POLYMORPHISM (C677T) IN THE 5,10-METHYLENETETRAHYDROFOLATE REDUCTASE (MTHFR) GENE: A PRELIMINARY STUDY ON NORTH INDIAN MEN

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ABSTRACT

An elevated level of plasma homocysteine, sulfur containing amino acid generated through demethylation of methionine has been widely accepted as a risk factor for cardiovascular disease (CVD). The increase can result from genetic and/or nutrient related disturbances in the remethylation or transsulfuration pathways for homocysteine metabolism. A common mutation (C677T) in the gene encoding for the enzyme 5, 10-methylenetetrahydrofolate reductase (MTHFR) or deficiency of the B vitamins namely folic acid, B₁₂, B₆ can lead to hyperhomocysteinemia.

In the present study, we have investigated the incidence of the (C677T) MTHFR polymorphism in the North Indian males. 141 angiographically proven coronary artery disease (CAD) patients and 55 age and sex matched healthy volunteers were examined for the association between MTHFR gene polymorphism and CAD. The MTHFR genotyping was performed using polymerase chain reaction (PCR) followed by restriction-isotyping with Hinf 1 endonuclease. A trend for higher 'T' allele frequency (0.19) was observed in patients than in controls (0.16). However no significant association was found between C677T mutation and CAD severity. The lack of statistical significance could be due to the small sample size studied. Hence a larger study including various ethnic groups is warranted.

KEY WORDS: Genetic polymorphism, hyperhomocysteinemia, atherosclerosis, cardiovascular disease.

INTRODUCTION

Cardiovascular disease and stroke, occurring as a consequence of atherosclerotic changes are on the rise in the developing countries including India. The genesis of atherosclerosis is multifactorial. Risk factors both modifiable (diabetes, renal insufficiency, nutrition, etc), and non-modifiable (genetics, age, sex, race, etc) influence homocysteine levels and hence the disease (1, 2).

Homocysteine (MW 135.1), sulfur containing natural amino acid formed as a by-product through demethylation of essential

amino acid methionine has been associated with atherosclerotic and thrombotic disorders (3,4,5). It can be remethylated to methionine, or the sulfur can be transferred to serine to form cysteine. Remethylation requires MTHFR enzyme, folate and vitamin B₁₂. The transsulfuration reaction requires cystathione-β synthase (CBS) enzyme and vitamin B₆ (6). The development of the concept that increased homocysteine levels contribute to the pathogenesis of vascular disease began in 1962 (7). In 1969 McCully postulated that vascular complication was the result of increased homocysteine levels (8). Homocysteine is a strong oxidant and its oxidation products (H₂O₂, superoxide anions, reactive O₂ species etc.) are toxic and may directly alter vascular cell function. Surface properties of endothelial cells may thus change from that of anticoagulant to pro-coagulant leading to increased adhesion of platelets and activation of coagulation factors (9).

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5-Methyltetrahydrofolate, the major form of folate in plasma, a carbon donor for the remethylation of homocysteine to methionine, is produced by the action of the enzyme MTHFR (10) (Fig 1). This thereby reduces the homocysteine concentration in the plasma. The MTHFR gene (2.2 kb long) is located on the short arm of chromosome 1. A common mutation C → T at bp 677 resulting in alanine (GCC) → valine (GTC) substitution in the functional MTHFR gene produces an enzyme with partially reduced activity. This autosomal recessive mutation results in a thermolabile MTHFR enzyme with low specificity. Homozygotes (T/T) are reported to have increased homocysteine levels and decreased MTHFR activity (35-50% of the normal) as against C/T heterozygotes (approximately 25% of the normal) (11). The plasma levels of homocysteine are however influenced by both genetic as well as environmental factors (12, 13).

MATERIALS AND METHODS

141 angiographically proven CAD patients and 55 age and sex matched healthy controls were analyzed for the MTHFR polymorphism and serum lipid levels.

The history of tobacco smoking, hypertension, diabetes mellitus and dietary habits were obtained from each subject.

Genomic DNA Isolation

High molecular weight genomic DNA was extracted from packed cells by the sarcosine method of Parzer and Mannhalter (14).

Amplification by PCR

198 bp fragment of MTHFR was amplified by PCR technique in a mixture containing MgCl₂, deoxyribonucleoside-triphosphates (dNTPs), Taq DNA polymerase, human genomic DNA and primers (forward: 5'-TGA AGG AGA AGG TGT CTG CGG GA-3', Reverse: 5'-AGG ACG GTG CGG TGA GAG TG-3'). The samples were cycled at 94°C for 1 minute, 68°C for 1 minute, 72°C for 2 minutes, 94°C for 5 minutes, 68°C for 2 minutes, 72°C for 1 minute, 36 times to 4, finally 72°C for 5 minutes in a thermal cycler [Minicycler (MJ Research)].

After amplification 8 µl of the sample was added to 1 µl Hinf-1 (10,000 U/ml) and NE buffer (500 mM NaCl, 10 mM Tris, pH 7.9, 100 mM MgCl₂

and 10 mM dithiothreitol) and incubated for 1 hour at 37°C. After digestion the product was separated on 1.5% agarose gel at 5 V/cm for 90 min. DNA bands were visualized with 0.5 µg/ml ethidium bromide and UV transillumination. Genotypes were identified by characteristic restriction fragment length patterns (15,16,17).

Analysis of serum lipids

3 ml of overnight fasting (10-12 hr) blood sample was collected in plain tubes for serum lipid analysis. The levels were analyzed on CX5 Beckman autoanalyzer using fully enzymatic, commercially available diagnostic kits (Randox, U.K.).

Statistical Analysis

Allele frequencies were calculated by allele counting. Hardy-Weinberg equilibrium and the differences in genotypic frequencies were examined by using Chi-square test. The significance of difference of continuous variables was tested by 't' test. All values were calculated as mean (mg/dl) ± standard deviation (SD). The clinical parameters were compared by applying 'Proportion test'.

RESULTS AND DISCUSSION

The study subjects were in Hardy-Weinberg equilibrium for the C677T mutation. The overall frequencies for 'C' and 'T' alleles were 0.816 and 0.184, respectively. The frequency of the three genotypes among the studied population was 70% (C/C, homozygous normal), 23.5% (C/T, heterozygous), and 6.6% (T/T, homozygous mutant), respectively. The 'T' allele (mutated) frequency was 0.192 for the patient population and 0.164 for the controls (Table 1). On comparing various genotypes for lipid levels no significant differences were observed for the total population or patient and control subjects (Table 2). No direct association was found between MTHFR polymorphism and CAD severity (Table 3).

The relation between (C677T) MTHFR polymorphism and CAD is not yet clearly established. There are reports in favour (16,17,18) and contradicting (6,19) this association. In the present study the MTHFR polymorphism does not seem to be directly associated with an increased risk for CAD although patients showed increased trend for 'T' allele frequency than controls.

Several reports have indicated that an interaction between the genetic and environmental factors (nutrition, antioxidant status, stress etc.) plays a major role in causing CAD (2,20). The present study showed a significantly higher incidence of diabetes ($p < 0.001$, sugar fasting > 100 mg/dl) and hypertriglyceridemia ($p < 0.001$, TG's > 170 mg/dl) in the patient group. From the blood pressure records 60/196 subjects were found to be hypertensive. 52/60 (86.66%) of these were patients and 8/60 (13.33%) were controls (Table 4). However they were maintaining their blood pressure within normal limits by oral antihypertensive drugs.

The 'T' allele frequency of 0.18 (Table I) among the studied subjects was more than that seen in Blacks (0.1) but less than that seen in Caucasians (0.3), White Americans (0.35) and

Japanese (0.33) (21, 22, 23) (Table 5).

The absence of plasma homocysteine levels and the small sample size, the major limitations of the present study, may be responsible for the statistically non-significant association between the (C677T) MTHFR mutation and CAD. Hence it calls for a study with larger sample size taking into consideration the plasma homocysteine levels, environmental factors and ethnicity.

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Table I
MTHFR [C677T] Polymorphism- Genotype and Allele Frequency in CAD Patients and Controls

S.No.	Genotype / Allele	Total n=196	Patients n=141	Control n=55
Genotypes				
1.	CC	137 (69.9)	98 (69.5)	39 (70.9)
2.	CT	46 (23.47)	32 (22.7)	14 (25.5)
3.	TT	13 (6.6)	11 (7.8)	2 (3.6)
Alleles				
1.	C	0.816	0.808	0.836
2.	T	0.184	0.192	0.164

The values in parenthesis are percentages

Table 2

Lipid and Lipoprotein Profile vs. MTHFR Genotypes (Total sample, Patients, Controls)

Total (n=196)	TC	TG	LDL	HDL	Ratio	VLDL
CC (137)	201±55 (102)	177±113 (102)	114±40 (102)	41.2±6 (102)	2.7±0.96 (102)	42±12 (102)
CT (46)	202±77 (32)	154±57 (11)	107±40 (32)	43.1±11 (32)	2.6±0.82 (32)	39±10 (32)
TT (13)	210±51 (11)	159±56 (11)	120±39 (11)	43.7±3 (24)	2.7±0.7 (11)	46±11 (11)
Patients (n=141)						
CC (98)	204±58 (76)	177±10.7 (76)	116±40 (76)	41±5.6 (76)	2.8±1.0 (76)	42±12 (76)
CT (32)	196±46 (24)	154±67 (24)	114±39 (24)	44.3±12 (24)	2.7±0.9 (24)	40±10 (24)
TT (11)	202±54 (9)	148±57 (9)	116±41 (9)	43.4±3.5 (9)	2.6±0.7 (9)	42±10 (9)
Controls (n=55)						
CC (39)	193±45 (26)	177±132 (26)	108±42 (26)	41.9±7 (26)	2.6±0.87 (26)	40±12 (26)
CT (14)	221±13.76 (8)	152±61 (8)	85±37 (8)	39.5±4 (8)	2.3±0.37 (8)	38±10 (8)
TT (2)	245±0 (2)	209±0 (2)	140±0 (2)	45±0 (2)	3.1±0 (2)	60±0 (2)

Values : Mean (mg/dl) ± SD

(n) : Number of subjects

Table 3
MTHFR Genotypes Vs CAD Severity

Genotype Groups	Coronary Score (n=39)		
	SVD	DVD	TVD
CC (n=29)	7 (17.94)* [24.14%]**	7 (17.94)* [24.14%]**	15 (38.50)* [51.72%]**
CT (n=10)	4 (10.25)* [40%]**	1 (2.56)* [10%]**	5 (12.82)* [50%]**
TT	—	—	—

n: no. of subjects
 SVD: Single vessel disease, DVD: Double vessel disease &
 TVD: Triple vessel disease
 (%) * Based on total number
 ** Based on group number

Table 4
Clinical Characteristics of the Study Subjects

Parameter	Total (n=196)	Patient (n=141) (%)	Control (n=55) (%)	P -value
Age, in years	51 ± 10.2	52 ± 10	50 ± 11	NS
Hyper tension (BP > 140/90mmHg)	60	52 (86.66)	8 (13.33)	< 0.001
Sugar fasting (>100mg/dl)	17	15 (88.2)	2 (11.76)	< 0.001
TG (> 170mg/dl)	74	57 (77.02)	17 (22.97)	< 0.001
HDL (< 35mg/dl)	15	9 (60)	6 (40)	NS
TC (>200mg/dl)	55	42 (76.4)	13 (23.64)	NS
Tobacco smoking	18	3 (22.22)	14 (77.77)	NS

NS: Not significant

Table 5

Allele Frequency of (C677T) Mutation Among Various Racial Groups

S.No.	Racial Group	'T' Allele Frequency	No. of subjects studied	Reference
1.	Caucasians	0.30	101	McAndrew 1996
2.	Africans - Americans	0.10	102	McAndrew 1996
3.	White Americans	0.35	151	Stevenson et al 1997
4.	Japanese	0.33	1140	Morita <i>et al</i> 1997

HOMOCYSTEINE METABOLISM

Compiled from Langman *et al* (1999) & Ueland and Refsum (1989)

FIGURE I

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