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Short Communication

Association between Glu298Asp/677C-T single nucleotide polymorphism in the eNOS/MTHRF gene and blood stasis syndrome of ischemic stroke☆

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ABSTRACT

Blood stasis syndrome of ischemic stroke (BSS-IS) is a common clinical phenotype that may be affected by certain mutagenic environmental factors or chemotherapeutic drugs; however, the role of susceptibility genes remains unclear. Previous studies have shown that ischemic stroke (IS) was closely associated with the Glu298Asp polymorphism in the eNOS gene and the 677C-T (Ala→Val) polymorphism in methylenetetrahydrofolate reductase (MTHRF) gene. Therefore, these two single nucleotide polymorphisms (SNPs) were selected to detect their associations with BSS-IS in this study. A SNP chip was employed to screen the SNP variation between both groups, and the results were verified using denaturing high-performance liquid chromatography (DHPLC) and restriction fragment length polymorphism (RFLP). The results confirmed that the TT genotype of Glu298Asp in the eNOS gene may be one of the risk factors associated with BSS-IS, while the genotype of 677C-T (Ala→Val) in the MTHRF gene may not be relevant to BSS-IS.

1. Introduction

Ischemic stroke (IS) is a multifactorial disease (Wang et al., 2009), and its molecular etiology involves interaction between many genes and environmental factors (Jood et al., 2005). Mutations in several candidate genes have been found to be associated with stroke (Munshi and Kaul, 2010). Sustained NO generation is primarily adjusted by eNOS in physiological conditions, and thus, polymorphism in the eNOS gene is closely associated with NO concentration in vivo (Wang et al., 1997). At present, it is generally accepted that eNOS is most closely associated with the incidence of IS (Berger et al., 2007; Saidi et al., 2010) and that NO concentration plays a critical role in the initiation and progression of IS (Nishikawa et al., 1993). Genotype and allele frequencies of polymorphism in eNOS between IS patients and healthy controls have been found to be significantly different in Indian populations (Munshi et al., 2010). Previous studies have shown that the Glu298asp polymorphism in eNOS gene within exon 7 (NCBI SNP cluster rs1799983; GenBank accession number NG_011992; protein accession number NP_000594) is closely associated with IS (Berger et al., 2007; Saidi et al., 2010). A mis-sense mutation of Glu298Asp causes a conformational change in eNOS, reduces its activity, and inevitably increases the subject's susceptibility to IS (Saidi et al., 2010).

Recently, an association between mutations of methylenetetrahydrofolate reductase (MTHFR) gene and ischemic stroke has been reported (Low et al., 2011). MTHFR 677C-T (Ala→Val) was a risk factor for ischemic stroke (Usacheva et al., 2012). A common polymorphism exists in the gene that encodes the MTHFR enzyme, as required for the conversion of homocysteine to methionine. Individuals who have T genotype of the MTHFR gene (677T) have lower enzyme activity and higher homocysteine and lower folate levels than those without this genotype (García-Pinilla et al., 2007). The 677C-T mutation might result in impaired folate metabolism, leading to high

Abbreviations: IS, ischemic stroke; BSS, blood stasis syndrome; BSS-IS, blood stasis syndrome of ischemic stroke; eNOS, endothelial nitric oxide synthase; MTHRF, methylenetetrahydrofolate reductase; SNPs, single nucleotide polymorphisms; DHPLC, denaturing high-performance liquid chromatography; RFLP, restriction fragment length polymorphism; TCM, traditional Chinese medicine; PCR, polymerase chain reaction; CI, confidence interval; OR, odds ratio.

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homocysteine concentrations, which was causally related to IS risk (Deloughery et al., 1996).

Traditional Chinese medicine (TCM) syndrome is the nature of a certain stage of the disease. IS includes several syndromes, in which blood stasis syndrome (Xueyu ZHENG) is one of the most common phenotypes (Sun, 2007). Blood stasis is not only an important risk factor but also one of the pathological products of IS. Although a significant association has been confirmed between single nucleotide polymorphism (SNP) in the eNOS gene or MTHFR gene and the risk of IS, the associations between blood stasis syndrome of IS (BSS-IS) and SNP in the eNOS and MTHFR gene still remain unclear. In this study, we investigated the frequencies of polymorphisms in eNOS and MTHFR gene in BSS-IS and non-BSS-IS patients. The eNOS gene allele frequency in these patients was evaluated and compared, and a detailed association among syndromes, SNP in the eNOS and MTHFR gene, and the susceptibility to IS was described.

2. Patients and methods

2.1. Patients

Patients were consecutively recruited from XiYuan Hospital in Beijing, China. A hospital based case–control study was used, and informed consent was obtained from each participant. The study protocol was reviewed and approved by the ethical committee of the Chinese Academy of Medical Sciences.

Diagnosis of IS and TCM syndrome was established according to clinical symptoms and signs. The clinical features of BSS include tingling, ecchymosis, dark purple tongue, and so on. The criteria for diagnosis of IS and BSS used in this study were based on guiding principles for clinical research of Chinese new herbal medicines (Zheng, 2002).

Patients with transient ischemic attack; those with cerebral embolism derived from brain tumor, brain trauma, cerebral parasitic diseases, metabolism disorders, rheumatic heart disease, and atrial fibrillation; and those with comorbid conditions, including other serious diseases and/or psychiatric disorders were excluded from the study. A detailed clinical questionnaire concerning different features of the disease was used.

Data regarding age, age at diagnosis, gender, medical history, and comorbid conditions were collected from all eligible patients.

2.2. Methods

2.2.1. DNA extraction

The Wizard Genomic DNA Purification Kit (Promega Co. Ltd.) was used for the isolation of genomic DNA. DNA samples collected from selected patients were analyzed for variants of the eNOS and MTHFR gene using melting curve analysis.

2.2.2. Gene amplification

The Cy5 fluorescent labeled primers and restriction enzymes (Sangon Biotech Shanghai Co. Ltd.) for each SNP used for polymerase chain reaction (PCR) analysis were as follows: nsp9a2, 5′-GGGG GGCAGAAGGAAGAGTT-3′; nsp9s3F, 5′-CY5-GCTGCA GGCCC CAG ATGA-3′ (in the eNOS gene Glu298asp amplification site). MTHFR: 5′-CAAAGGCCACCCCGAAGC3, 5′-AGGACGGTGCGGTGAGAGTG3′ (in the MTHFR gene 677C-T (Ala→Val) amplification site).

We identified and optimized multiple PCR amplifications by single amplification and performed PCR using 0.3 U of rTaq polymerase (5 U/μl, Takara), 400 μmol/l dNTPs (2.5 mM, Promega), and 0.1 μmol/l of each primer in a total volume of 25 μl. For Glu298Asp SNP/677C-T in the eNOS and MTHFR gene, multiple PCR amplification was performed as follows: amplification at 94 °C for 2.5 min, at 94 °C for 30 s, at 57 °C for 30 s, at 72 °C for 30 s, followed by 35 cycles, and a final extension at 72 °C for 5 min. Amplification was verified by 2% agarose gel electrophoresis. PCR products were not purified, but directly hybridized.

2.2.3. SNP detection

We designed an oligonucleotide probe for detection of the genes after consulting the NCBI gene database. Probe sequences are listed in Table 1.

A clean glass slide was processed in a 95% acetone and 10% pyridine (containing 2% PDC) solution and spotted at 4 °C after airing. We prepared the spotting solution by dissolving synthetic oligonucleotide probes and determined the final concentration of the probes at 0.1 μmol/l. The layout design of the SNP microarray is described in Table 2.

The amplified samples of the eNOS and MTHRF gene were amplified at 94 °C and hybridized at 48 °C for 2 h. The products were scanned using a ScanArray 4000 scanner at 649 nm, and the signal strength was recorded. Scan results were analyzed with scanner image processing software. If the ratio was >2 but <0.5, the product was considered a homozygote and if the ratio was ≥ 0.5 but ≤ 2, the product was considered to be a heterozygote.

To verify the validity of the results, we detected the variants of eNOS gene using denaturing high-performance liquid chromatography (DHPLC) (WAVE™, Transgenomic, UK) in 25 randomly selected BSS-IS patients and 25 controls. The altered mobility down the acetonitrile column was monitored and analyzed by WAVEMAKER™ Version 4.1.25 software. The sample was considered to have the same genotype as the homozygote if a single peak was observed, whereas multiple peaks indicated heterozygotes. At the same time, we also detected the genotype of 677C-T (Ala→Val) in the MTHRF gene in 20 randomly selected patients using restriction fragment length polymorphism (RFLP).

2.2.4. Statistical analysis

SPSS 12.0 statistical software was used for statistical analysis. The allele and genotype frequencies between both groups were compared based on the χ^2 test or Fisher's exact test. The correlation between genotype and BSS-IS was presented as an odds ratio (OR) and 95% confidence interval (CI). p values were considered significant at a level of <0.05.

3. Results

The study population comprised 89 BSS-IS patients (64 males, 25 females; mean age, 68.6 ± 10.5 years) and 102 non-BSS-IS patients (74 males, 28 females; mean age, 67.5 ± 9.9 years). No significant differences were observed between both groups in terms of gender and age ($p > 0.05$). Both groups had showed no differences in terms of emerging diabetes, coronary heart disease, and hypertension. A comparison of the basic characteristics between the two groups is shown in Table 3.

After the genomic DNA was diluted at 1:40, the average optical density at 260 nm using a spectrophotometer was found to be 0.30 and the average concentration was 583.65 ng/μl. The electrophoretic bands of the samples were clear, and the specificity was relatively high using single PCR amplification (Fig. 1). Multiple PCR amplification

Table 1
Probe design for SNP chips.

Gene name	Probe name	Probe sequence
MTHFR	Nsp4na	5′-NH2-(T)10-GATGAAATCGaCTCCCG-3′ 27
	Nsp4ma	5′-NH2-(T)10-TGAAATCGgCTCCCGG-3′ 26
eNOS	Nsp9na	5′-NH2-(T)10-TTCTGGAGGcTCATCTGG-3′ 28
	Nsp9ma	5′-NH2-(T)10-ATCTGGAGGaTCATCTGGG-3′ 29
	Nsp9nat	5′-NH2-(T)10-aCTGGAGGcTCATCTGG-3′ 27
	Nsp9mat	5′-NH2-(T)10-ATCTGGAGGaTCATCTGG-3′ 28
	Positive probe	5′-NH2-(T)10-AACCCGCTCAATGCCTGGAGATTTGG-3′
	Negative probe	5′-NH2-(T)10-GCAAGCAAGGAATTGTCCGAAAAGG-3′