Original Article

Methylenetetrahydrofolate reductase gene polymorphism in Indian stroke patients

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Background and Aims: In view of the prevailing controversy about the role of Methylenetetrahydrofolate reductase (MTHFR) C677T mutation in stroke and paucity of studies from India, this study has been undertaken to evaluate MTHFR C677T gene polymorphism in consecutive ischemic stroke patients and correlate these with folic acid, homocysteine (Hcy) and conventional risk factors. **Settings and Design:** Ischemic stroke patients prospectively evaluated in a tertiary care teaching hospital. **Materials and Methods:** Computerized tomography proven ischemic stroke patients were prospectively evaluated including clinical, family history of stroke, dietary habits and addictions. Their fasting and postprandial blood sugar, lipid profile, vitamin B12, folic acid and MTHFR gene analysis were done. **Statistical Analysis:** MTHFR gene polymorphism was correlated with serum folic acid, Vitamin B12 and Hcy levels; family history of stroke in first-degree relatives; and dietary habits; employing Chi-square test. **Results:** There were 58 patients with ischemic stroke, whose mean age was 50 (4-79) years; among them, 10 were females. MTHFR gene polymorphism was present in 19 (32.8%) patients, 3 were homozygous and 16 were heterozygous. Both serum folate and B12 levels were low in 29 (50%) patients and Hcy in 48 (83%). Hypertension was present in 28 (48%) patients, diabetes in 12 (21%), hyperlipidemia in 52 (90%), smoking in 17 (29%), obesity in 1 (1.7%) and family history of stroke in first-degree relatives in 13 (22.4%). There was no significant relationship of MTHFR gene polymorphism with folic acid, B12, Hcy levels, dietary habits and number of risk factors. Vitamin B12 level was low in vegetarians (P<0.003). In 3 patients with MTHFR TT alleles, Hcy was elevated in all 3, low folic acid in 2 and family history of stroke in 1 patient. **Conclusion:** MTHFR gene polymorphism was found in one-third of patients with ischemic stroke and was insignificantly associated with higher frequency of elevated Hcy.

Key words: Diet, folic acid, homocysteine, Infarction, methylenetetrahydrofolate reductase, stroke, vegetarianism, vitamin B12

Introduction

More than 110 heritable disorders, 175 genetic loci and 2,050 unique mutations predisposing to stroke are known. Although ischemic stroke can result from merely one genetic defect, the interaction of unfavorable genetic factors such as Leiden V, Methylenetetrahydrofolate reductase (MTHFR) 677T, apoliprotein E (ApoE) 4 and angiotensin converting enzyme (ACE) D/D genotypes with other risk factors such as hypertension, diabetes, smoking and alcohol consumption can influence the occurrence of stroke.[1-3] Elevation of homocysteine (Hcy) is a recently established risk factor for cardiovascular disease.[4-6] The role of homocysteine in stroke however remains controversial, though most case-control studies suggest a positive association between homocysteine and stroke.[5,7,8] But the association has not been established in nested case control studies.[9,10] MTHFR is an enzyme in modulation of plasma homocysteine by converting it into methionine. Polymorphism of MTHFR C677T leads to a reduction in enzyme activity and subsequent elevation of plasma homocysteine. The C677T mutation has been reported to be associated with ischemic stroke in some studies[11,12] but not in others.[13] Variation in ethnicity and methodological differences could account for this discrepancy.

The population frequency of C677T homozygosity ranges from 1% or less among blacks to 20% amongst Italians and US Hispanics.[7] In the pooled analysis of several thousand Japanese, the frequency of C677T homozygosity was 11% and limited data are available for other Asian population.[14] We report preliminary results of a study evaluating MTHFR C677T mutations in Indian ischemic stroke patients and correlate these with folic acid and homocysteine levels in the context of other risk factors.

Materials and Methods

In this study, computerized tomography proven ischemic stroke patients attending to the department of Neurology during January to October 2005 were prospectively evaluated. The project was duly approved by the local ethics committee. The patients

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with endocrine disorders other than diabetes mellitus (DM); cardiogenic stroke; liver failure; pregnancy; malignant disease; vasculitis; and those with exposure to nitric oxide, methotrexate or other folic acid inhibitors were excluded. Those who received B12 or folic acid supplementation in the past 1 year were also excluded.

The patients were subjected to detailed medical history and clinical examination. The medical history also included family history of stroke in the first-degree relatives. Dietary history was taken by food frequency questionnaire employing recall of food items in 1 week and daily intake of vitamin B12 was calculated.

The risk factors of stroke such as hypertension (≥140/90 mmHg), diabetes (blood sugar ≥ 106 and 2 h postprandial > 200 mg/dl) and hyperlipidemia [high density lipoprotein (HDL) <40 mg/dl, low density lipoprotein (LDL) >160 mg/dl, triglyceride >150 mg/dl, very low density lipoprotein (VLDL) >30 mg/dl and total cholesterol >200 mg/dl][15] were recorded. For diagnosis of hyperlipidemia, values of fasting blood lipid after 1 month of stroke were considered. History of smoking (pack/year), tobacco and alcohol intake were also noted. Body mass index was calculated as weight in kg/height in m^2. The neurological examination included the severity of stroke as defined by Canadian neurological scale.[16] The patients were subjected to 12-lead electrocardiogram (ECG) and echocardiography, blood counts, red blood cell (RBC) morphology, hemoglobin, mean corpuscular volume (MCV), serum protein, serum creatinine and blood urea nitrogen (BUN) estimation.

After 3 months of stroke, fasting blood was drawn by venipuncture and stored at -70°C. It was centrifuged and serum was analyzed for B12,[17] folic acid[17] and homocysteine,[18] using ADVIA Centaur assay (Bayer Corporation). It is a competitive immunoassay using direct chemiluminescent technology. The lower limit of folic acid in normal was 5.38 ng/ml and B12 was 211 pg/ml. The upper limit of Hcy in normal was 13.9 mol/l as per laboratory standard.

MTHFR gene analysis

Deoxyribonucleic acid (DNA) was extracted from peripheral leukocyte with the use of commercially available kit (NUCLEON, Stotlab Ltd., USA). MTHFR C-T677 substitution was identified with the use of restriction enzyme digestion of the polymerase chain reaction (PCR) amplified products.[19] 5'-TGAA GGAGAA GGTGTCTGCG GGA-3' (exonic) and 5'AGG ACGG TGC GGTG AGAGTG-3' (intronic) primers were used. This generates a 198-bp fragment. The C-T substitution creates *HinfI* recognition sequence and digestion of the nutrient product results in 175- and 23- BP fragments. The fragment size was determined by gel electrophoresis.

Statistical analysis

MTHFR gene polymorphism was correlated with various demographic, clinical and laboratory parameters, i.e., folic acid, B12 and homocysteine levels employing Chi-square and Fisher exact tests using SPSS 10 version software. Two-sided '*P*' value was considered significant if it was <0.05.

Results

There were 58 patients with ischemic stroke, whose mean age was 50 (range 4-79) years; among them, 10 were females. The stroke was in middle cerebral arterial (MCA) territory in 45, posterior cerebral arterial (PCA) territory in 10 and involved multiple territories in 3 patients. The infarctions were cortical in 23, subcortical in 26, both cortical and subcortical in 2 and were located in brainstem in 7 patients. Carotid artery stenosis in the range of 50-80% was present in 4 and more than 80% in 9 patients.

MTHFR gene polymorphism (C677T) was noted in 19 (32.8%) patients, 3 patients were homozygous (TT) and 16 were heterozygous (TC) [Figure 1]. Both serum folate and B12 levels were low in 29 (50%) patients and serum homocysteine was elevated in 48 (83%) patients. The distribution of conventional risk factors in stroke patients included hypertension in 28 (48%), diabetes mellitus in 12 (21%), hyperlipidemia in 52 (90%), smoking in 17 (29%) and obesity in 1 (1.7%) patient. Family history of stroke in first-degree relatives was present in 13 (22.4%) patients. Fourteen patients consumed alcohol. In the patients with hyperlipidemia, total cholesterol was raised in 7, triglyceride in 29, VLDL in 25 and low HDL in 38 patients. However, LDL above 160 mg/dl was present in 8 (13.8%) patients only.

The relationship of MTHFR gene polymorphism with folic acid, homocysteine and family history of stroke is shown in Table 1. Comparison of serum folic acid ($x^2 = 0.09$, df = 1, $P = 0.78$), B12 ($x^2 = 0.16$, df = 1, $P = 0.69$), homocysteine ($x^2 = 0.07$, df = 1, $P = 0.79$), dietary habits ($x^2 = 0.31$, df = 1, $P = 0.58$) and the number of risk factors ($x^2 = 0.003$, df = 1, $P = 0.95$) in patients with MTHFR polymorphism and without it revealed no significant difference [Figure 2]. However, serum B12 levels were significantly lower in vegetarians ($x^2 = 9.21$, df = 1, $P = 0.003$), but vegetarianism was not correlated with folic acid ($x^2 = 0.35$, df = 1, $P = 0.77$) and serum homocysteine ($x^2 = 0.89$, df = 1, $P = 0.43$). The distribution of various conventional risk factors in relation to MTHFR polymorphism is summarized in Table 2.

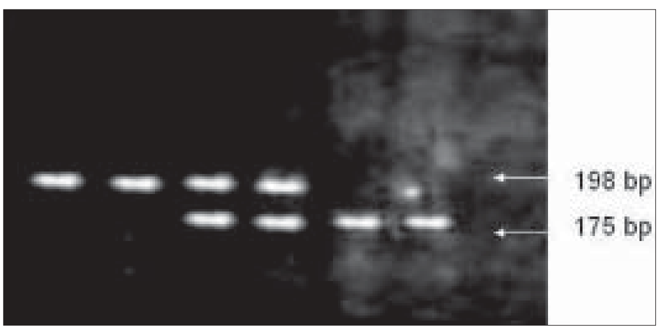

Figure 1: Methylenetetrahydrofolate reductase gene polymorphism in stroke patients: Lane 1 and 2 CC (Wild), Lane 3 and 4 CT (Heterozygous) and Lane 5 and 6 TT (Homozygous)

Table 1: Clinical and biochemical characteristics of the stroke patients with methylenetetrahydrofolate reductase (MTHFR) polymorphism

Age	Sex	Veg/non veg	Location and type of stroke	MTHFR	FA (N≥5.38 ng/L)	Hcy (N≤13.9 µmol/l)	FH stroke
52	M	V	MCA SC	TT	4.93	49.58	-
55	M	NV	MCA cortical	TT	3.59	16.44	+
59	M	V	R. Cerebellar	TT	6.30	45.30	-
57	M	V	PCA occipital	CT	5.73	22.7	-
42	M	NV	MCA SC	CT	5.44	17.30	-
51	M	NV	MCA B/L SC	CT	4.76	20.17	-
56	M	NV	MCA SC	CT	4.24	41.36	+
56	M	V	PCA brainstem	CT	24.00	31.25	-
40	M	NV	PCA cortical	CT	9.02	18.45	-
44	M	NV	MCA cortical	CT	7.51	15.40	-
10	F	V	MCA cortical	CT	4.37	13.69	-
60	F	V	MCA SC	CT	4.87	-	+
4	M	NV	MCA	CT	-	8.8	-
61	F	NV	MCA SC	CT	3.38	35.94	-
32	F	NV	MCA cortical	CT	3.54	17.16	-
79	F	V	MCA SC	CT	12.8	27.73	-
30	M	NV	MCA SC	CT	3.32	18.74	-
61	M	NV	MCA cortical	CT	2.84	-	+
70	M	V	Multiple	CT	7.07	17.26	-

MCA = Middle cerebral artery, PCA = Posterior cerebral artery, SC = Subcortical, M = Male, F = Female, V = Vegetarian, NV = Non-vegetarian, FH = Family history, N = Normal, TT = Homozygous, CT = Heterozygous

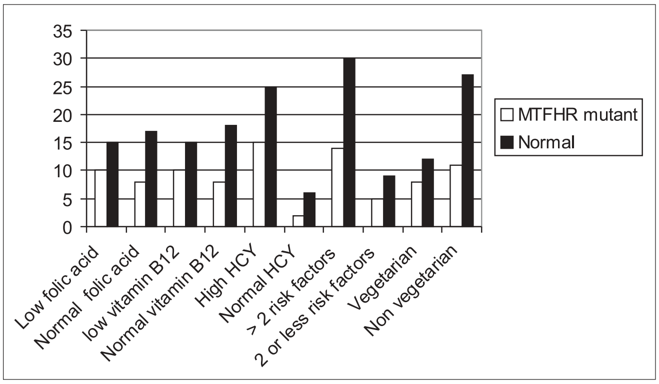

Figure 2: Relationship of various risk factors with methylenetetrahydrofolate reductase gene polymorphism

Table 2: Various risk factors in respect of methylenetetrahydrofolate reductase (MTHFR) gene polymorphism

MTHFR polymorphism	TT (n=3)	TC (n=16)	CC (n=39)	X^2/df/p
Family history of stroke	1	3	9	0.34/2/0.84
Diabetes mellitus	0	4	8	0.96/2/0.62
Hyperlipidemia	3	15	34	0.89/2/0.64
LVH	1	3	9	0.34/2/0.84
BMI>25	1	7	10	1.75/2/0.42
Sedentary	3	8	26	10.13/2/0.001
Tobacco	1	8	18	3.06/2/0.22
Alcohol	1	3	10	0.20/2/0.91
Hypertension	3	7	18	3.42/2/0.18

LVH = Left ventricular hypertrophy, BMI = Body mass index, TT = Homozygous, TC = Heterozygous, CC = Wild type

Discussion

In our study of 58 patients with ischemic stroke, 19 (32.8%) had MTHFR C677T gene polymorphism, 3 were homozygous (TT), 16 heterozygous (TC) and remaining 39 were normal (CC). The frequency of MTHFR gene mutation is quite variable in different geographic and ethnic groups: 38% in French Canadian, 5-15% in Canadian,[19,20] 11% in Japanese and 12.3% in Chinese populations.[7,21] In India, the reported frequency of MTHFR gene polymorphism in arterial stroke is 28-32% patients.[22,23]

In our study, 3 patients were homozygous and had high Hcy in all, low folic acid in 2 and history of stroke in 1. In the heterozygous group, serum Hcy was elevated in 12, reduced folic acid in 8 and family history of stroke in 3 patients. The comparison of different risk factors in TT, TC and CC alleles of MTHFR gene mutation in stroke patients did not reveal any statistically significant difference; however, lower levels of serum folate and higher level of homocysteine in the TT and TC alleles were noted. Significantly lower folate levels in MTHFR homozygotes were reported in patients with late onset vascular disease.[24,25]

Normal MTHFR helps in maintaining a pool of folate and methionine and possibly prevents elevation of homocysteine. In our study, homocysteine levels were highest in TT alleles compared to TC and CC, although the difference was not significant. The requirement of 5-methyltetrahydrofolate as a methyl group donor for conversion of methionine to homocysteine might explain marginal rise of serum homocysteine in homozygous patients. Hyperhomocysteinemia is a risk factor for thrombotic disorder and MTHFR polymorphism has been reported to be a risk factor of myocardial infarction, cerebral stroke and silent brain infarction.[11,19,26,27] The influence of MTHFR genotype on Hcy is greater in individuals with low serum folate and B12 levels.[28]

In our study, insignificantly higher frequency of hyperhomocysteinemia was noted in patients with MTHFR gene polymorphism. Hyperhomocysteinemia is multifactorial; dietary vitamins such as B12 and pyridoxine also have an important influence. In our study, vegetarians had low serum B12 compared to non-vegetarians. Folic acid levels in vegetarians and non-vegetarians were not significantly different. It is possible that in a typical Indian diet, inclusion of high pulses and vegetables may make up for the folate deficiency even in the MTHFR polymorphic patients. Moreover, the levels of serum B12 and pyridoxine may also be influenced by genetic predisposition, which have not been evaluated in the present study.

In our study, MTHFR gene polymorphism was found in one-third of patients with ischemic stroke and was associated with insignificantly higher frequency of hyperhomocysteinemia compared to those without polymorphism. Presence of MTFHR polymorphism may facilitate the effect of other risk factors of stroke. The lack of significance in our study may be due to small sample size; hence further study is needed in larger population.

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