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The Association of MTHFR C677t Polymorphism with Ischemic Stroke in Kashmiri Population: A Case Control Study

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Abstract **Original Research Article**

Introduction: MethylTetraHydroFolateReductase (MTHFR) C 677 -T Polymorphism is a widely studied genetic mutation all over world not only because of its association with the prevalence of stroke, but also because of the associated hyperhomocysteinemia and potentially preventive nutritional interventions. ***Methadology:*** We conducted a hospital based case control study with 75 confirmed cases of Ischemic (ISC) stroke against 100 matched controls, and employed the PCR-RFLP technique to study MTHFR C677T Polymorphism. ***Results:*** Among the controls we found the frequency of MTHFR CC, CT and TT genotypes to be 66 (66%), 29 (29%) and 5(5%) respectively. No statistically significant differences in the allelic and genotypic frequencies between ISC patients and controls were found in this polymorphism **(p=0.18; OR=0.61; CI=0.31-1.2).** ***Conclusion:*** MTHFR C677T Polymorphism was not associated significantly with ischemic stroke. Furthermore its association with the various clinical parameters of ISC cases was not found to be significant.

Keywords: MethyleneTetra Hydro Folate Reductase, Polymorphism, Ischemic.

INTRODUCTION

Stroke is defined as a focal (or at times global) neurological impairment of sudden onset, lasting more than 24 h (or leading to death) and of presumed vascular origin [1]. According to WHO, 15 million people worldwide suffer a stroke annually and of these 5 million die while another 5 million are left disabled [1]. In the year 2013, stroke was identified as the second common cause for global mortality after IHD [2], however, when considered separately from other CVDs, it ranks no. 5 among all causes of death, behind diseases of the heart, cancer, chronic lower respiratory disease, and unintentional injuries/accidents [3]. Stroke remains the most common cause of disability in developed countries [4,5]. It is estimated that by 2020, 19 out of 25 million annual stroke deaths will be in developing countries [6].

About 85% of strokes are due to cerebral ischemia (ISC) and 15% are due to primary intracerebral hemorrhage (ICH) [7,8]. Some 8% to 12% of ischemic strokes result in death within 30 days [9]. Of the thousands of stroke survivors each year, approximately 30% require assistance with activities of daily living, 20% require assistance with ambulation, and 16% require institutional care [9].

Although the non-modifiable risk factors (age, African and Asian race, male sex) and acquired risk factors (hypertension, cigarette smoking, diabetes, atrial fibrillation, and obesity) account for much of the risk of ischemic stroke [10], yet, stroke risk remains insufficiently explained by these factors. This infers that other influences, including genetic ones, are involved in stroke risk [11]. Genetic predisposition to stroke can be categorized as either a single gene disorder (with minor contribution) or as a polygenic disorder (major genetic contributor for predisposition to stroke) [12]. Among polygenic disorders one important genetic polymorphism studied is MTHFR (Methylene Tetra Hydro Folate Reductase) gene polymorphism [13]

Many studies have shown that the plasma homocysteine level is associated with the risk for atherosclerosis and thrombotic diseases [14]. Skepticism although has crept in recently [15] , but in

spite of contradicting arguments, the significant possibility of this association cannot be negated [16]. Several mechanisms have been proposed for this association. Homocysteine is reported to be actively involved in the oxidative stress event [17], endothelial dysfunction including induction of NADPH oxidase and decreasing bioavailability of nitric oxide (NO) [18] enhanced platelet adhesion to endothelial cells[19] and also promotion of growth of vascular smooth muscle cells [20]. This in turn may be accomplished by Protein N-homocysteinylation contributing finally to vascular inflammation, atherogenesis, hypercoagulation status, and vulnerability to establishment of atherosclerotic plaques [21]. Regarding predisposition to ICH, increased promotion to plaque rupture has been proposed as a possible mechanism [22].

MTHFR is a key enzyme in homocysteine metabolism which catalyzes irreversibly conversion of 5,10-MTHF to 5-MTHF, which is the main circulatory form of folate. Human MTHFR gene is located on chromosome 1p36.3 [23] C667T polymorphism of MTHFR gene is a C-to-T (Cytosine to Thymine) transition at nucleotide 677 (C677T) in exon 4, which results in an alanine (Ala) to valine (Val) substitution in the MTHFR enzyme [24] and makes the enzyme thermo-labile and less active which leads to hyperhomocysteinemia and hypomethylation in homozygous mutant state and this makes it an important marker for thrombotic events [24] Functionally, MTHFR enzyme activity is reduced 35% with the heterozygous CT genotype and 70% with the variant TT genotype [25] Polymorphisms in the MTHFR gene, including mainly C677T and to some extent A1298C A/V, have been shown to be associated with increased homocysteine levels and have shown evidence of causality of both stroke subtypes [26] In first decade of this century reports of the association came from India [27] as well. However, some studies have produced controversial results, thus failed to confirm previous associations [28]

Identification and management of possible risk factors to prevent stroke is an important strategy to reduce human and economic burden of stroke [29]. A potential advantage of delineating the at-risk population with these mutations could be primary preventive interventions including nutritional supplementation. E.g, a meta-analysis suggested that folic acid supplementation could significantly reduce the risk of stroke by 18% [30]. It has been also reported that vitamin B12 deficiency is positively correlated with hyperhomocysteinemia, which is partly attributed to low levels of folate [31]. B vitamin supplementation has been shown to decrease plasma homocysteine levels, although the effect on cardiovascular end points has been mostly negative [13]. Despite the inherent contradictions, the fact of the matter is that vitamin treatment remains potentially a cheap and safe mode of primary prevention of stroke.

METHODOLOGY

We followed a candidate gene case-control approach, which is generally taken to study genetic risk factors and associations for stroke [32] , to evaluate the association of C677T polymorphism (in Exon 4) of MTHFR gene in patients of Ischemic stroke and in the control population and to assess the clinical implications, if any, of the gene polymorphism in stroke patients including association with the subtype of stroke, severity and other risk factors of stroke. The ischemic stroke patients with Atrial fibrillation on ECG or a known valvular heart disease predisposing to cardiac emboli or documentation of left ventricular clot or wall motion abnormalities on precordial echocardiography were not included in this study.

Patients attending the Department of Neurology SKIMS for ISC stroke management were screened for the disease. A pretested, semi-structured questionnaire was used to collect the information on clinical and laboratory parameters. A total of 75 ISC cases with prior consent were included in our study with 100 age and gender matched controls. The data collected included Gender, Age, Smoking status, Hypertension, Diabetes Mellitus, Dyslipidaemia, Vascular Type of ISC [33] and NIHS Scoring for severity of ISC [34]. The study was approved by the ethical committee of the institute (No. SIMS 1 131/IEC-SKIMS/2017-239; Dated: 03-01-2017).

The collected blood samples were subjected to DNA polymorphism analyses using PCR - RFLP technique [35] (Figure 1).

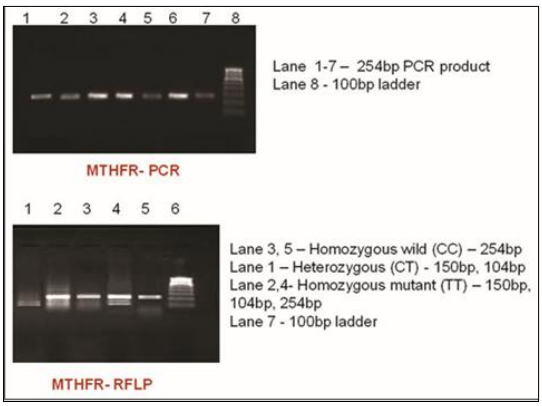

Fig-1: Analysis of polymorphism using PCR - RFLP technique

Statistical analysis was performed by using SPSS software (V.11.5). Chi Square test for homogeneity of proportions was used to determine significance of mutation pattern and Odds ratio was used to determine association of presence of polymorphism with various Clinico-epidemiological characteristics such as age, gender, diabetes, hypertension and smoking status. Statistical significance was considered with p-value ≤ 0.05.

RESULTS

A total of 75 ICH, 75 ISC cases and 100 healthy controls were included in this study No significant gender- or age-related differences were observed between the groups (p>0.05) (**Table 1**).

Table-1: Age and Gender distribution among Cases and Controls.

Variable		Cases ISC (n=75)	Controls (n=100)	p-values
Age (years)	≤55	45	49	0.16
	>55	30	51	
Gender	Male	32	56	0.09
	Female	43	44	

The distribution of *MTHFR C677T* genotypic and allelic frequency in ISC cases and controls is given in Table 2.

Table-2: The distribution of MTHFR C677T genotypic and allelic frequency in ISC cases and control.

Genotype	**ISC Cases n=75(%)**	**Controls n=100(%)**	***OR (95% CI); P¥; Fψ***
MTHFR C677T *CC* *CT +TT*	57(76%) 18+0=18(24 %)	66(66%) 29+5=34(34 %)	1.0 (Reference) 0.61(0.31 - 1.2);0.18
Allele(2N)	***(2N)*=150**	***(2N)*=200**	***OR (95% CI); P¥; Fψ***
C *T*	132 18	161 39	0.56(0.30 - 1.02);0.07

¥ = Pearson's P Value, ψ = Fisher Exact P Value.

No statistically significant differences in the allelic and genotypic frequencies between ISC patients and controls were found in this polymorphism (p=0.18; OR=0.61; CI=0.31-1.2).

Furthermore the association between *MTHFR C677T* polymorphism with that of the clinical parameters of ISC cases was also carefully analysed (Table 3), and none of these clinical parameters among ISC cases were found to be significantly associated with *MTHFR C677T* polymorphism.

Table-3: Association between MTHFR C677T polymorphism and various clinical variables of ISC patients.

Parameter	**No of ISC cases (n=75)**		
	CC(57)	***CT (18)***	***OR (95% CI); p-value***
Age ≤55years(45) >55years(30)	37 20	8 10	0.43(0.14-1.26);0.16
Gender Females(43) Males(32)	36 21	7 11	0.37(0.12-1.10);0.10
Hypertension Hypertensive(54) Non- Hypertensive (21)	39 18	15 3	2.30(0.59-8.98);0.24
Diabetes Diabetic(18) Non-diabetic(57)	12 45	6 12	1.87(0.58-6.03);0.34
Smoking Smoker(46) Non-Smoker(29)	36 21	10 8	0.72(0.24-2.13);0.58
Dyslipidaemia Present(34) Absent(41)	28 29	6 12	0.51(0.17-1.56);0.28
Vascular Type Large Vessel (55) Small Vessel (20)	40 17	15 3	2.12(0.54-8.30);0.36
NHISS Grading Mild (15) Moderate (36) Severe (20) Very Severe (4)	12 26 15 4	3 10 5 0	P=0.64 1.0 (Reference) 0.65(0.15-2.79);0.73 0.75(0.14-3.3.79);1 NA;0.58

DISCUSSION

In the present study, we examined whether the MTHFR C677T polymorphism is associated with patients with ischemic stroke. We did not find any significant association between MTHFR C677T polymorphism and ISC cases (p=0.18; OR=0.61; CI=0.31-1.2) in our Kashmiri population. The result is similar to that of a number of studies. In a study by Somarajan B et al, MTHFR C677T gene polymorphism was neither associated with hemorrhagic nor ischemic stroke in Indian population [36]. Our results are in tune with the results of many similar studies conducted populations worldwide including those in China [37] ,Brazil [38] ,Tunisia [39] , Turkey [40] , Utah [41] , Colarado [42], Ohio [43] and others [44,45]. In our valley a similar study was done in 2015 by Nissar *et al.* where 70 ischemic stroke patients were studied against 160 controls and conclusion was drawn that the MTHFR C677T polymorphism is not involved in increasing the risk of stroke development in Kashmiri population [46].

However, these results are in contradiction to a list of other studies, spanning right from the earliest studies in this respect, including, Canadian [47], Japanese [48,49], Italian [50], Chinese [22], and English [51] studies, to the later ones confirming the association, including, those from Iraq [52], Malaysia [53], Behrain [54], and Tunisia [55]. Among Indian studies, a study in North India has suggested that primary hyperhomocysteinemia to be an important risk factor for ischemic stroke mostly due to MTHFR C677T homozygosity, Inusha Panigrahi *et al.* , 2006 [56]. Similar conclusions were drawn by Kalita J *et al.* ,2006 [57]. A recent South Indian study by S. Das *et al.* also confirmed the same [58]. Various metaanalysis have been conducted in this respect and most have confirmed a positive association including Kelly PJ *et al.* in 2002 [59], Juan *et al.* in 2004 [60], S. Cronin *et al*. in 2005 [61] , Indranil Banergee *et al.* in 2006 [32], Xia-Yu Xin *et al.* in 2009 [62] , Li. P *et al.* in China in 2013 [63], Sunaina Yadav *et al.* in South Asian population [64], Amit kumar *et al.* in 2014 in Asian and Caucasian population [65], XiaoYan Zhu *et al.* in 2014 in Chinese population [66], Yanli Song *et al.* in 2015 [67], Cui T in 2014-15 [68] and recent metaanalyses done in 2017 by Abhinand *et al.* [69] and Wei, Loo Keat *et al.*[70].

The variability may be due to different genetic makeup of different populations, that includes not just the frequency of the polymorphism, but susceptibility as well [64]. E.g., studies suggest the polymorphism to be more pronounced in ischemic stroke among Asian patients, but fail to show a positive relationship among Caucasians [45] The polymorphism 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 [71]. The susceptibility of the polymorphism to cause stroke may also involve gene-gene interactions, e.g., at least one MTHFR 677T allele combined with at least one MTHFR 1298C allele [72], MTHFR CT genotype/T allele and 'CT-3/3'(OR=7.42; p=0.001) genotypic combination [73], and MTHFR 677TT and F2 20210GA [74] ; haplotypic pattern variations [75] ; or gene-environment interactions, e.g., smoking and MTHFR C-677T polymorphism [76]. In addition only specific type of ischemic stroke (small vessel strokes) may have a positive association which is not studied in most of the studies, and consequently leads to variable results [77,78]

As many investigators have found an association, we also investigated the association of MTHFRC677T genetic polymorphism with the various risk factors in our stroke population. MTHFR C677T genetic has been found to be associated with - increased risk of hypertension in Indians [79]; increased risk of essential hypertension in Caucasian.[80]; increasing the smoking induced risk for ischemic stroke [76] and increasing risk of T2DM [81]. However we did not get any significant association between MTHFR gene polymorphism with any of the risk factors in ISC group. This is in agreement with many investigators, including, for example, Khalid B. *et al*., who in a prospective study during 3 years, on 165 patients of strokes, in 2014, concluded that there was no correlation between MTHFR and other risk factors of stroke including age, sex, hypertension, diabetes, smoking, alcoholism and cholesterol [82].

We also tried to evaluate the association of MTHFR polymorphism with the subtype of ISC cases. Studies had suggested the sub-type effect for Large artery stokes [83], but growing evidence suggests that MTHFR C677T polymorphism is associated with multiple small artery occlusion[84]. Our study did not reveal any such association. This is in agreement with a study by Somarajan B.I. *et al*. in which no such association was found with vascular territory [36]. Also we did not find any significant association with the severity of ISC stroke as has been proposed by some researchers [7].

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