX4* gene that could potentially alter the function of the enzyme. There is a C-to-T single-nucleotide polymorphism at the 718 position (rs713041) located in the 3′-untranslated region of the *GPX4* gene close to the predicted selenocysteine insertion sequence structure (SECIS, a region of the mRNA required for incorporation of the amino acid Sec into GPX4).[17] This polymorphism is involved in the modulation of the GPX4 synthesis by altering the affinity of the selenocysteine insertion machinery for its SECIS element.[20] Bermano and coworkers[19] have observed that the C variant at the 3′-untranslated region of the *GPX4* gene was stronger than the T variant at driving biosynthesis of a selenoprotein reporter. These data support a hypothesis that the T and C allelic variants of the *GPX4* gene differ in their capacity to promote selenocysteine incorporation into GPX4, thereby the polymorphism influences an expression and concentration of the enzyme. The polymorphism has been found to be associated with colorectal cancer susceptibility[19] and survival after diagnosis of breast cancer.[21]

**Possible role of the GPX4 gene in the development of CS**

A possible role of glutathione peroxidase-4 in the development of CS has been summarized in Figure 1. The hypothesized scheme was developed on the basis of the assumption that the 718C variant of the *GPX4* gene is responsible for an increased activity or level of this enzyme in the brain. We propose that all considered effects of GPX4 on the risk of CS can be mediated by three following mechanisms.

*A competition of GPxs for selenium incorporation.* Koyama with coworkers[22] have found that reduced serum level of selenoprotein P was associated with a higher risk of stroke. Gautrey with coworkers[23] have shown that the C718T variant of the *GPX4* gene alters the pattern of selenoprotein synthesis under low selenium intake. Méplan with coworkers[15] have observed that carriers of the genotype 718CC maintain GPX4 concentrations better than carriers of genotype 718TT do when selenium intake falls after withdrawal of the selenium supplement. Interestingly, the authors have also showed that the polymorphism influences the expression of other selenoproteins such as GPX1 and GPX3. This finding could be explained by a competition among selenoproteins for available selenium in the form of selenocysteine required for biosynthesis of the selenoproteins and for the selenium-incorporation machinery.[24,25] It has been proposed that variations in the 3′-untranslated region of the *GPX4* gene can influence the position of GPX4 in the hierarchy of demands on the components of the selenoprotein-synthesis apparatus, with consequent effects on the synthesis of other selenoproteins.[15] Thus, a competition for selenium incorporation would be responsible for the decrease of the biosynthesis or activity of GPxs 1 and 3 leading to accumulation of $H_2O_2$ that has been suggested to exert neurotoxic effects promoting oxidative stress and inflammation, which are critical factors of brain damage induced by cerebral ischemia.[26,27] Furthermore, it is known that GPxs are involved in the regulation of platelet activity, endothelial function, platelet-dependent thrombosis and propensity for vascular thrombosis, and a deficiency of this enzyme has been associated with platelet-dependent thrombosis and arterial ischemic stroke.[28,29] Certainly, the hypothesis of competition between GPxs 1, 3 and 4 for selenium incorporation needs to be investigated in detail in further clinical and experimental studies.

*Proinflammatory properties of glutathione peroxidase-4.* Notably, the 718C allele of the *GPX4* gene was found to be associated with the increased levels of lymphocyte lipoxygenase products implying that

the polymorphism can modulate inflammatory processes through the regulation of the biosynthesis of leukotrienes, thromboxanes and prostaglandins.[17] It has been revealed that the inflammation has an important role in the pathogenesis of CS, and various inflammatory markers have been investigated as predictors of the disease.[26] Therefore, we suggest that hypertensive individuals with the 718C variant of the *GPX4* gene are susceptible to CS because of the increased concentration or activity of the enzyme promoting proinflammatory processes and increasing the brain injury.

*GPx activity and neuroprotective effects of $H_2O_2$.* It is generally agreed that ischemic insult facilitates an excessive generation of hydroxyl radicals and, therefore, GPxs have an important role in the defense against $H_2O_2$-induced damage.[30–33] Interestingly, experimental data also suggest that an increased production of $H_2O_2$ can represent a critical component of the neuroprotective processes that occur during or after ischemic stroke. In particular, the animal study[7] in which pharmacological inhibition of GPx activity significantly reduced brain infarct damage suggested that under reduced oxygen supply, $H_2O_2$ may exert either physiological or protective roles in the brain through its metabolic degradation to oxygen by catalase compensating for the lack of oxygen that occurs in the tissue after an ischemic event. Two other studies[34,35] have observed that $H_2O_2$ may reduce the release of some neurotransmitters through activation of ATP-sensitive K-channels, the mechanism that has been suggested to underlie the beneficial effects of $H_2O_2$ during metabolic stress.

In conclusion, the present study demonstrated for the first time that the *GPX4* gene could be involved in the development of CS in patients with EH. However, further investigations in larger populations are required to confirm the contribution of the *GPX4* gene to the risk of CS, and to assess the relationship between the *GPX4* genotype and the enzyme expression/activity. Moreover, genetic studies should be focused on an individual selenium intake in order to identify gene–gene and gene–environment interactions responsible for stroke development in hypertensive subjects. Before drawing a definitive conclusion regarding the biological effects of increased GPX4 activity, further functional studies are required to clarify the physiological actions of GPx in human brain and to determine the role of this enzyme in the development of CS. A better understanding of the genetic factors underlying the susceptibility of hypertensive patients to CS can result in the identification of early prognostic markers of individual risk of cerebrovascular complications of EH.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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