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Research article

METHYLENETETRAHYDROFOLATE REDUCTASE C677T GENE POLYMORPHISM AND HOMOCYSTEINE LEVELS ARE RISK FACTORS FOR MYOCARDIAL INFARCTION.

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ABSTRACT: Increased plasma total homocysteine (tHcy) levels shown to be a risk factor for coronary artery disease (CAD). The common methylenetetrahydrofolate reductase C677T (MTHFR C677T) polymorphism has been reported to be a strong predictor of mild hyperhomocysteinaemia (HHcy). We assessed whether this mutation was associated with increased risk of myocardial infarction (MI) and plasma levels of tHcy. The study group consisted of 210 angiographically proven MI patients, and 202 age and sex matched healthy individuals as controls. MTHFR (C677T) gene polymorphism was detected based on the polymerase chain reaction and restriction digestion with HinfI. Total homocysteine plasma concentration was measured using immunoassay. T allele frequency was not found to be significantly higher in patients than in the control group: T *vs. C* was $\chi 2$=0.19, OR 1.0, CI 95% 0.8–1.4, p=0.6; and TT *vs. CC* was $\chi 2$=0.24, OR 1.2, CI 95% 0.6–2.3, p=0.6.We found significantly elevated levels of mean homocysteine in the patient group when compared to the control group (p =0.00). Our findings showed that MTHFR C677T polymorphism is not a risk factor for myocardial infarction in South Indian population and higher levels of homocysteine in patients indicated that the severity of the disease is independent of homocystein levels.

Key words: CAD, MI, homocysteine, MTHFR polymorphism.

INTRODUCTION

Myocardial infarction (MI) is the death of heart muscle from the sudden blockage of a coronary artery by a blood clot. Coronary arteries are blood vessels that supply the heart muscle with blood and oxygen. Myocardial infarction is a leading cause of mortality and disability of adults in urban and rural India, and occurs at a younger age than in western populations (world health report 2002. Geneva: WHO, 2005). For example, about 30% of all estimated MI mortality occurs at ages 45–59 years in India, versus only 14% in high income countries. Male smoking of cigarettes or, more commonly, bidis—small unfiltered cigarettes hand rolled in a temburni leaf—is well established in India and has been recently documented as a major cause of death among Indian men (Gajalakshmi et al., 2003; Gupta and Mehta., 2000), with much of this resulting from vascular and respiratory disease. Smoking of cigarettes and bidis has been associated with a twofold risk for fatal MI in India (Gupta and Mehta. 2000). The classical symptoms of MI are shortness of breath, anxiety, chest pain typically radiating to the left arm or left side of the neck, vomiting and palpitations. Important risk factors are previous history of vascular disease such as atherosclerosis, angina -heart attack or stroke and age – especially men over 40 and women over 50 years (Braunwald, 1997).

One of the candidate genes for the development of atherosclerosis regardless of localization is methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20). MTHFR is a regulatory enzyme of the homocysteine (Hcy) metabolism, and is also necessary in the metabolism of tetrahydrofolate, as well as in the synthesis of purine, DNA, and RNA. Elevated plasma total homocysteine (tHcy) concentration is now widely accepted as a major independent risk factor for cerebrovascular, peripheral vascular disease and may explain, at least in part, the occurrence of coronary artery disease (CAD) in patients who do not have dyslipidemia, hypertension, and other conventional risk factors (Geisel et al., 2001; Boushey et al., 1995).

Hcy is a sulphur-containing amino acid and is formed by demethylation of the essential amino acid methionine. It can be either degraded by transsulfuration, involving the vitamin B6-dependant enzyme cystathionine *β*-synthase, or remethylated to methionine, involving the cobalamine (vitamin B12- dependant enzyme) methionine synthase. Elevations of tHcy might result from nutritional deficiencies, including folate, pyridoxal phosphate (vitamin B6), and methylcobalamin (vitamin B12), or from genetically determined abnormalities of Hcy metabolism (e.g., MTHFR), or a combination of these (Van der Put et al., 1995; Kang et al., 1996). MTHFR is an enzyme that reduces 5, 10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, the main circulating form of folate and the methyl donor for the remethylation of Hcy to methionine. The MTHFR gene has been mapped to the chromosomal region 1p36.3. A thermolabile form of MTHFR has been identified (Kang et al.,1991; Kang et al.,1993) and found to be caused by a missense mutation in its encoding gene, with the cytidine residue at nucleotide position 677 being replaced by thymidine (MTHFR C677T), resulting in the substitution of valine for alanine in the enzyme. The homozygous 677TT genotype of this mutation has been found to specify a variant enzyme with reduced activity and to be associated with elevated tHcy levels, particularly in the setting of low folate levels, as compared to the wild-type (677CC) and heterozygous (677CT) genotypes. Also, data have shown that the frequency of the mutation varies among different populations (Engbersen et al., 1995; Jacques et al., 1996). These considerations have raised the possibility that the relatively common C677T mutation in MTHFR might be an important genetic risk factor for cardiovascular disease through its effects on homocysteine metabolism (Frosst et al., 1995; Kluijtmans et al., 1996).

Several studies (Wald et al., 2002; Ford et al., 2002; Zee et al., 2007) found an association of raised homocysteine levels with CHD. However, this association was weaker in prospective studies (Ford et al., 2002) and randomized controlled trials of homocysteine-lowering therapy failed to prove a causal relationship between homocysteine and cardiovascular risk (Kaul et al., 2006). There are only very limited studies regarding the role of hyperhomocysteinemia in the development of premature MI (Lewis et al., 2005; Gallagher et al., 1996; Schmitz et al., 1996). Plasma homocysteine levels are modulated by genetic and nutritional factors among which the MTHFR gene has attracted special attention. In particular, the 677C->T mutation in the gene coding for MTHFR, characterized by the replacement of cytosine with thymidine, leads to 50% reduced MTHFR activity and is associated with moderately elevated homocysteine levels. There are conflicting data regarding the association between the MTHFR 677C->T polymorphism and the risk of CHD (Zee et al., 2007, Ma et al., 1996; Jee et al., 2000; 22–24). A meta-analysis (Klerk et al.,2002) of case-control observational studies reported a higher risk of CHD of individuals with the MTHFR 677TT genotype, while another met analysis later (Lewis et al.,2005) did not support this association in Europe, North America or Australia. Assuming that factors associated with increased thrombotic tendency, such as homocysteine, are more likely to have a greater impact in patients with premature MI, we explored the role of homocysteine and its main genetic modulator MTHFR 677C->T polymorphism in myocardial infarction patients .

The aim of this study was to investigate the frequency of MTHFR C677T mutation and its association to myocardial infarction and to fasting plasma tHcy concentrations in south Indian population.

MATERIALS AND METHODS

Subjects

The study was carried out on 202 MI patients (male: female = 181: 21) admitted to Osmania General Hospital, ICCU, Cardiology Division, Hyderabad, India. The patients were 54- 68years of age. All patients were admitted with acute coronary syndrome and underwent coronary angiography. On the basis of typical ECG changes, elevated cardiac markers and clinical history, the diagnosis was confirmed as MI by the cardiologists. All these patients were without previous history of coronary artery disease. The study was approved by the Institutional Ethical Committee and written informed consent was obtained from all the subjects. Blood samples were collected from patients after 12 to 14 hours of fasting. Simultaneously, blood samples were collected from 210 healthy, age- and sex-matched (male: female = 184: 26) controls (blood donors from the same hospital) aged between 56 and 67 years and all controls were non-hypertensive.

Information has been collected using a questionnaire on height, weight, body mass index, cigarette smoking, alcohol consumption and hypertension. Exercise was defined by the individual doing one hour daily in the form of brisk walking or gym activity. Hypertension was defined according to JNC-VII guidelines. In keeping with these, hypertension was defined as a systolic blood pressure> 140 mmHg and/or a diastolic blood pressure > 90 mmHg based on the average of two blood pressure measurements, or a patient's self reported history of hypertension. Smokers were defined as those reporting daily smoking. Ex-smokers and occasional smokers were classified as non-smokers. Since patients were found to drink alcohol in different forms and many were reluctant to admit the exact amount consumed, we defined alcohol usage as consumption of at least three alcoholic drinks in a week.

Blood sampling and biochemical analysis:

Venous blood (5 ml) was collected in a plain EDTA test tube and serum was separated. Total cholesterol (TCL), triglycerides (TGL), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) were estimated using a semi-automatic analyser (ERBA, CHEM-7, and Transasia Biomedicals, India) using commercial kits (ERBA).

Plasma concentration of homocysteine was determined by Axis Shield diagnostic kit. Plasma samples for homocysteine were placed on ice after collection in order to minimize increases in homocysteine concentrations from synthesis by red cells.

DNA preparation and genotyping

Genomic DNA was extracted from EDTA peripheral blood leukocytes by the standard phenol-chloroform method (Poncz *et al.*, 1982). The region in exon 4 of the MTHFR gene containing the C677T polymorphism was amplified using the following primers: forward primer 5'TGA AGG AGA AGG TGT CTG CGG GA3', Reverse 5'AGG ACG GTG CGG TGA GAG TG3') as described by Frost *et al.*(1995). The mixture was initially denatured at 94 °C for 4 min, followed by 30 cycles with 30 s at 94 °C, 45 s at 62 °C, 45 s at 72 °C and a 12 min final extension at 72 °C. The amplified PCR products were checked on 2% of agarose gels followed by staining with 1µg/mL of ethidium bromide. . The amplified PCR products of 198 bp were digested with *Hinf*I (NEB UK) restriction enzyme according to manufacturer's specifications. The restriction digestion products were separated on a 3%agarose gel and visualized by ethidium bromide staining. The presence of 198 bp is homozygous CC genotype and 198 bp and 173 bp are heterozygous CT genotype and 173 bp bands represented homozygous mutant TT genotype.

Statistical analysis

Genotype and allele frequencies in MI Patients and control groups were compared by Chi square testing. The characteristics of patients and controls were evaluated by comparing biochemical findings using the Student t-test. Allele frequencies for the MTHFR polymorphic site were estimated by gene counting. Analysis of variance (ANOVA) was performed for lipid profiles between controls and MI patients using SPSS v.11.5 (SPSS Inc., Chicago, USA) statistical analysis software. A two-tailed p value of $p < 0.05$ was considered statistically significant.

RESULTS

The present study group include 202 MI patients and 210 controls. The demographic and clinical characteristics are summarized in table 1. The mean age of patients and controls was 63.2 ± 3.96 and 61.3 ± 4.6 years, respectively. The patient group had a prevalence of higher BMI, smokers, hypertensives and alcohol consumpers compared with controls. The comparison of lipid profiles between patients and controls is presented in table 2. The value of HDL-cholesterol was significantly lower in MI patients compared to controls. Although serum triglyceride, total cholesterol, LDL-cholesterol, VLDL-cholesterol and homocysteine levels were found to be significantly higher among pateints ($p = 0.0001$).

Allele and genotype frequencies of patients and controls are presented in table 4. The distribution of genotypes between patients and controls is presented in table 5.We found the prevalence of the CC genotype to be 49% in the control against 47.5% in the patient group, whereas the TC genotype was found to be 41.9% in the control and 42.1% in the patient group, TT genotype were 9.1% and 10.4% respectively in controls and patients. Neither T allele nor the C allele was not significantly associated with MI: T *vs. C* was $\chi 2=0.19$, OR 1.0, CI 95% 0.8–1.4, p=0.6; and TT *vs. CC* was $\chi 2=0.24$, OR 1.2, CI 95% 0.6–2.3, p=0.6. Significantly high levels of homocysteine were observed in the patient group as compared to the control, irrespective of the genotypes involved (CC, TC and TT) (Table 6).

Table 1: Demographic characteristics of the South Indian study population

Characteristics	Control group	MI patient group
Number of subjects	210	202
Age (mean years ± SD)	61.3 ± 4.6	63.2 ± 3.96
Sex ratio (male: female)	184: 26	181: 21
Body mass index (mean kg/m^2 ± SD)	22.4 ± 2.5	28.1 ± 1.7
Smokers	11(5.2)	65 (32.1)
Alcoholic	17 (8.0))	75 (37.1)
Exercise	117 (55.7)	16 (32.3)
Hypertension	0	66 (29.5)

Key: MI = myocardial infarction; SD = standard deviation.

Table 2: Comparison of lipid profiles between controls and MI patients

Parameters	Controls (mean mg/ml ±SD)	Patients (mean mg/ml ± SD)	t value	p value
T.CHOL	168.3 ± 24.0	239.8 ± 29.0	27.3	0.0001
TGL	137.8 ± 1.5	177.3 ± 11.6	48.9	0.0001
LDL	98.5 ± 11.9	173.6 ± 28.4	35.2	0.0001
HDL	42.3 ± 5.3	33.8 ± 3.6	18.3	0.0001
VLDL	27.3 ± 1.3	36.6 ± 1.9	58.1	0.0001

Key: Values represent mean ± standard deviation. HDL = high-density lipoprotein; LDL = low-density lipoprotein; MI = myocardial infarction; TCL = total cholesterol; TGL = triglycerides; VLDL = very-low-density lipoprotein.

Table 3. Distribution of Methylenetetrahydrofolate (MTHFR) genotypes and allelic frequencies of the total Study group.

Study group	MTHFR genotypes			Total	Allelic frequencies		Total
	CC	TC	TT		C	T	
Controls (%)	103(49)	88(41.9)	19(9.1)	210	0.7	0.3	420
Patients (%)	96(47.5)	85(42.1)	21(10.4)	202	0.69	0.31	404

Table 4. Comparison of genotypic and allelic frequencies of MTHFR gene in MI patients and controls.

Genotypes & Alleles (Patients vs. Controls)	Chi-square ($\chi 2$)	Odds ratio	CI 95%		
			L.limit	U.limit	p value
TT vs CC	0.24	1.2	0.6	2.3	0.6
TT vs. TC	0.14	1.1	0.5	2.2	0.7
TT vs. CC+ TC	0.21	1.1	0.6	2.2	0.6
T vs. C	0.19	1.0	0.8	1.4	0.6

Table 5. Comparison of inflammatory markers in patient and control using Student's t- test.

Parameters	Patients (Mean ± SD)	Controls (Mean ±SD)	t value	p value
Hs- CRP(mg/L)	6.2 ± 1.1	0.82 ± 0.19	27.3	0.0001
Homocystein (µmol/L)	14.8 ± 2.1	7.2 ± 1.32	48.9	0.0001

Table 6. Comparison of Homocystein levels (Mean ± SD) µ.mol/L in controls and Patients according to MTHFR genotypes

Group	Homocystein levels & Genotypes			F value	p value
	CC	TC	TT		
Controls	5.8 ± 1.0	6.7 ± 1.2	9.2 ± 1.7	55.0	0.011
Patients	11.0 ± 1.8	15.2 ± 2.1	18.2 ± 2.4	120.7	0.001

DISCUSSION

Myocardial infarction (MI) is a Complex disease caused by interaction of a number of genetic and environmental factors (Figen and Senk, 2005). Indians are more prone to MI at younger age compared to other populations. Approximately one fourth of all myocardial infarctions are silent without chest pain or other symptoms (Ramraj and Alper, 2008).

A thermolabile form of MTHFR has been identified (Kang et al., 1993) and found to be caused by a missense mutation in its encoding gene, with the cytidine residue at nucleotide position 677 being replaced by thymidine (MTHFR C677T, rs no.1801133), resulting in the substitution of valine for alanine in the enzyme.The implication of the MTHFR gene in CAD pathogenesis has been extensively studied in several ethnic groups. In the present study we found no significant association of T allele in MI patients compared to controls. Other epidemiological studies that estimated the risk of CAD associated with the T allele showed conflicting results. Notwithstanding, Kluijtmans *et al.* (1996) on summarizing the results from 8 studies, encountered no significant difference for the T allele between patients and controls. On the other hand, Kerkeni et al. (2006) and Alam et al. (2008) found this substitution to be a significant risk factor.

The present study investigated the role of this polymorphism in the development of MI. The TT genotype and T allele frequencies were not found to be significantly high in patients ($p < 0.6$) when compared to controls. The results of the study are in accordance with previous studies which support that insignificant difference for the T allele between MI subjects and the control group (Anderson *et al.*, 1997; Hsu *et al.,* 2001).

Goracy et al., (1999) stated that C677T polymorphism of MTHFR gene is not a risk factor for myocardial infarction in polish population. Pint´o et al (1997) and Sibani et al (2000) reported insignificant difference between C677T polymorphism of MTHFR gene and coronary artery disease which is also a risk factor for Myocardial infarction (MI). On the other hand Tripathi et al, (2010) reported that the T allele significantly associated with myocardial infarction ($p = 0.016$, OR1.93, 95% CI1.08-3.44).

In a health report released by US physicians, the frequency of the MTHFR genotypes was similar between patients and control subjects (Ma et al.,1996) .The discrepancy between the results may be due to differences in nutritional intake of cofactors required for the MTHFR pathway, such as vitamin B12 or folate, or other ethnic differences (e.g., weight, BMI).

In the present study we noted that the severity of the disease is independent of homocysteine levels. The results of the study are in accordance with previous studies which support that Wang *et al.* (1999) found no correlation between the level of homocysteine and severity of the disease, whereas Rassoul *et al.* (2008), on the contrary, discovered a positive association.

The present study included 202 patients and 210 controls and our selection criteria were specific for patients with a first MI without previous history of vascular disease. However, patients with cancer, neurological and all kidney diseases were excluded from the study. The classical risk factors and lipid profiles were significantly more unfavourable in patients in comparison with controls, which is in consistent with previous studies (Rallidis et al., 2008). Infact; Studies on the MTHFRC677T polymorphism are very limited in India. Moreover, there are no reports on the MTHFRC677T polymorphism in MI from an Indian population. To the best of our knowledge, this is the first study to investigate the association of this polymorphism in MI in a South Indian population. Since these are preliminary data with a relatively small number of subjects, it warrants further study in a larger cohort.

CONCLUSION

Our data suggest that the MTHFR gene T allele is not a risk factor for MI patients. This requires to be confirmed in a larger cohort.

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