Original Research Article

A study of C677T polymorphism of Methylenetetrahydrofolate reductase (MTHFR) gene and it's susceptibility in Coronary artery disease

ABSTRACT:

Background: MTHFR has been implicated in several diseases like breast cancer and leukemia, where the deficiency of ferric acid has been shown to increase the disease progression as it is a highly polymorphic gene. However there are very few reports of its role in CAD. Hence our aim was to genotype the CAD patients and healthy controls with a particular polymorphism at the C677T region of the gene.

Methods: After determining the biochemical and clinical parameters, we tried to correlate these parameters with the MTHFR C667T genotypes which were done by PCR-RFLP. Interesting results were observed- with these parameters, we also found a significant association of the polymorphism to disease progression.

Results: The presence of the *MTHFR C*677T was significantly associated with CAD compared to healthy controls. The percentage was greater with other common risk factors such as age, sex, diabetes mellitus, hypertension, smoking in CAD patients than in the normal subjects.

Conclusion: This study investigated the role of genetic polymorphism of methylenetetrahydrofolate reductase (MTHFR) as a potential genetic marker associated with coronary artery disease.

Key words: Coronary artery disease, genotypes, methylenetetrahydrofolate reductase, single nucleotide polymorphism, gene frequency, demographs.

Introduction

Cardiovascular disease remains the major cause of morbidity and death in developed countries. Coronary artery disease (CAD) due to atherosclerosis is associated with increased mortality and morbidity. Various risk factors have been found to be associated with the development of CAD. The role of diabetes mellitus, smoking and hyperlipidaemia as risk factors for CAD is well established. The major classic risk factors like diabetes, hypertension, and the like, and non-modifiable risk factors such as age, sex, and family history cannot fully explain why some

individuals are prone to coronary artery disease and others are not. Pathological and epidemiological studies suggest that only about one half to two-thirds of the variation in anatomic extent of atherosclerosis and risk for atherosclerotic vascular disease can be explained by the classic risk factors. Therefore, many emerging risk factors have been investigated.

Coronary artery disease has a complex etiology generated by combined effects of both, genetic and environmental factors (1). The polymorphic genes, encoding products involved in atherosclerotic process, predispose individuals to a greater or lower extent to CAD. However, traditional risk factors, such as cigarette smoking, hypercholesterolemia, hypertension and overweight, interacting with the genetic risk factors (in cumulative or synergistic ways), may increase or not the risk of the disease. It is known that interactions between genetic and environmental factors are very important in subjects with a high-risk genetic profile (2). Genetic factors have greater contribution to the development of CAD at younger age (3).

It has been predicted that cardiovascular diseases will increase rapidly in India, and this country will be the host to more than half the cases of heart disease in the world within the next 15 years (4). Global Burden of Disease Study estimated that India faces the greatest burden due to coronary artery disease (CAD) (5).

Genetic polymorphism of methylenetetrahydrofolate reductase (MTHFR) has been the subject of increasing attention as a potential genetic marker associated with atherosclerosis (6, 7). The human 5,10-MTHFR gene is located at the end of the short arm of chromosome 1 (1p36.3), and the total length of the gene cDNA (complementary DNA) is 2.2 kb. MTHFR plays a crucial role in the metabolism of folates and irreversibly converts 5, 10-methylenetetrahydrofolate to 5 methyltetrahydrofolate. 5-methyltetrahydrofolate is the predominant circulatory form of folates and donates a methyl group for remethylation of homocysteine to methionine. Consecutively, methionine is metabolized to yield S-adenosylmethionine (SAM), the main methyl donor for important methylation reactions that are required for DNA repair. Impaired MTHFR activity may lead to homocysteine accumulation in plasma, and this condition may contribute towards progressive atherosclerosis through several mechanisms, including arterial endothelial function impairment, oxidative stress induction and promotion of inflammation and thrombosis. (8, 9)

A common thermolabile mutation in the MTHFR gene, consisting of a cytosine (C) to thymidine (T) subtitution at nucleotide position 677, leads to the exchange of a highly conserved alanine to valine (677C→T, alanine →valine), resulting in reduced activity of this enzyme, affecting folate

distribution. The MTHFR 677 TT genotype led to elevated homocysteine levels and DNA hypomethylation in folate-depleted subjects. (10,11). Low serum folate levels are known to cause several cancers (12,13) by influencing DNA methylation (14,15).

MTHFR is one of the metabolic pathways for CAD and is a key regulatory gene of the remethylation pathway. The C677T polymorphism (rs1801133) in MTHFR has been implicated in vascular disease (16). The T677 allele is distributed widely among populations showing a high heterogeneity (17). Its frequency varies in different geographical regions and ethnic groups. A number of studies have reported the frequencies of C677T in European and American Caucasian populations.

The objective of this study was also to investigate whether there is any difference in allele prevalence in MTHFR C677T polymorphism between subjects with CAD and subjects without CAD, as evaluated by means of coronary catheterization.

Material and Methods

This study was approved by the Institutional Ethics Committee of Mahavir Hospiatal and research centre Hyderabad India. The cases (n=100) were consecutively selected from Cardiology OPD and Ward/CCU of Mahavir Hospital and all patients were angiography confirmed CAD patients. Controls (n=110) were age and sex-matched healthy individuals. Details of type of cardiac problems along with angiography findings, blood pressure, history of smoking, hypertension, diabetes, *etc*. were also recorded. The levels of lipid parameters like total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C.) LDL-C. and triglycerol (TG) were measured.

DNA extraction and MTHFR C677T polymorphism analysis.

Blood samples were drawn from the cases and controls and collected in tubes containing EDTA. The DNA samples were extracted from whole blood by a salting-out procedure. The DNA samples were analyzed for the C677T missense mutation using a polymerase chain reaction with locus-specific primers, followed by subsequent analysis of a restriction fragment length polymorphism created by the mutation, as described below.

PCR Conditions for *MTHFR 677*: Initial- denaturation-94°C for 8 Min, Denaturation-94°C for 1 Min, Annealing-63°C for 1 Min, Extension -72°C for 1 Min, Final extension-72°C

for7Min4°C, Forever Repeated for 40 cycles. The primer sequences were 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and 5′- AGGACGGTGCGTGAGAGTG-3′.

For MTHFR 677, the PCR yielded a 198 bp product, which on digestion with *Hinf* I produced 175 and 23 bp fragments for TT condition (homozygous polymorphic) and a 198,175 and 23 bp fragments for CT condition (heterozygous polymorphic). An undigested product length of 198 bp was retained by the wild types.

The 677C→T substitution creates a *Hinf*I recognition sequence, which digests the initial polymerase chain recognition product of 198 base pairs (bp) into 175- and 23-bp fragments. The presence of the mutation was determined by digestion of the initial polymerase chain reaction product with HinfI at 37°C for 24 h. The digested DNAs were separated on 3% agarose gel in 1x Tris borate EDTA buffer, followed by staining with ethidium bromide solution and the MTHFR C677T genotypes were typed by visualization under ultraviolet light.

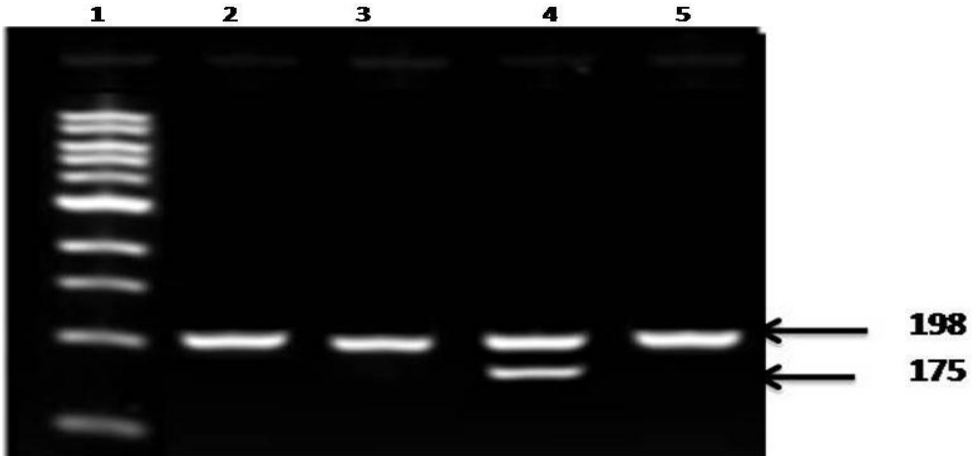

FIG 1: MTHFR PCR products after restriction digestion with Hinf1 on 3% agarose gel. Lane 1 = 100bp DNA Ladder, Lane 2,3,5 = CC genotypes, Lane 4 = CT genotypes

Statistical analyses.

The data are presented as Mean±SD. Statistical analyses, using SPSS version 10.0, included the x^2 test for genotype and allele frequency comparison. Odds ratios and 95% confidence intervals were calculated as a measure of the relationships between CAD and MTHFR genotypes. The clinical characteristics were compared by the Student's *t*-test and C677T allele frequencies were estimated by gene counting methods. A *p*-value of less than 0.05 was regarded as being statistically significant.

Results

Demographic results

The mean age of cases (56males, 44 females) was 56.4± 2.6. The maximum number of cases n=45 (45 %) was seen in age group 50-60 yr. Of the 100 cases 60(60%) were smokers, 40 (40%) non smokers, 25(25 %) were alcoholics, and a majority of 75 (75 %) were non-vegetarians. The majority in the study group were found not to consume fruits and salads. 30 (30%) hypertension, 25 (25%) diabetes mellitus and 25 (25%) had family history of diabetes mellitus. Majority of the cases (50, 50 %) had single vessel disease, 30 (30%) had double vessel and 20 (20 %) had triple vessel disease.

The mean age of controls (60 males, 50 females) was 55.4± 2.8. Among the 110 controls, 38 (34.54%) were smokers, 72 (65.45%) were non smokers, 25 (22.72%) hypertension, 11 (10%) diabetes mellitus, 15 (13.63) family history of diabetes mellitus.

Table 1: Demographic details involved in this study.

	Subjects with CAD (n=100)	**Subjects without CAD (n=110)**	**p-value**
Age (mean±SD)	56.4±2.6	55.4± 2.8	0.008
Males	56 (56%)	60 (54.54%)	0.83
Females	44 (44%)	50 (45.45%)	0.83
Smoking	60 (60%)	38 (34.54%)	0.004
Hypertension	30 (30%)	25 (22.72%)	0.23
Diabetes mellitus	25 (25%)	11 (10%)	0.006
Family history	25 (25%)	15 (13.63%)	0.04

Clinical parameters

General characteristics and levels of biochemical parameters like TC, HDL-, LDL-cholesterol, VLDL and TG of the study groups are shown in Table 2. Cases had significantly higher levels of TC and LDL cholesterol compared to control group.

The mean ± SD of Cholesterol was found to be 200 ± 47.1 in the patients and 148 ± 34.7 in the controls, the LDL was found to be 125 ± 39 in the patients and 111 ± 33 in the controls, HDL was 40.11 ± 14.12 in the patients and 42.50 ± 15.42 in the controls, and VLDL was 26.52 ± 13 in patients and 22.97 ± 11.21 in normal controls of our study. Triglycerides were found to be 153.18 ± 68.02 in the patients and 140.25 ± 68.15 in the controls. The difference was significant for cholesterol and LDL when cases of CAD were compared with controls.

Table 2: Shows the mean levels of lipid profile in the cases of CAD and controls.

	Subjects with CAD (n=100)	Subjects without CAD (n=110)	P-Value
Mean total cholesterol (mg/dl)	200 ± 47.1	148 ± 34.7	< 0.001
Mean LDL cholesterol (mg/dl)	125 ± 39	111 ± 33	< 0.005
Mean HDL cholesterol (mg/dl)	40.11 ± 14.12	42.50 ± 15.42	NS
Mean VLDL cholesterol (mg/dl)	26.52 ± 13.22	22.97 ± 11.21	NS
Mean TG	153.18 ± 68.02	140.25 ± 62.15	NS

Genotype results:

DNA extracted from blood samples was checked for quality and quantity on 1% agarose gel. After checking for the purity of DNA, PCR was carried out in 20 µl reactions.PCR for the *MTHFR* gene gave a 198-bp fragment following enzymatic digestion of the PCR product using *Hinf* I restriction enzyme and incubated at 37°C for 24 h. Then it was electrophoresed in a 2% agarose gel at 90V for 30mins, and visualized in gel documentation. Three results were obtained for *MTHFR* 677, the PCR yielded a 198 bp product, which on digestion with *Hinf* I produced a 175 and 23 bp fragments for TT condition (homozygous polymorphic) and a 198,175 and 23 bp

fragments for CT condition (heterozygous polymorphic). An undigested product length of 198 bp was retained by the wild types.

A total of 100 cases were genotyped, in MTHFR 677, CC genotype was found in 69 (69%), CT genotype was found in 24(24%) and TT genotype was found in 7 (7%). In controls CC genotype was found in 95 (86%), CT was found in 15 (14%) and TT was not found in controls in this study. The genotype distribution in cases showed deviation from HWE (X^2 = 4.852, P value = 0.028) while the controls were in HWE (X^2 = 0.589, P value = 0.443).

Table 3: MTHFR C677Tgenotype distributions in CAD Patients and controls

Poymorphism	Genotype / Alleles	CAD n 100 (%)	Control n 110 (%)	Odds Ratio	95% CI	P-value
C677T	CC	69 (69%)	95 (86%)	0.35	0.17-0.70	0.003
	CT	24 (24%)	15 (14%)	2.0	0.98-4.07	0.05
	TT	7 (7%)	0 (0 %)	17.7	0.99-3.14	0.05
	C	162	205	0.31	0.16 - 0.58	0.0003
	T	38	15	3.2	1.70 - 6.03	0.0003

Correlation between MTHFR C677T and exogenous factors

MTHFR genotypes (CC, CT and TT) were correlated with demographic factors like gender, smoking and lipid profile to investigate the effect of genetic polymorphism in modulating the risk of developing CAD. When MTHFR genotypes were correlated with gender, it was noted that there was no significant difference between males [CC, CT, and TT genotypes were in 69.65 % (39 patients), 21.42 % (12 patients), and 8.93 % (5 patients)] and females [CC, CT, and TT genotypes were in 68.18 % (30 patients), 27.28 % (12 patients), and 4.54 % (2 patients), respectively]. We also investigated the association between MTHFR genotypes versus smoking. In patients we observed that smokers with CAD were 66.66 % (40 patients) and 23.33 % (14 patients) with CC and CT genotypes than nonsmokers [29 patients (72.5 %) 10 patients (25 %)] While as TT genotype shows higher frequency [6 patients (10%)] in smokers and 1 patient (2.5%) was found with same genotype in non smoker. (Table 4)

The lipid profile (Total cholesterol, HDL-C and LDL-C, VLDL-C and Triglycerides) along with different genotype frequencies of MTHFR (C677T) polymorphism were calculated for CAD patients and have been expressed as mean ± standard deviations (Fig. 2).

Table 4: Association between MTHFR C677T with exogenous risk factors

MTHFR genotype	Males (n=56)	Females (n= 44)	Statistics
CC (69)	39	30	OR 1.07, 95% CI 0.45-2.5, P 0.87
CT (24)	12	12	OR 0.72, 95% CI 0.28-1.82, P 0.49
TT (7)	5	2	OR 2.0, 95% CI 0.37-11.15, P 0.40
MTHFR genotype	**Smokers (n=60)**	**Non smokers (n= 40)**	**Statistics**
CC (69)	40	29	OR 0.75, 95% CI 0.31-1.82,P 0.53
CT (24)	14	10	OR 0.91, 95% CI 0.35-2.32,P 0.84
TT (7)	6	1	OR 4.33, 95% CI 0.50-37.45,P 0.18

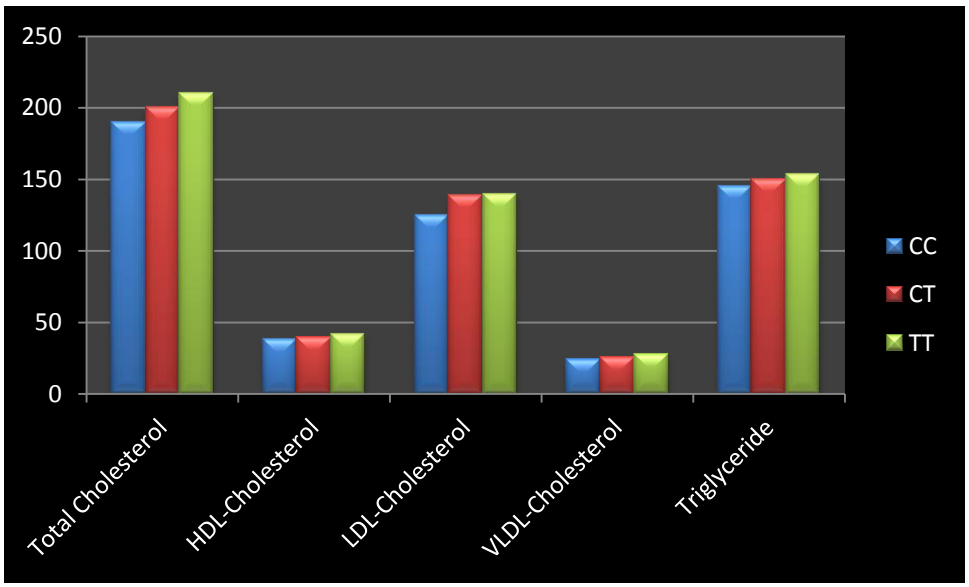

Fig. 2: Showing the lipid profile (TC, HDL-C, LDL-C, VLDL-C and TG) along with genotype frequencies of MTHFR gene (C677T) polymorphism in CAD patients.

DISCUSSION

Coronary artery disease (CAD) due to atherosclerosis is associated with increased mortality and morbidity. Various risk factors have been found to be associated with the development of CAD. A person's genetic makeup, reflected by his or her family history, may influence the risk of various forms of cardiovascular disease. *MTHFR* (*C677T*) gene is found to be polymorphic in both cases and controls. In present study we found CC (OR: 0.35, 95% CI; 0.17-0.70, p: 0.003), CT (OR: 2.0, 95% CI; 0. 0.98-4.07, p: 0.05) and TT (OR: 17.7, 95% CI; 0.99-3.14, p: 0.05) genotypes were associated with CAD cases compared to controls and T and C alleles were highly significant (P: 0.0003) among cases as compared to controls. The significant deviation from HWE observed in the cases in our study could be due to several factors such as disparity in survival of carriers of the marker, genetic drift, as suggested by Zafarmand et al. 2008 (18).

The primary objectives of this study was to assess the frequency distribution of the MTHFR C677T mutation in a large population of patients with angiographically documented severe CAD, in comparison to subjects with absence of CAD. An attempt was made to clarify the relative contribution of this genetic factor to lipid level, with particular reference to the interaction with an environmental factor potentially modifiable. There was a significant association between C677T polymorphism and CAD (OR: 2.0, 95% CI; 0.98-4.07, p: 0.05) (Table 3). The present study comprised of maximum number of cases in age group of 50 to 60 years, and all the cases above the age of 45 years. The C677T polymorphism may predict CAD risk only in certain ethnic groups (19) and the significant association of polymorphisms in the *MTHFR* gene has been observed in this group.

The mean ± SD of Cholesterol was found to be 200 ± 47.1 in the patients and 148 ± 34.7 in the controls, the LDL was found to be 125 ± 39 in the patients and 111 ± 33 in the control, HDL was 40.11 ± 14.12 in the patients and 42.50 ± 15.42 in the controls, and VLDL was26.52 ± 13 in patients and 22.97 ± 11.21 in normal controls of our study, triglycerides were found to be 153.18 ± 68.02 in the patients and 140.25 ± 68.15 in the controls. Cases had significantly higher levels

of TC and LDL cholesterol compared to control group. The difference was significant ($P<0.001$) for cholesterol, and LDL ($p<0.001$), when cases of CAD were compared with controls.

The *MTHFR* C677T polymorphism has been investigated for its association with several complex diseases in several studies across different populations; however, results are not consistent. One of the possible reasons for this could be attributed to variation in allelic frequency distribution in different population groups. The 677T allele frequency was found to be highest in European populations ranging from 24.1% to 64.3% and, hence, it is presumed to be originated in Europe in the late state of human evolution. However, zero frequency of this allele is reported from African population (20). In Indian population, distribution of 677T allele ranges from complete absence to 23.7% and highest frequency was reported from North-Indian population. Moreover, frequency of T allele is found to be relatively higher among caste populations as compared to that of tribal populations of India. Linguistically, Indo-European speakers have relatively higher T allele frequency followed by Tibeto-Burman, Dravidian, and Austro-Asiatic speakers (21).

Whereas it is now becoming clear that, at least in most of the populations studied so far, the C677T mutation cannot be considered as a single genetic risk factor for CAD. The genotype and phenotype characteristics study in our population confirms that the MTHFR polymorphism is a major determinant of coronary artery disease, but also clearly shows that it is not important as a single factor.

Conclusion

In conclusion, we have observed that the presence of the *MTHFR C*677T was significantly associated with CAD .The percentage was greater with other common risk factors such as age, sex, diabetes mellitus, hypertension, smoking in CAD patients than in the normal subjects. In addition, the levels of TC, LDL-C were more in CAD patients than controls as compared to HDL-C and VLDL.

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