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Methylenetetrahydro folatereductase enzyme polymorphism associated with hyperhomocysteinemia in patients with stroke

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Abstract

Cerebrovascular stroke is complex heterogeneous multifactorial disorder associated with number of risk factors, including diabetes mellitus,hypertension, tobacco smoking or chewing; etc. MethylenetetrahydroFolate reductase (MTHFR) enzyme is one of the main regulatory enzymerequired for the metabolism of homocysteine. An impaired function of this metabolic pathway leads to accumulation of homocysteine in blood. The elevation of plasma total homocysteine and deficiency of B-vitamins such as folic acid and vitamin B_{12} factors associated with increased cardiovascular cerebrovascular and thromboembolic risk. A single nucleotide polymorphism of the MTHFR gene C677T causes reduction in enzyme activity. In the current study, we determined the prevalence of C677T mutation andcorrelated them to plasma total homocysteine, folic acid and vitaminB_{12}.

Total 50 cerebral stroke patients and 50 normal, healthy subjects acting as controls were taken for the study. The total homocysteine level in plasma was determined by high performance liquid chromatography (HPLC). Plasma level of folic acid and vitamin B_{12}were estimated by competitive immunoassay using direct chemiluminescence technology. ARMS PCR was used toexamine MTHFR C677T polymorphism.

Result shows concentration of significantly low plasma Folatelevels,whereas vitamin B_{12} does notshow any significant differencein patients than controls.Total homocysteine concentrations in plasma were significantly higher in stroke as compared to controls. The variation in gene of MTHFR C677T and reduced level of B-vitamins have been related to hyperhomocysteinemia.

Keywords: Methylenetetrahydro Folatereductase, Hyperhomocysteinemia, Cerebral stroke, Folic acid, B_{12} vitamin.

Introduction

Elevation of plasma total homocysteine concentration were recognised as major alterations in pathological vascular lesions and thromboembolic events develop in anteriorly located infarctions leads to arteriosclerosis and cerebrovascular stroke.[1]It is complex multifactorial disorder that causes clotting of blood and therefore rapid loss of brain functions, whose incidence increases by number of risk factors, including blood pressure, hypertension, tobacco smoking or chewing, diabetes mellitus etc.[2]

The Indian population has a higher than average incidence of cerebrovascular stroke events and evidence indicates that abnormalities of homocysteine metabolism may be a contributing factor.[3]

Over two decades, scientific study evident that homocysteine is an independent risk factor for vascular disorders like cardiovascular, cerebrovascular stroke, thromboembolism etc.[4,5]

The enzyme methylenetetrahydro Folatereductase (MTHFR 1.1.1.171) is involved in homocysteine metabolism which catalyzes the reduction of 5, 10-methylenetetra hydro Folate to 5-methyltetrahydro Folateand acts a carbon donor, 5-methyltetrahydro Folate, is predominant form of Folate in circulation, and act as methyl donor for the conversion of homocysteine to methionine by the vitamin B_{12} dependent enzyme, methionine synthase (MS). Impair transmethylation of homocysteine to methionine results in accumulation of homocysteine. A point mutation occurring at 677 nucleotide base pair where replacement of Cytosine to Thymine incorporates Alanine-to-Valine substitution at position 223 of N terminal catalytic domain which decreases enzyme activity.[6,7]

Individuals with a deficiency of B-vitamins develop severe hyperhomocysteinemia, and this may cause high risk for vascular lesions and thromboembolic events.[8] Homozygous state of a polymorphic allele in the MTHFR gene is responsible for the thermolabile phenotype which severely attains enzyme MTHFR activity.[9]

To explore lacuna of information about MTHFR gene mutation in Indian population, we took present study and attempted to correlate role of MTHFR mutation with hyperhomocysteinemia in Folate and B12 deficient individuals from south Maharashtra.

Material and Methods

The present study is carried out at Department of Biochemistry, Government Medical College, Miraj and in collaboration with BJMC, Government Medical College, Pune. The study was approved by the local ethics committee. Total 50 cerebrovascular stroke subjects were selected for the study based on clinical evidence and computed tomography scan of the brain. Total 50 numbers of healthy controls with no family history of cerebrovascular stroke were included in the study as controls. The written informed consent was obtained from all subjects. Blood samples were withdrawn from patients after an overnight fast in

vaccutainer containing EDTA and plasma was promptly separated. Plasma samples were analyzed for total homocysteine; folic acid and vitamin B_{12}. Packed red cell with buffy coat was preserved at -20ºC for DNA extraction.

Homocysteine level in plasma was determined by high performance liquid chromatography (HPLC), coupled with fluorescence detector with internal standard.[10] Plasma level of folic acid along with vitamin B_{12} was determined by competitive immunoassay using direct chemiluminescence technology.[11] The results were expressed as mean±SD and analyzed by 'z' test.

DNA extraction was carried out by salting out method from frozen venous EDTA blood samples.[12] DNA samples where analyses of polymorphism of C677T mutation in MTHFR enzyme by ARMS PCR.[13]

Table 1:The mutation specific ARMS-PCR primersfor MTHFR polymorphism[14]

Name	Name of primer (Mutation/ wild specific/ common/ control)	Primer Sequence (5'---3')	Length of Product size
MTHFR	MTHFR:223A (677C)	GAAGGAGAAGGTGTCTGCGGTAGC	197 bp
	MTHFR:223V (677T)	GAAGGAGAAGGTGTCTGCGGAAGT	197 bp
	MTHFR Common	AGGACGGTGCGGTGAGAGTG	
	Control for	CCC ACC TTC CCC TCT CTC CAG GCA AAT GGG	360 bp
	Control RES	GGG CCT CAG TCC CAA CAT GGC TAA GAG GTG	

Frozen DNA samples were dissolved for PCR reaction to get 25 µL reaction mixtures. 25-µL volume containing 1.5 U of Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 µM each dNTP and 1.5 pmol/50µL MTHFR 677C or MTHFR 677T specific primer, 1.5 pmol/50µL common primers, 8 nmol/L control primer and 2.5µg of DNA. 360 bp fragments of the α_1-antitrypsin gene function as internal control. Amplifications were performed in Quanta Biotech Thermocycler.

The PCR conditions are denaturation for 5 min at 95 °C; followed by 30 cycles at 95 °C for 1 min, 54 °C for 2 min, and 72 °C for 1 min; and a final extension step of 10 min at 72 °C.

Total 10 µl of amplified product was mixed with 2 µl of gel loading dye and separated in a 2% agarose gel that contained 0.1 mg/L ethidium bromide. The samples were electrophoresedfor 1 hour at 100 volts, using 1x TAE (Tris-Acetate-EDTA) running buffer. The amplicons were sized using a 100-bp DNA marker.

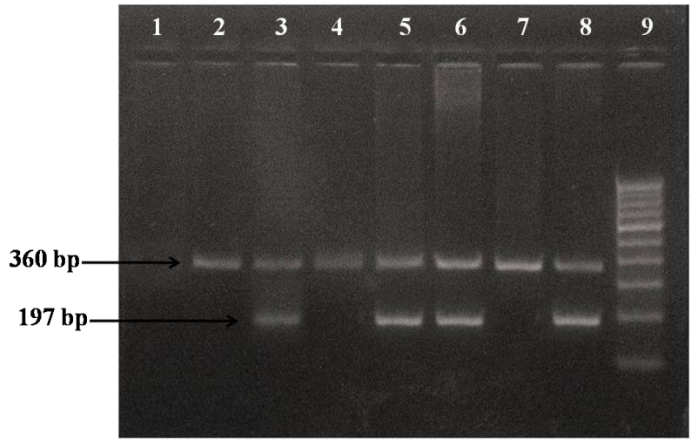

Fig.1:Genotypes ofMTHFR C677T: detected by ARMS- PCR

1-NegativeDNA control, 2-Known TT sample, 3-Known CT sample, 4 &7-Negative for C allele
5, 6 &8-Positiive forCallele, 9-100 bp DNA ladder.

Statistical analysis: Genotype frequencies with Hardy-Weinberg equilibrium were tested by X^2 test. The Hardy-Weinberg law defines a simple relationship between the frequencies of genes in the population and frequencies of genotypes.[15]

Result

A total of 50 cerebral stroke patients were enrolled with 50 age and sex matched healthy control. We found the mean plasma level of total homocysteine (21.55±3.05 µmol/L) in the study group was significantly higher (P <0.0001) in patients than controls (12.62±1.79 µmol/L). On the other hand plasma Folate level was significantly low (P = 0.0324) in patients (5.61±0.79 ng/mL) as compared to controls (6.75±0.95 ng/mL). Mean vitamin B_{12} level in patients was 240.20±11.91pg/ml and in control was 272.34±13.05 pg/ml the difference was statistically not significant (P = 0.2602), as depicted in the **Table 2**.

Table 2: Levels of biochemical parameters in study groups

Parameters	Controls (50)	Patients (50)	'P' Value
Homocysteine (µmol/L)	12.62±1.79	21.55±3.05	P < 0.0001
Folic acid (ng/ml)	6.75±0.95	5.61±0.79	P = 0.0324
Vitamin B_{12} (pg/ml)	272.34±13.05	240.20±11.91	P = 0.2602

Table 3 shows frequency distribution MTHFR C677T in cerebral stroke patients as well as controls. The occurrence of each polymorphism in control and study group was CC: 68.00% and 60.00%, CT: 28.00% and 32.00%, and for TT: 04.00% and 08.00% respectively.

Table 3: **Frequency distribution of MTHFR C677T mutation**

Subjects	MTHFR C677T Mutation		
	CC	CT	TT
Control (n=50)	34 (68.00%)	14 (28.00%)	02 (04.00%)
Patients(n=50)	30(60.00%)	16 (32.00%)	04 (08.00%)

Subjects were further classified for the comparison of MTHFR C677T genotypes with values of folic acid, vitamin B_{12} and total homocysteinelevels in cerebrovascular stroke patients and controls are summarized in **Table 4**.

In CC genotype, there was no significant difference in plasma Folate level (P = 0.8854) and vitamin B_{12} (P = 0.6487) butsignificantly higher mean value for total homocysteine (P < 0.0001) was observed among patients than controls.

In CT genotype, there was no statistical significant difference found in Folate (P = 0.0288) and vitamin B_{12} (P = 0.3257), whereas level of total homocysteine (P = 0.0011) were significantly higher in patients than controls.

In TT genotype, the plasma Folate (P = 0.0125) was significantly decreased, whereas total homocysteine (P = 0.0131) was significantly high. There was no statistical difference in vitamin B_{12} (P = 0.7150) among patients and controls.

Table 4: Levels of biochemical parameters in study groups with distribution of MTHFR C677T mutation

	CC		CT*		TT*	
	Controls (34)	Patients (30)	Controls (14)	Patients (16)	Controls (02)	Patients (04)
Folic acid (ng/ml)	6.17±0.87	6.08±0.86	6.60±0.93	5.64±0.79	6.00±0.85	4.86±0.68
VitB_{12} (pg/ml)	284.63±13.60	264.76±2.65	257.55±12.30	220.53±10.54	224.42±10.72	205.88±9.84
tHcy (µmol/L)	10.52±1.49	21.99±3.11*	12.86±1.79	20.30±2.87 *	14.13±2.00	22.14±4.05*

*Statistically significant

Discussion

In last few years, the role of variable genes in the pathogenesis of cerebrovascular stroke has been examined. In particular, the G1691A polymorphism of the factor V gene, the G20210A polymorphism for prothrombin gene, the C677T and A1298C polymorphism of the methylenetetrahydro Folatereductase (MTHFR) gene are among the most frequently studied polymorphisms for ischemic stroke and arteriosclerosis.[16,17]

In this study, we found that both high plasma concentrations of homocysteine and low plasma concentrations of vitamins Folate and B_{12} are associated with an increased risk of acute coronary disease and cerebrovascular stroke. Similar combined predictive value of these two parameters was found in various co-workers study.[18,19,20]

Hyperhomocysteinemia may be due to excessive breakdown of methionine in association with abnormal functioning of vitamin coenzymes and increased body demand of folic acid, vitamin B_{12}, B_6, B_2 either singly or in combination for maintaining of homocysteine[21] in blood, also excess dietary intake of B-vitamins shows proportionate increased formation of homocysteine. Therefore, formation of homocysteine and reutilization of homocysteine to methionine is not maintained.

The present study shows that, the MTHFR 677 TT genotype was stratified according to their plasma homocysteine level and plasma levels of B-vitamins such as folic acid and B_{12}. Here 677TT polymorphism showed significant high concentration of homocysteine, low concentrations of Folatebut statistically no difference in vitamin B_{12} concentration as compared to controls, the similar results are also reported from other regions,[22,23,24] hence, plasmatotal homocysteine with B-vitamins enhanced both thromboembolic and arteriosclerotic process by proliferation of vascular cells, this promotes prothrombolic activity in vascular cell wall. The effect of homocysteine involved in the correlation between allele and arteriosclerosis in the artery demonstrated hyperhomocysteinemia which is a causal risk factor for cerebrovascular diseases. Our results are in agreement with other studies.[25,26] In other words, elevated level of homocysteine interacts with otherconventional risk factors and in combination with these factors increases the risk of arteriothrombotic vascular disease.

Hyperhomocysteinemia was most frequent in patient with cerebral stroke having MTHFR 677TT genotype, may act as an initiator of blood coagulation in vivo. Hence, the MTHFR 677TT genotype may induce cerebral stroke through elevation in homocysteine level.[27]

The polymorphism of MTHFR gene together with elevated plasma homocysteine level was shown to be associated with developing thrombosis, including cerebrovascular stroke. Hence we propose that MTHFR C677T gene polymorphism is associated with increase plasma homocysteine levels, which is a genetic risk factor for cerebrovascular stroke.

Among Indians, homozygous and heterozygous mutations are observed, but due to lack of research not much data for homocysteine is reported as compared to western population, where homozygous type is predominant.

In conclusion, the MTHFR C677T polymorphism is an independent, non modifiable risk factor, which can lead to cerebrovascular stroke. Individuals having Folate and B_{12} deficiency are at higher risk,hence should be screened for genetic parameters like MTHFR C677T polymorphism so that appropriate preventive measures can be undertaken and susceptibility for developing disease are reduced.

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