ORIGINAL ARTICLE

MTHFR Gene (C677t) Polymorphism in Ischemic Stroke, its Subtypes and Hemorrhagic Stroke in a South Indian Population

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ABSTRACT

Objective: We investigated the association of MTHFR C677T polymorphism with ischemic stroke, its subtypes and hemorrhagic stroke in a South Indian Population from Andhra Pradesh**. Methods:** Six hundred and twenty ischemic stroke patients, 220 hemorrhagic stroke patients and 620 age and sex matched healthy controls, were included in the present study. The polymorphism was determined using PCR-RFLP technique. **Results:** The strength of association between genotypes and stroke types was measured by the odds ratio with 95% confidence interval and chi-squared analysis. We found significant association of the CT genotype with ischemic stroke as well as haemorrhagic stroke ($p<0.05$). Further, evaluating the association of this polymorphism with stroke subtypes, we found significant association with intracranial large artery ($p<0.05$), lacunar stroke ($p<0.05$) and undetermined etiology ($p<0.05$). However, there was no significant difference in genotypic or allelic frequencies between ischemic and hemorrhagic strokes. **Conclusion:** Our study suggests that MTHFR (C677T) is an important risk factor for ischemic stroke, its subtypes and hemorrhagic stroke in the South Indians from Andhra Pradesh but it cannot help in distinguishing between the two types of stroke.

Keywords: MTHFR, C677T polymorphism, Ischemic stroke, Hemorrhagic stroke

INTRODUCTION

Stroke is recognised as a major public health problem and has been identified as the second common cause for mortality worldwide.[1] Estimates by WHO suggest stroke cases to be 80% in low and middle income countries; particularly in India and China.[2] Thus, identification and management of possible risk factors to prevent stroke is an important strategy to reduce human and economic burden of stroke.[3] There are numerous studies reporting variation in candidate genes responsible for stroke and the frequency of the studied genes differs across and within ethnic populations due to complex environment gene interactions.[4] One such gene is methylene tetrahydrofolate reductase (MTHFR) which plays a prominent role in methionine metabolism and converts 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulatory form of folate.[5] This polymorphism makes the enzyme thermo-labile and less active which leads to hyperhomocysteinemia in homozygous mutant state and this makes it an important marker for thrombotic events.[6] Meta-analysis on MTHFR gene suggests TT homozygosity to be associated with ischemic cardiovascular disease (ICD) and venous thromboembolism (VTE).[7,8] Studies suggesting role of C677T with ischemic stroke are controversial, with few reporting positive association and others negative but with negligible number of studies in hemorrhagic stroke.[9-17] This suggests that there is need for comparative studies reporting on types of stroke which can provide valuable insights into stroke research.

Thus, in the present study we evaluated the association of MTHFR (C677T) polymorphism with ischemic stroke, its subtypes and hemorrhagic stroke in a South Indian population from Andhra Pradesh. Further, ischemic and hemorrhagic strokes were also compared for this polymorphism.

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METHOD

Subjects

Six hundred and twenty ischemic stroke patients (males: females=434:186) and 220 hemorrhagic stroke

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patients (males: females=158:62) presenting with new stroke evaluated in the neurology department of Nizam's Institute of Medical Sciences, Hyderabad (A.P, India) between September 2007 and December 2013 were included in the study. The study was approved by the institutional ethics committee and all the patients were examined by a qualified stroke neurologist. Ischemic and hemorrhagic strokes were differentiated by computed tomography (CT) scans and magnetic resonance imaging (MRI). Patients with major cardiac, hepatic, renal, endocrinological disorders, skeletal disorders and cancerous diseases were excluded from this study. The ischemic stroke was classified into subtypes according to the TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification.[18] As control group 620 healthy individuals (males: females=428:192) that had no clinical evidence of any cerebrovascular disease matched for sex and age were recruited from the same demographic area. Information on demographic features and risk factors was collected using a structured questionnaire. Hypertension, alcohol use, diabetes and smoking were defined as reported previously.[19] All the samples were collected only after obtaining the written informed consent.

DNA Isolation and Genotyping

Two millilitres of blood was used for genomic DNA extraction using standard phenol-chloroform method and the polymorphism was analysed using PCR - RFLP technique[20] (Figure 1).

Statistical Analysis

Hardy–Weinberg equilibrium was tested and association between genotypes and stroke was examined by Odds ratio with 95% confidence interval (CI) and chi-square analysis using Open EPI6 software (Open Epi Version 2.3.1). Allelic frequencies were calculated according to the number of different alleles observed and the total number of alleles examined. A stepwise multiple logistic regression (MLR) analysis was performed using SPSS18 to confirm the results of pairwise comparisons. Statistical significance was defined as $p<0.05$.

RESULTS

Six hundred and twenty ischemic stroke patients, 620 age and sex matched controls and 220 hemorrhagic stroke patients were included in the study. The clinical characteristics of ischemic stroke, hemorrhagic stroke patients and controls have been given in Table 1. Mean age was 51.3 years for ischemic stroke patients, 53.8 years for

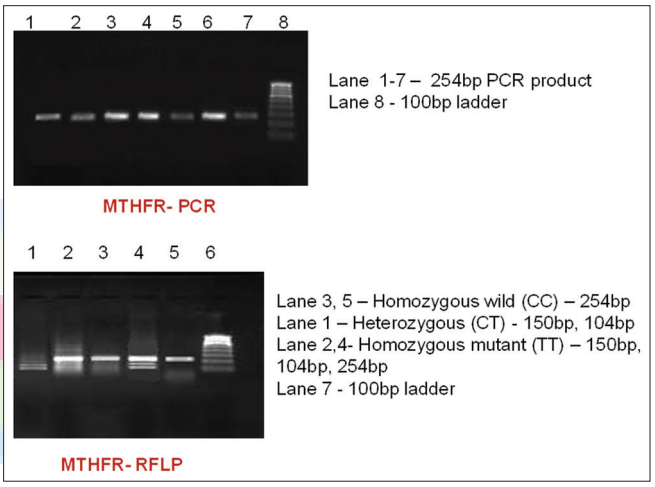

Figure 1: Analysis of polymorphism using PCR - RFLP technique

Table 1: Clinical characteristics of ischemic stroke patients, hemorrhagic stroke patients and controls

Characteristics	Ischemic stroke patients (n=620)	Controls (n=620)	p value	Haemorrhagic stroke patients (n=220)	p value
Age	51.3 (13.4)	49.08 (16.9)		53.8 (3.9)	
Male: female	434:186	428:192		158:62	
Systolic Bp (mmHg) (Mean±S.D)	142.3 (19.3)	126.7 (16.2)	p<0.001	142.02 (16.8)	p<0.001
Diastolic Bp (mmHg) (Mean±S.D)	85.2 (12.4)	78.7 (16.2)	p<0.001	87.5 (19.5)	p<0.001
Total Cholesterol (Mean±S.D)	194.2 (40.7)	195.8 (45.9)	NS	178.7 (39.2)	NS
Triglycerides (Mean±S.D)	173.4 (30.7)	138.2 (44.3)	p<0.001	176.84 (40.2)	p<0.001
Random Glucose (Mean±S.D)	135.7 (9.6)	119.4 (21.4)	p<0.001	128.7 (6.4)	p<0.001
HDL cholesterol (Mean±S.D)	49.5 (16.9)	59.3 (23.3)	p<0.001	53.7 (19.1)	NS
Hypertension	53.4%	32.1%	p<0.001	55.8%	p<0.001
Diabetes	47.9%	28.8%	p<0.001	33.4%	p<0.001
Smokers	49.5%	31.9%	p=0.001	38%	p<0.001
Alcohol use	37.3%	25.8%	p<0.001	42%	p<0.001
Family history of stroke	28.4%	6.9%	p<0.001	5.9%	p<0.001

NS: Non-significant. Age, systolic BP, diastolic BP, total cholesterol, high density lipoprotein (HDL) cholesterol, random glucose and triglycerides are given as mean (SD). *p* values were calculated using Students' paired t-test (SPSS 18)

hemorrhagic stroke patients and 49.08 years in controls. Risk factor profile of the ischemic patients revealed hypertension in 53.4%, diabetes in 47.9%, smoking in 49.5%, alcohol use in 37.3% and family history of stroke in 28.4% subjects. In the control group 32.1% had hypertension, 28.8% were diabetic, 31.9% smokers, 25.8% were alcohol users and 6.9% had a family history of stroke. Profiles of patients for the various risk factors in hemorrhagic stroke revealed hypertension in 55.8%, diabetes in 33.4 %, smoking in 38%, alcohol use in 42% and family history of stroke in 5.9% of patients.

The genotypic distribution of MTHFR C677T polymorphism and allelic frequencies of C and T alleles in ischemic stroke, hemorrhagic stroke patients and controls have been given in Table 2. There was statistically significant difference in the CT genotypic distribution between ischemic stroke patients and controls (p<0.05). Significant association of this polymorphism with ischemic stroke was observed following dominant and co-dominant genotypic models (p<0.05). The results were further confirmed by MLR analysis (p<0.05). However, no significant difference was observed in the allele frequency of C and T alleles in ischemic stroke patients and controls (p=NS) (Table 3). A statistically significant difference in the CT genotypic distribution between hemorrhagic stroke patients and controls was also observed (P<0.05). Significant association of this polymorphism with hemorrhagic stroke was found following dominant and co-dominant genotypic models (p<0.05). The results were further confirmed by MLR analysis (P<0.01). However, no significant difference was observed in the allele frequency of C and T alleles in hemorrhagic stroke patients and controls (p=NS) (Table 4).

Examining the association with ischemic stroke subtypes we found significant association with intracranial large artery (ILA) (p<0.05), lacunar stroke (p<0.05) and undetermined etiology (UDE) (p<0.05) (Table 5). Table 6 represents the analysis of the genotypic and allelic frequencies between ischemic and hemorrhagic stroke patients which reveals statistically no significant difference between the two types of stroke (p=NS).

Table 2: Distribution of MTHFR (C677T) genotypes and allelic frequencies in ischemic stroke patients, hemorrhagic stroke patients and controls

Variable	CC (%)	CT (%)	TT (%)	Total	C	T	Total
Ischemic stroke	468 (75.5)	132 (21.3)	20 (3.2)	620	1068 (0.86)	172 (0.14)	1240
Control	497 (80.2)	99 (15.9)	24 (3.9)	620	1093 (0.88)	147 (0.12)	1240
Hemorrhagic stroke	156 (71)	54 (24.5)	10 (4.5)	220	366 (0.83)	74 (0.17)	440

Table 3: Analysis of MTHFR (C677T) genotypes and alleles among ischemic stroke patients and controls

Genotype	OR (95% CI)	χ^2	p-value
TT vs CC	0.9 (95%C.I; 0.5-1.6)	0.2	NS
CT vs CC	1.42 (95% C.I; 1.06-1.9)	5.6	p<0.05
TT vs CT	0.62 (95% C.I; 0.32-1.19)	2.0	NS
Dominant CC vs CT+TT Co-dominant	0.76 (95% C.I; 0.6-0.9)	3.9	p<0.05
CT vs CC+TT recessive	1.42 (95% C.I; 1.1-1.9)	5.8	p<0.05
TT vs CC+CT alleles	0.8 (95% C.I; 0.5-1.5)	0.4	NS
C vs T	0.8 (95% C.I; 0.7-1.05)	2.2	NS
T vs C	1.2 (95% C.I; 0.9-1.52)	2.2	NS

NS: Non-significant, OR: Odds ratio, CI: Confidence interval

Table 4: Analysis of MTHFR (C677T) genotypes and alleles among hemorrhagic stroke patients and controls

Genotype	OR (95% CI)	χ^2	p-value
TT vs CC	1.32 (95% C.I; 0.62-2.83)	0.5	NS
CT vs CC	1.73 (95% C.I; 1.2-2.5)	8.3	p<0.05
TT vs CT	0.7 (95% C.I; 0.34-1.71)	0.4	NS
Dominant CC vs CT+TT	0.60 (95% C.I; 0.42-0.9)	8.0	p<0.05
Co-dominant CT vs CC+TT	1.71 (95% C.I; 1.17-2.5)	8.0	p<0.05
Recessive TT vs CC+CT alleles	1.2 (95% C.I; 0.6-2.5)	0.19	NS
C vs T	0.7 (95% C.I; 0.5-0.9)	7.0	NS
T vs C	1.5 (95% C.I; 1.11-2.03)	7.0	NS

NS: Non-significant, OR: Odds ratio, CI: Confidence interval

DISCUSSION

In this study we evaluated the role of MTHFR C677T gene polymorphism with the risk of ischemic stroke and hemorrhagic stroke in a South Indian population from Andhra Pradesh. This polymorphism is a known risk factor for myocardial infarction, stroke and silent brain infarction.[6,9,21,22] Weisberg et al. (1998) reported that in heterozygotes for this polymorphism, the MTHFR activity decreases by approximately 30%, and in homozygous mutant individuals, the activity decreases by about 60%.[23] The frequency of this polymorphism varies across the globe and among different ethnicities. It has been reported to be 38% in French Canadian, 11% in Japanese, 12.3% in Chinese, 0-1.2% in Indians and uncommon among Africans.[24]

Multiple studies on the association of this variant shows inconsistent result related to ischemic stroke. Studies suggest the polymorphism to be more pronounced in ischemic stroke among Asian patients[10] but fail to show a positive relationship among Caucasians.[21,25,26]. Meta-analysis study by Cronin *et al.* (2005) reports a graded increase in ischemic stroke with increase in T allele dose.[14] This finding is supported by studies from Japan and China which show a similar increase in ischemic stroke due to TT genotype.[10,27] However, earlier studies from India do not document significant association although there is high frequency for TT genotype among ischemic stroke patients.[28] A possible explanation for this could be due to low prevalence

Table 5: MTHFR (C677T) genotypic and allelic frequencies in ischemic stroke patients classified according to TOAST classification

TOAST Classification	No. of patients	Genotype (%)			Allelic frequencies		Odds ratio	95% CI	p value
		CC	CT	TT	C	T			
Large artery atherosclerosis	303								
A. Intracranial large artery	225	160 (71.1)	58 (25.8)	7 (3.1)	378 (0.84)	72 (0.16)	1.8	(1.3-2.7)	p<0.05
B. Extracranial large artery	78	59 (75.6)	16 (20.5)	3 (3.9)	134 (0.86)	22 (0.14)	1.4	(0.75-2.5)	NS
Small artery occlusions (Lacunar)	83	58 (69.9)	21 (25.3)	4 (4.8)	137 (0.83)	29 (0.17)	1.8	(1.1-3.1)	p<0.05
Cardioembolism	76	56 (73.7)	18 (23.7)	2 (2.6)	130 (0.85)	22 (0.15)	1.6	(0.9-2.9)	NS
Other determined etiology	50	37 (74)	10 (20)	3 (6)	84 (0.84)	16 (0.16)	1.4	(0.7-2.8)	NS
Undetermined etiology	108	98 (90.7)	9 (8.3)	1 (1.0)	205 (0.95)	11 (0.05)	0.5	(0.23-0.94)	p<0.05

NS: Non-significant

Table 6: Analysis of MTHFR (C677T) genotypes and alleles among ischemic stroke and hemorrhagic stroke patients

Genotype	OR (95% CI)	χ^2	p-value
TT vs CC	0.7 (95% C.I; 0.30-1.45)	1.1	NS
CT vs CC	0.81 (95% C.I; 0.6-1.2)	1.2	NS
TT vs CT	0.81 (95% C.I; 0.36-1.8)	0.2	NS
Dominant CC vs CT+TT	1.3 (95% C.I; 0.9-1.8)	1.8	NS
Co-dominant CT vs CC+TT	0.83 (95% C.I; 0.6-1.2)	0.9	NS
Recessive TT vs CC+ CT alleles	0.7 (95% C.I; 0.32-1.52)	0.8	NS
C vs T	1.241 (95% C.I; 0.907-1.697)	1.825	NS
T vs C	0.806 (95% C.I; 0.5892-1.103)	1.825	NS

NS: Non-significant, OR: Odds ratio, CI: Confidence interval

of mutant genotype in our population.[29] However, Frederiksen *et al.* (2004) reports negative association of the polymorphism with both ICD and VTE.[30] The association of this polymorphism with hemorrhagic stroke has been documented in a Turkish Caucasian population.[31] Further, a recent meta-analysis also confirms the polymorphism to be strongly associated with hemorrhagic stroke and suggests T allele and TT genotype to be strong risk factors for the development of hemorrhagic stroke.[32,33]

Among Indians, positive association of C677T but not A1298C polymorphism in MTHFR gene with ischemic stroke has been reported in a Tamil South Indian Population.[13] Similarly another study by Alluri et al. found strong association for the polymorphism in arterial stroke among young adults.[12] But a recent study from North India reports negative association in both ischemic and hemorrhagic stroke.[15] However, in our study we found the CT genotype to be significantly associated with both ischemic and hemorrhagic stroke. Evaluating the association of this polymorphism with stroke subtypes classified according to TOAST criteria, we found significant association with ILA, lacunar stroke and UDE. However, we did not find significant difference in the genotypic distribution and allelic frequency between ischemic and hemorrhagic stroke.

A recent study by Kumar et al. (2005) reports the frequency of TT genotype to be 9% among the Caucasians, 15-16% among Chinese and Japanese and among Indians it has been reported to be present at a frequency of 2.9%.[34] Further, it has been reported that the frequency of T-allele is lower among South Indians when compared to populations from UK and USA and higher when compared to population from Sri Lanka.[35] Altogether, the frequency of TT genotype has been found to be lower among Indian subjects, which is consistent with our present finding. A meta-analysis by Kluijtmans et al. involving atherothrombosis, reports the three genotypes of MTHFR C677T to confer different levels of atherothrombotic risk in "genetically vulnerable" populations and also the CT genotype to actively confer atherothrombotic risk.[36]

Further, it has been seen that individuals harbouring CT genotype have been reported to have higher levels of homocysteine (Hcy) than CC genotypes and hyperhomocysteinaemia has long been known to be a risk factor for stroke. Although in the present study we could not estimate the Hcy levels for all the patients, a number of studies have positively correlated the Hcy levels with the genotypes and elevation was found to be more pronounced among TT homozygote mutants. In contrast to this, two studies report that TT genotype is not associated with Hcy concentrations.[37,34] A study involving Indian population from all over the country with different ethnicities reports that individuals with vegetarian diet have higher Hcy levels irrespective of MTHFR genotypes.[34] Despite a clear view on the role of Hcy levels in vascular diseases, its role cannot be ignored since it has been instrumental in detection of various disease conditions including stroke and thus estimating the Hcy levels and correlation with stroke genotypes would have been an added advantage of the study.

In conclusion, our study reveals that CT genotype of MTHFR (C677T) polymorphism is a risk factor for ischemic stroke, its subtypes (ILA, lacunar and UDE) and hemorrhagic stroke among the South Indians from Andhra Pradesh.

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