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Research paper

# MTHFR (C677T) CT genotype and CT-apoE3/3 genotypic combination predisposes the risk of ischemic stroke

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ABSTRACT

The predisposition to ischemic stroke (IS) might involve interactions of several genes and environmental factors. The present study was aimed to evaluate the influence of polymorphisms in methylenetetrahydrofolate reductase (MTHFR-C677T) and apolipoprotein-E (apo-E) as risk factors for IS patients in south Indian population. 200 IS patients and 193 age and sex matched controls were genotyped for MTHFR-C677T and apoE by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method. Statistically significant association was observed for MTHFR CT genotype (IS-Pooled: OR = 4.29; $p = 5.01 \times 10^{-5}$; IS-Males: OR = 4.13; p = 0.001; IS-Females: OR = 8.62; p = 0.027; IS-Large Vessel Disease (LVD)- Pooled: OR = 4.14; p = 0.0002) and T allele (IS-Pooled: OR = 4.82; $p = 1.49 \times 10^{-5}$; IS-Males: OR = 4.33; p = 0.0002; IS-Females: OR = 7.99; p = 0.031; IS-LVD-Pooled: OR = 4.13; p = 0.0001). Further, reduced frequencies of CC genotype (IS-Pooled: OR = 0.20; $p = 9.80 \times 10^{-6}$; IS-Males: OR = 0.25; p = 0.001; IS-Females: OR = 0.12; p = 0.027; IS-LVD-Pooled: OR = 0.23; p = 0.0001) and C allele (IS-Pooled: OR = 0.21; $p = 1.49 \times 10^{-5}$; IS-Males: OR = 0.23; p = 0.0002; IS-Females: OR = 0.13; p = 0.031; IS-LVD-Pooled: OR = 0.24; p = 0.0001) were observed in IS patients than the controls. No association was observed for apoE genotypes/alleles in IS/LVD cases. Our study demonstrated the presence of risk for MTHFR CT genotype/T allele and 'CT-3/3' (n = 33 vs. 5; OR = 7.42; p = 0.001) genotypic combination in the development of IS in south India. Further, follow-up study of these stroke cases i.e., in later stages of the disease whether they are developing the neurological disorders such as Alzheimer's Disease (AD) and vascular dementia (VaD) is needed to draw a fruitful conclusion in connection between neurological disorders and with these two polymorphisms, before translating it into clinical practice.



## 1. Introduction

Stroke has emerged as the second commonest cause of mortality worldwide and is a major public health problem. It has been estimated that stroke affects 15 million people worldwide (WHO, 2011). Globally, the prevalence rate of stroke is estimated to be about 400–800/100,000 persons (Banerjee et al., 2005). For India, the overall age adjusted prevalence rate for stroke is estimated to lie between 84 and 262/100,000 in rural and between 334 and 424/100,000 in urban areas (Gupta et al., 2008) with an average prevalence rate being 90–222/100,000 (Dalal et al., 2007). WHO's estimates suggest that by 2050, 80% of stroke cases in the world would be in low and middle income Asian countries viz., India, China, Bangladesh, Pakistan, China, Japan and Korea (Banerjee et al., 2008). The contribution of genetic factors to stroke pathogenesis is evidenced by the association of specific gene variants with stroke risk (Gao et al., 2006; Szolnoki and Melegh, 2006). The human candidate gene studies in Ischemic Stroke (IS) have so far been inconsistent and could be attributed to the confounding effects of ethnic and phenotypic heterogeneity (Saeed, 2004; Schulz et al., 2004). Methylenetetrahydrofolate reductase (MTHFR) is the key enzyme that catalyzes the reduction of

*Abbreviations:* MTHFR, methylenetetrahydrofolate reductase; apoE, apolipoprotein-E; VaD, vascular dementia; AD, Alzheimer's disease; PCR, polymerase chain reaction; IS, ischemic stroke; TOAST, Trial of ORG 10172 in Acute Stroke Treatment; OR, odds ratio; WHO, World Health Organization; LVD, large vessel disease; SVD, small vessel disease; DALY, disability-adjusted life-years; DNA, deoxyribonucleic acid; SBP, systolic blood pressure; DBP, diastolic blood pressure; TGL, triglycerides; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; SD, standard deviation.

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5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which is required for methionine synthase enzyme to convert homocysteine to methionine. MTHFR gene is located on chromosome 1 (1p36.3) and contains 11 exons. A common missense mutation in the MTHFR gene, C → T substitution at nucleotide 677 is responsible for reduced MTHFR activity and is associated with a moderate increase in plasma hyperhomocysteinemia (Hcy) concentrations (Chen et al., 2005; Frosst et al., 1995). Mild to moderate Hcy is known to be due to genetic factors like mutation in MTHFR gene or due to deficiency of vitamin B12 or folic acid. The apolipoprotein-E (apoE) is a 35-kDa glycosylated polymorphic protein located on chromosome 19 and is a key lipoprotein in lipid and cholesterol metabolism. ApoE has been implicated in the initiation and progress of atherosclerosis and thereby leading to the development of vascular diseases and its expression in the brain was also thought to contribute to the growth and repair of the nervous system (Ignatius et al., 1986; Davignon et al., 1988). It has three common isoforms E2, E3 and E4 encoded by the alleles e2, e3 and e4 giving rise to six genotypes such as E2/2, E2/3, E2/4, E3/3, E3/4, E4/4. Of these the genotype E3/3 occurring in about one half to two thirds of world populations. Genotype E2 allele is linked with lower blood cholesterol levels, whereas E4 allele is consistently linked with higher levels (Davignon et al., 1988). Neurological diseases like stroke, Alzheimer's disease (AD) and vascular dementia (VaD) share the same and common risk factors. Post stroke condition can lead to many neurological disorders but mainly AD and VaD (Gorelick et al., 2011). In the present work, we investigated to determine the genotype and allele frequencies of the MTHFR C677T, apoE and to evaluate the influence of genotype on risk in south Indian IS patients.

## 2. Materials and methods

### 2.1. Enrollment of samples

We enrolled 200 ischemic stroke (IS) patients (154 men, 46 women: mean age of 57.5 ± 13.8 years) presenting with new or recurrent stroke attending the stroke clinics located in Madurai, Tamil Nadu, south India during the period of 2012–2013. Patients were diagnosed by neurologic examination and CT or MRI proven or both. The stroke subtype assignment was as per the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification (Adams et al., 1993). IS patients with vascular risk factors such as hypertension (HT) and diabetes mellitus (T2DM) were included and patients with hemorrhagic stroke, transient ischemic attack and cerebral venous thrombosis were excluded. Age and gender matched 193 healthy volunteers (144 men, 49 women: mean age: 55.9 ± 13.2 years) without any neurological disorders were randomly selected from the same geographic region as controls. Controls with previous history of stroke, myocardial infarction, HT, T2DM and peripheral arterial disease were excluded. HT was diagnosed according to the JNC VI-VII criteria (Britov and Bystrova, 2003) and T2DM was diagnosed if fasting plasma glucose was >110 mg/100 ml or patient was on antidiabetic medications. Smokers were defined as those reporting daily smoking. Ex-smokers and occasional smokers were classified as non-smokers. Information on demographic details, medical history, biochemical profile and other established risk factors such as smoking and alcohol consumption were recorded by using a standardized questionnaire. Written informed consent was obtained from each subject according to a protocol approved by the Institutional Ethical Committee (IEC), Bharathidasan University.

### 2.2. DNA extraction

Two ml of blood was collected in an EDTA vacutainer and the genomic DNA was extracted using modified salting out method (Miller et al., 1988). The concentration of the extracted DNA was estimated spectrophotometrically using a UV-Spectrophotometer (Milton Roy, USA). DNA samples were stored at −80 °C until further use. Biochemical parameters were tested according to standard methods using semi-automated biochemical analyzer (ERBA Mannheim, Germany; model no: Erba Chem 5 v2).

### 2.3. Genotyping of the MTHFR C677T polymorphism

The method described by Frosst et al. (1995) was used for the detection of MTHFR 677C → T polymorphism. A segment of 198 base pairs on exon 4 of MTHFR gene was amplified using 5′-TGAAGGAGAAGGTGTC TGCGGGA-3′ as forward primer and 5′-AGGACGGTGCGGTGAGAGTG-3′ as reverse primer. The C → T polymorphism at codon 677 introduces a restriction site for enzyme *Hinf1*. The PCR was carried out in a total volume of 25 μl consisting of 17.3 μl of milli Q water, 2.5 μl of 10× PCR buffer, 3 μl of genomic DNA (200 ng/ml), 0.5 μl of 10 mM dNTP (CinnaGen, Iran), 0.2 μl of 5 U *Taq* polymerase (Genet Bio, Korea), 0.75 μl each of forward and reverse primer (10 mM). The mixture was subjected to amplification with an initial denaturation at 94 °C for 4 min followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s. This was completed by a final extension at 72 °C for 10 min in a thermal cycler (Agilent, USA). Amplified products were electrophoresed on a 1.5% agarose gel containing 0.5 μg/ml Ethidium Bromide and documented in Gel Documentation system (Vilber Lourmat, France) (Fig.1a). Restriction digestion with *Hinf1* was carried out on 3 μl buffer, 2 μl (10 U) of *Hinf1*, 10 μl water and 15 μl of amplicons and incubated at 37 °C for 4 to 6 h. The digested product was sized by 3% agarose gel containing 0.2% Ethidium Bromide and genotype was determined. Wild type (677 CC) shows a single band of 198 bp. The presence of the 'T' allele introduces a cut among heterozygous (677 CT) and 3 bands of 198, 175 and 23 bp were seen. The homozygous (677 TT) have two bands of 175 bp and 23 bp (Fig. 1b).

### 2.4. Genotyping of apoE

For apoE genotyping, the following primers were used: Forward (5′-ACAGAATTCGCCCCGGCCTGGTACAC-3′) and Reverse (5′-TAAGCTTGGC ACGGCTGTCCAAGGA 3′). PCR amplification was done according to the modified method described earlier (Hixson and Vernier, 1990). The PCR was carried out in a total volume of 25 μl containing about 13.8 μl of milli Q water, 2.5 μl of 10× PCR buffer, 2.0 μl of 10% DMSO, 5 μl of genomic DNA (200 ng/ml), 0.5 μl of 10 mM dNTP (CinnaGen, Iran), 0.2 μl of 5 U *Taq* polymerase (Genet Bio, Korea), 0.5 μl each of forward and reverse primer (10 mM). Genomic DNA was amplified for 35 cycles. Each cycle consisted of 1 min at 95 °C, 1 min at 65 °C and 1 min at 72 °C (Fig. 1c). PCR products were digested with *Hhal* over a period of 3 h at 37 °C. The fragments obtained were separated by electrophoresis on a 4% agarose gel prepared in TBE buffer, and visualized by Ethidium Bromide fluorescence. The genotype of each person was determined from the PCR-RFLP banding patterns (Fig. 1d).

### 2.5. Statistical analysis

The statistical analysis was performed using STATA 11.1 (Stata Corp Lp, College Station TX, USA). The allelic frequencies were calculated by the gene counting method. Multivariate logistic regression analyses were performed for genotype, allele frequencies and lipid profile factor associated with ischemic stroke. Student's *t*-test was used to find the significance difference between groups with age, blood pressure, Total cholesterol (TCL), Triglycerides (TGL), High density lipoprotein (HDL), Low density lipoprotein (LDL), serum creatinine and continuous variables were expressed as mean and standard deviation (SD). Pearson's Chi Square test was used to measure the association between the gender, within groups and was expressed as frequency and percentage. The correlation values of plasma HDL, LDL, TG and TC level with the apoE polymorphism between IS patients and controls were calculated using an unpaired *t*-test. Statistical significance was considered at $p < 0.05$.