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MTHFR C677T Polymorphism and Risk of Ischemic Stroke in Kashmiri Population

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Abstract

Methylenetetrahydrofolate reductase (MTHFR) is a critical enzyme in folate metabolism as it is involved in DNA synthesis, DNA repair and DNA methylation. One of the common functional polymorphisms of *MTHFR is* 677 C→T which has been shown to impact various diseases, including stroke. To investigate the *MTHFR C677T* genotype frequency in stroke cases in the Kashmiri population, we designed a case- control study, where 70 stroke cases were studied for *MTHFR C677T* polymorphism against 160 controls taken from the general population employing the PCR-RFLP technique. We found the frequency of the three different genotypes of *MTHFR* C677T in stroke case of Kashmiri population, i.e. CC, CT and TT, to be 71.4%, 17.1% and 11.4%, as compared to healthy controls, where they were 75.6%, 16.9% and 7.5%, respectively. There was no significant association between the *MTHFR* TT genotype and stroke. We conclude that the *MTHFR C677T* polymorphism is not involved in increasing the risk of stroke development in Kashmiri population.

Keywords: Stroke; *MTHFR;* Polymorphism; RFLP; Restriction digestion; Kashmir

Introduction

Methylenetetrahydrofolate reductase (*MTHFR*) is a key enzyme regulating the metabolism of folates, which are important nutrients required for both DNA synthesis as well as its methylation [1,2]. *MTHFR* irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulating folate and the one-carbon donor for remethylation processes [3].

MTHFR C677T polymorphism is one of the most important polymorphisms regulating the function of MTHFR enzyme. This polymorphism results in an alanine-to-valine substitution at codon 222 of the protein [4], which has a profound effect on the biological activity MTHFR enzyme. It is known to drastically reduce the activity of the enzyme and also decrease its thermal stability [1]. Individuals with the variant Val/Val genotype (TT) have no more than 30% of normal enzyme activity, and heterozygotes (CT) have 65% of normal enzyme activity [4,5]. The alanine-to-valine substitution also results in lower levels of 5-methyltetrahydrofolate, an accumulation of 5,10-methylenetetrahydrofolate increased plasma homocysteine levels and consequently homocysteinuria [4,6-9].

The MTHFR C677T polymorphism has been linked to various other diseases also like rheumatoid arthritis (RA) [10], coronary artery disease (CAD) [11], epilepsy [12] and Parkinson's disease [13]. Stroke is the second most common cause of death and disability world-wide. It is a multi factorial disease influenced by both environmental and genetic factors [14]. Several studies from around the globe have reported on the association of *MTHFR C677T* polymorphism and the risk of ischemic stroke. Many studies including few meta-analyses have found the homozygous TT variant form to be directly associated with the increased risk of stroke [15-19], but these studies do not conclude the association consistently and some studies have shown no association at all [20]. However, a recent meta-analysis by Li et al., [20] has suggested that *MTHFR C667T* genetic polymorphism to be significantly associated with increased risk of ischemic stroke.

A number of studies evaluating the role of *MTHFR C677T* polymorphism in the stroke patients have also been carried out in India, some studies failed to report anything conclusive [21,22] but a few studies however were able to report that the MTHFR C677T polymorphisms showed association with both homocysteine levels as well as stroke [23-26].

Since no such kind of study has been carried in Kashmiri population; therefore, we carried out a case-control study in our population to determine if this *MTHFR C677T* polymorphism is associated with an altered risk of ischemic stroke in our Kashmiri Population or not.

Methodology

Subjects

We chose 70 patients who were admitted in Sher I Kashmir Institute of Medical Sciences (SKIMS), Soura, Kashmir for the treatment of ischemic stroke. Chief complaints of studied patients included left / right sided weakness, altered sensorium and speech disturbances. Blood samples of 160 age- and sex-matched cases were also collected to serve as the external population controls for the study. The study was carried for the period of two years from March 2013 to March 2015.

Data on all stroke patients were obtained either from personal interviews with patients or from guardians, medical records. All patients and/or guardians were informed about the study and their will to participate in this study was taken on predesigned questionnaire (Available on request). The collection and use of samples for this study were previously approved by the SKIMS Ethics Committee.

DNA extraction & genotype analysis

DNA extraction of the samples was performed by commercial DNA

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extraction kit (Fermentas, USA). Previously reported primers: forward primer 5'-GGTCAGAAGCATATCAGTCATGAG-3' and the reverse primer 5'-CTGGGAAGAACTCAGCGAACTCAG-3', were used for the amplification of the 494-bp target region within the *MTHFR* gene [27].

PCR was carried out in a final volume of 25 µL containing 50 ng genomic DNA template, 1X PCR buffer (Biotools, Spain) with 2 mM $MgCl_2$, 0.4 µM of each primer (Genescript, India), 50 µM dNTPs (Biotools, Spain), and 0.5 U DNA polymerase (Biotools, Spain). For PCR amplification, the standard program was used as follows: one initial denaturation step at 94°C for 7 min, followed by 35 cycles of denaturation for 30 s at 94°C, 30 s of annealing at 58°C, and 30 s of extension at 72°C, followed by a final elongation cycle at 72°C for 5 min.

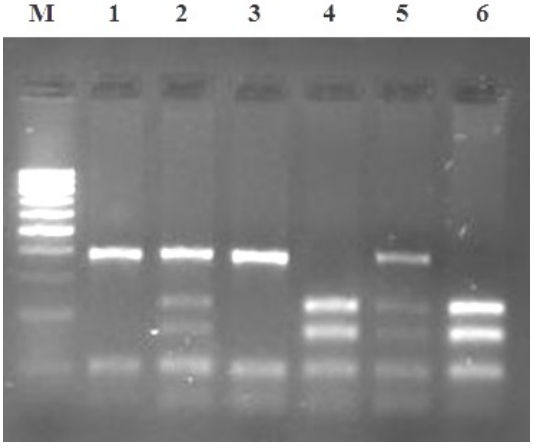
For RFLP, the PCR product of *MTHFR* was digested with *HinfI* (2 U at 37°C for 16 h) (Fermentas, USA). In the case of *MTHFR C677T* polymorphism, the Ala/Ala (CC) wild-type was identified by 394-bp and 100-bp bands, while the Val/Val (TT) variant was identified by 229-bp, 165-bp and 100-bp bands and the heterozygous Ala/Val (CT) variant displayed all four bands (394 bp, 229 bp, 165 bp and 100 bp) (Figure 1). DNA fragments were electrophoresed through a 2-3% agarose gel for resolution.

Statistical analysis

Statistical analysis was performed by using SPSS Software (IBM). Observed frequencies of genotypes in stroke patients were compared to controls using chi-square or Fisher exact tests when expected frequencies were small. The chi-square test was used to verify whether genotype distributions were in Hardy-Weinberg equilibrium. Odds ratio was used to determine association between any of the recorded Clinico-epidemiological characteristics of patients with any one of the three genotypes of *MTHFR* gene. Statistical significance was considered when $p \leq 0.05$.

Results

A total of 70 cases and 160 control subjects were included in this study with prior consent. Furthermore, out of 70 stroke cases, 45 were males and 25 cases were females (M/F ratio=1.8), 42 were rural and 28 were urban and 38 were smokers and 32 were non-smokers (Table 1). Among control subjects, 88 were males and 72 were females (M/F ratio=1.22). No significant gender- or age-related differences were observed between the groups (p>0.05).



Note: 1) Lane M: 100-bp ladder. 2) Lanes 1 and 3 show wild-type (CC) form (394 bp and 100 bp). 3) Lanes 2 and 5 show heterozygous (CT) variant form (394 bp, 229 bp, 165 bp and 100 bp). 4) Lanes 4 and 6 show homozygous (TT) variant form (229 bp, 165 bp and 100 bp).

Figure 1: Representative gel of *MTHFR C677T* polymorphism, representing amplicon digest with HinfI (G|ANTC/CTNA|G); where variant (TT) is cleaved but not wild-type (CC).

Variable	Cases n=70	Controls n=160	*P*-Value
Age			
> 50	39	104	0.1
≤ 50	31	56	
Gender			
Males	45	88	0.1
Females	25	72	
Dwelling			
Rural	42	104	0.5
Urban	28	56	
Smoking Status			
Ever	38	85	0.8
Never	32	75	

Table 1: Frequency distribution analysis of selected demographic and risk factors in Stroke cases and controls.

MTHFR Genotype	Cases (n= 70)	Controls (n=160)	OR (95% CI), $P^{¥}$, $F^{ψ}$	χ^2, *P* Value (Overall)
CC– (Wild)	50(71.4%)	121(75.6%)	1	0.98,0.6
CT – (Heterozygous)	12(17.1%)	27 (16.9%)	1.07(0.5-2.2)0.8, 0	
TT – (Variant)	8(11.4%)	12 (7.5%)	1.6(0.6-4.1),0.32, 0.44	
¥Pearson's P Value, ψFisher Exact P Value. Significant P values are shown in bold				

Table 2: Genotype frequencies of *MTHFR* gene polymorphism in stroke cases and controls.

The frequency of Ala/Ala (CC), Ala/Val (CT) and Val/Val (TT) genotype in ischemic stroke cases were found to be 71.4%, 17.1% and 11.4%, as compared to healthy controls, where they were 75.6%, 16.9% and 7.5%, respectively. There was not any varied difference in the genotype frequency of C677T *MTHFR* between ischemic stroke cases and the matched controls. So, this study suggests that there is no significant correlation between the *Val/Val (TT)* variant of *MTHFR* gene and ischemic stroke in our Kashmiri population (Table 2).

Discussion

This is the first study to report on the association of *MTHFR* genotype with the risk of development of stroke in Kashmiri population, as data on *MTHFR* genotypes and susceptibility to ischemic stroke in our population did not exist at all. A number of studies have been reported across the globe on the modulation of risk of stroke by *MTHFR C677T* polymorphisms, because of the fact that plasma homocysteine (main substrate of MTHFR enzyme) levels are considered a major risk factor for various vascular diseases, including stroke [28,29]. This is because MTHFR plays a key role in regulating the homocysteine levels by utilizing it to make S-adenosyl methionine (SAM). A common C to T substitution in the *MTHFR* gene, at 677 nucleotide results in the conversion of alanine to valine in MTHFR enzyme [27] which in turn leads to reduction in the enzyme activity and decreased usage of homocysteine in cells thereby ensuing elevation of the plasma homocysteine level [21]. Hyperhomocysteinemia has many adverse effects including endothelial dysfunction with associated platelet activation and thrombus formation [30].

The *MTHFR* gene is centralized on chromosome 1p36.22 and is known to encompass 19.3 kb of DNA stretch 11 exons. The gene encodes 74.6-kD protein composed of 656 amino acids [31]. MTHFR enzyme is cytosolic and catalyzes the conversion of 5,10-methylene tetrahydrofolate (THF) to 5-methylTHF, which in turn is used as cosubstrate for methionine synthesis and subsequent production of S-adenosyl methionine (SAM). MTHFR is also linked to the production of dTMP via thymidylate synthase and to purine synthesis and, therefore, plays a role in the provision of nucleotides essential for DNA synthesis [32]. Thus, any defect in the *MTHFR* gene will be reflected in a defect in the methylation pattern of DNA as well as in its synthesis. Chen et al. [33] has reported that the low activity of *MTHFR 677TT* genotype being advantageous as it ensures an adequate thymidylate pool for DNA synthesis when in folate sufficient cells.

In this molecular case-control study, C677T polymorphism in the *MTHFR* gene and its association with susceptibility to ischemic stroke were demonstrated. We demonstrated that *MTHFR C677T* gene polymorphism is not significantly associated with risk of ischemic stroke in Kashmiri population. We did not found any varied difference in the genotype frequency of C677T *MTHFR* between ischemic stroke cases and the matched controls. The frequency of Ala/Ala (CC), Ala/Val (CT) and Val/Val (TT) genotype in ischemic stroke cases were found to be 71.4%, 17.1% and 11.4%, as compared to healthy controls, where they were 75.6%, 16.9% and 7.5% respectively. So, this study suggests that there is no significant correlation between the *Val/Val (TT)* variant of *MTHFR* gene and ischemic stroke in our Kashmiri population. Our results are consistent with the previous study on the north Indian population [22].

Brattstrom et al. [34] in their meta-analyses of 13 case-control studies reported that the homozygous MTHFR *677TT* individuals generally had fasting homocysteine levels higher than in heterozygous MTHFR 677CT or normal individuals with MTHFR 677CC genotype. They also concluded that this polymorphism is only a modest risk factor for arterial thrombosis. A similar meta-analysis by Li et al. [35] found a statistically significant association of ischemic stroke with T allele of MTHFR gene, suggesting that the *MTHFR C667T* genetic polymorphism to be significantly associated with increased risk of ischemic stroke. Also, another meta-analysis by Kang et al. (19) found out that *MTHFR C667T* genetic polymorphism is associated with increased risk of hemorrhagic stroke, and the T allele may be an important risk factor for hemorrhagic stroke. Another recent study of Zhou et al. [36] in Chinese population also observed that *MTHFR C677T* gene polymorphism influences the risk of ischemic stroke by modulating serum homocysteine levels in individuals. Similar kinds of results implicating T allele of MTHFR in elevating the risk of ischemic stroke were reported by numerous researchers in their respective populations of varied racial background [16,35,37,38] (Table 3). Klerk et al. [39] in their study on European Vs Asian population showed that the association between *MTHFR C677T* polymorphism and coronary heart disease was weaker among Europeans than that in Asian populations.

	***MTHFR* Genotype**	**Cases**	**Controls**	**Significant Association**
Our Study 2014		**(n= 70)**	**(n=160)**	
	CC– (Wild)	50(71.4%)	121(75.6%)	No
	CT – (Heterozygous)	12(17.1%)	27 (16.9%)	
	TT – (Variant)	8(11.4%)	12 (7.5%)	
[25]		**(n= 120)**	**(n=120)**	
	CC– (Wild)	67 (55.8%)	90 (75.0%)	Yes
	CT – (Heterozygous)	49 (40.8%)	30 (25.0%)	
	TT – (Variant)	4 (3.3%)	0(0.00%)	
[22]		**(n= 207)**	**(n=188)**	
	CC– (Wild)	137 (66.2%)	129(68.6%)	No
	CT – (Heterozygous)	65 (31.4%)	54 (28.7%)	
	TT – (Variant)	5 (2.4%)	5 (2.7%)	
[38]		**(n= 84)**	**(n=100)**	
	CC– (Wild)	35 (41.6%)	60(60.0%)	Yes
	CT – (Heterozygous)	43 (51.2%)	35 (35.0%)	
	TT – (Variant)	6 (7.2%)	5 (5.0%)	
[16]		**(n= 70)**	**(n=50)**	
	CC– (Wild)	26 (37.1%)	27 (54.0%)	Yes
	CT – (Heterozygous)	30 (42.9%)	20 (40.0%)	
	TT – (Variant)	14 (2.0%)	3 (6.0%)	
[37]		**(n= 72)**	**(n=292)**	
	CC– (Wild)	26 (37.1%)	27 (54.0%)	Yes
	CT – (Heterozygous)	30 (42.9%)	20 (40.0%)	
	TT – (Variant)	14 (2.0%)	3 (6.0%)	

Table 3: Genotype frequencies of *MTHFR* gene polymorphism in Stroke Cases and Controls.

Therefore, we conclude that MTHFR C677T gene polymorphism is not significantly associated with risk of ischemic stroke in Kashmiri population.

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