Clinical Importance of MTHFR Gene Polymorphism in Coronary Artery Disease: A Study from India

Mamta P Sumi[a], Sameer Ahmad Guru[a], Rashid Mir[a], Musadiq A Bhat[a], Girish MP[b], Alpana Saxena[a]

a. Department of Biochemistry, Maulana Azad Medical College, University of Delhi, India

b. Department of Cardiology, GB Pant Hospital, University Of Delhi, India

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ABSTRACT

Introduction: The influence of *MTHFR C677T gene* polymorphisms on the risk of development of coronary artery disease (CAD) in north Indian population was analysed. Homozygosity for this 677T variant was shown to be associated with increased plasma Hcy levels, particularly when folate status is low and it has been described as a risk factor for CAD. In this study we have attempted to elucidate the role of *MTHFR C677T gene* polymorphism in CAD in the north Indian population, and analyse their influence on the risk of development of CAD. **Methodology:** The study included hundred (100) coronary artery disease (CAD) patients who showed presence of greater than 50% luminal stenosis in at least one major coronary artery at angiography and hundred (100) age and sex matched control subjects with good general health and no history of chronic diseases including cardiovascular diseases. The *MTHFR* gene polymorphism were investigated by PCR-RFLP. **Results**: Genotypic frequencies of MTHFR C677T gene polymorphism differed significantly between CAD patients and control subjects and this differential distribution reached a good statistical significance (p<0.0001). Analysis of risk of CAD associated with different genotypes of *MTHFR C677T* polymorphism, using co-dominant, dominant and allele specific inheritance model which indicated a high risk of CAD associated with the TC,CC, TC+CC genotypes and mutant C allele of this polymorphism. There was no significant differential distribution of this *gene* polymorphism observed with respect to disease severity of CAD patients. However, a significant differential allelic frequency distribution was observed with p value <0.0001. **Conclusions:** In conclusion, this study demonstrates a significantly heightened risk of CAD associated with the inheritance of mutant genotypes of *MTHFR C677T* gene polymorphisms in the north Indian population.

INTRODUCTION

Coronary artery disease (CAD) is a leading cause of death in developed countries and is rapidly assuming epidemic proportions in developing countries as well. Several risk factors have been found to be associated with the development of CAD. Genetic and environmental factors play a vital role in the pathogenesis and progression of CAD (1). Methylenetetrahydrofolate reductase (MTHFR) is an enzyme plays an significant role in homocysteine metabolism by catalyzing the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5-Methyltetrahydrofolate is the major circulatory form of folate and carbon donor for remethylating of homocysteine to methionine. (2) The human MTHFR gene is located at chromosome 1p36.3 and consists of 11 exons with a length 1980 bp(3) The C to T missense mutation in exon 4 at codon 677 of the MTHFR gene (677C→T), which causes an alanine (A) to valine (V) substitution in the MTHFR protein, reduces enzyme activity, and results in increased plasma homocysteine(4). Elevated plasma level of homocysteine has been predictable as an independent risk factor for coronary artery disease. (5) A point mutation in the gene encoding MTHFR has been associated with the elevations of homocysteine levels in homozygous carriers (TT genotype) and is considered as an independent risk factor for vascular diseases(6). Homozygosity for this 677T variant was shown to be associated with increased plasma Hcy levels, particularly when folate status is low and it has been described as a risk factor for CAD. In this study, we have attempted to elucidate the role of *MTHFR C677T gene* polymorphism in CAD in the north Indian population, and analyse their influence on the risk of development of CAD.

METHODOLOGY

Study population

The present study was conducted on 100 north Indian consecutive patients aged 40-70 years who underwent elective coronary angiography and angioplasty (PTCA) at Gobind Ballabh Pant Hospital New Delhi, India from January 2016 to May 2017.

Inclusion criteria: Inclusion criteria for CAD patients were presence of greater than 50% luminal stenosis in at least one major coronary artery. Inclusion criteria for age and sex matched

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control subjects were good general health and lack of chronic diseases including cardiovascular diseases in the medical history.

Clinical characteristics of CAD patients:

Presence of major cardiovascular disease risk factors viz diabetes mellitus, hypertension, positive family history was noted in the cohort of CAD patients. Diabetes mellitus was defined as presence of active treatment with insulin / oral antidiabetic agent or those individuals on dietary control for diabetes for whom documentary proof of diabetic diagnosis as per American Diabetes Association criteria was confirmed. Hypertension was defined as a systolic blood pressure of ≥140 mm Hg and /or diastolic blood pressure of ≥90 mm Hg on at least 2 separate occasions or presence of antihypertensive treatment. The study was approved by Institutional Ethical Committee of Maulana Azad Medical College, New Delhi, India. Written informed consent was obtained from each patient and healthy volunteer.

Peripheral blood sample collection:

A peripheral blood sample (5 ml) was collected in EDTA containing vials from both patients and control subjects. Peripheral blood leucocytes were isolated using RBC lysis buffer. 300 µLof a peripheral blood sample was diluted with 900 µL of RBC lysis buffer and incubated for 20 mins at room temperature. Centrifuged the RBC lysed blood suspension at 3500 RCF for 5 mins, discarded the supernatant and stored at -80°C till analysis.

MTHFR C677T gene polymorphism Study:

Restriction Fragment Length Polymorphism Polymerase Chain Reaction (PCR-RFLP)

The *MTHFR C677T gene* polymorphism was studied by PCR-RFLP technique. Briefly, genomic DNA was extracted from peripheral blood leukocytes of CAD patients and control subjects using a DNA extraction kit (Gene Aid, India) following the manufacturer's protocol. DNA concentration was determined by measuring absorbance at 260 nm. The quality and integrity of DNA was determined by the A260/280 ratio. DNA quality was also checked by ethidium bromide stained 2% agarose gel electrophoresis.

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The *MTHFR C677T gene* polymorphism was analysed by PCR RFLP using *MTHFR* gene specific forward primer (5'-TGAAGGAGAAGGTGTCTGCGGGA-3') and reverse primer (5'-AGGACGGTGCGGTGAGAGTG-3') for PCR amplification and digestion by restriction enzyme *HinfI*. PCR was performed in 25 μl of total reaction volume. The PCR protocol was as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 57°C for 45 seconds, extension at 72°C for 90 seconds and a final extension step at 72°C for 5 minutes. The PCR amplicons on 2 % agarose gel electrophoresis containing ethidium bromide showed amplified fragments of 198 bp size.

This was followed by restriction digestion of the PCR amplicons at 37°C for 15 mins with fast digest restriction enzyme *HinfI* (Thermo Scientific, USA) for study of *C677T MTHFR* gene polymorphism. The 677T allele contains anHinfI site resulting in 175 bp and 23 bp fragments, whereas a C at position 677 (677C) does not. The PCR and digestion products were analysed in 3% TBE agarose gels. Samples were categorised as homozygous for the thermolabile variant (677TT), heterozygous for wild type and variant (C677T), or wild type (677CC). The 23 bp fragments were not visible on agarose gels. (image no 1)
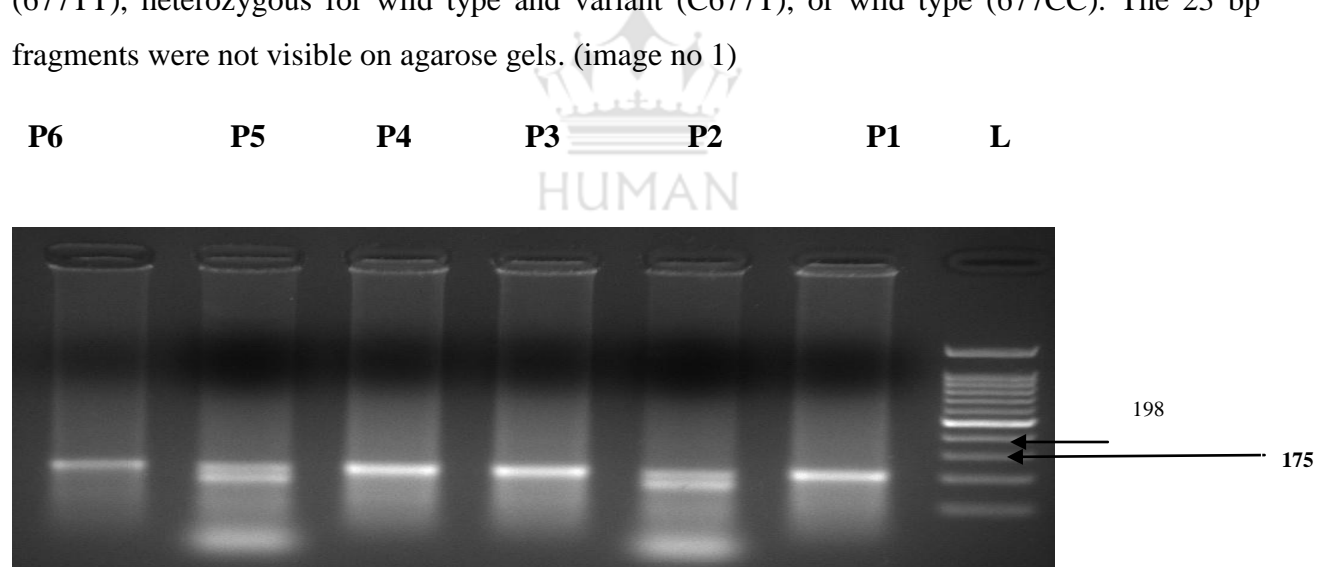

Image no.1: Agarose Gel Electrophoresis Picture after digestion:

Representative gel photograph showing RFLP for the MTHFR gene *HinfI* polymorphism. The product is of 198bp (allele C), *Hinf I* generates products of 175 and 23bp (allele c).

Lane P1, P3, P4 and P6: Undigested Lane P2 and P5: Digested L: ladder.

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Statistical Analysis

Compliance with Hardy-Weinberg equilibrium was ascertained in the control subjects by the chi-square test. Odds ratio was computed using codominant and dominant models for assessment of risk of development of CAD. All statistical analyses were performed using Graph Pad Prism 7.03 software package. A p-value of <0.05 was considered as significant.

RESULTS:

Study population:

In this case control study, a total of 100 CAD patients from GB Pant Hospital, New Delhi, India, 100 age and sex matched healthy controls subjects were included. The genotypic distributions *C677T MTHFRgene* polymorphism in the control subjects were in accordance with Hardy-Weinberg equilibrium. Of the 100 CAD patients, 67 were aged ≤ 55 years while 33 were of more than 55 years age. Amongst the CAD patients 81 were male and 19 were female.

Association of C677T MTHFR gene polymorphism with CAD patients:

Genotypic frequencies of MTHFR C677Tgene polymorphism differed significantly between CAD patients and control subjects and this differential distribution reached a good statistical significance (p<0.0001). Significantly, in MTHFR C677T SNP analysis the frequency of mutant homozygous (TT) and heterozygous (CT) genotypes were greater in CAD patients as compared to control subjects (43% and 35% vs 13% and 15% respectively). The mutant T allele frequency was greater in CAD patients than in the normal controls (0.605vs 0.2278) respectively as shown in table no.1.and fig 1.

Table 1: Frequency of MTHFR C677T gene polymorphism genotypes in CAD patients and control subjects:

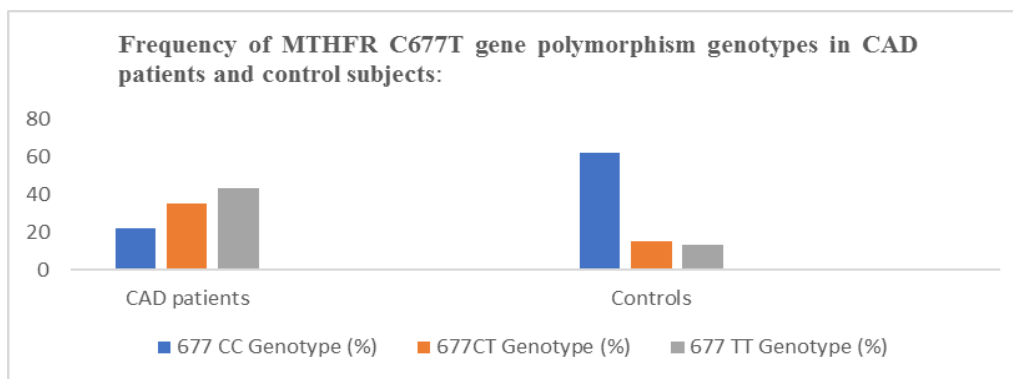| CAD Patients and Control (Total)						
Subjects (No)	677 CC Genotype (%)	677CT Genotype (%)	677 TT Genotype (%)	(C)Allele frequency	(T) Allele frequency	Chi square	p value
CAD patients (100)	22	35	43	0.395	0.605	42.71	<0.0001
Controls (100)	62	15	13	0.7722	0.2278		

Fig 1: Frequency of MTHFR C677T gene polymorphism genotypes in CAD patients and control subjects:

Fig 1: Frequency of MTHFR C677T gene polymorphism genotypes in CAD patients and control subjects:

MTHFR C677T genotypes are associated with an increased risk of CAD:

The risk of CAD associated with inheritance of different genotypes of MTHFR C677Tpolymorphism was evaluated by computation of odds ratios in the codominant model, dominant model and allele specific model of analysis. In case of codominant model both mutant homozygous TT and heterozygous CT genotypes were independently associated with significantly higher CAD risk in comparison to wild type CC genotype with odds ratio of 6.5758 (95% CI 3.0259 to 14.2901p =<0.0001) and 9.3217 (95% CI 4.2378 to 20.5046 p =<0.0001) respectively. Additionally in the dominant model inheritance of either mutant homozygous TT or heterozygous CT genotypes, when compared to wild type CC genotype, also showed a highly significant association with higher CAD risk (odds ratio 7.8506,95 % CI-4.0967 to 15.0444, p=< 0.0001) (table 3). Furthermore, analysis was performed in allele specific model inheritance while mutant (T) allele showed a significant association with higher CAD risk compared to wild type (C) allele with odds ratio 5.0217% CI 2.7177 to 9.2791, p=<0.0001) (table no2).

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Table no 2: Risk of Coronary artery disease associated with *MTHFR C766T* gene polymorphism genotypes.

All CAD Patients					
677C/T Genotype	**CAD PATIENTS**	**CONTROLS**	**OR**	**95% CI**	**p value**
CC	22	62			
CT	35	15	9.3217	4.2378 to 20.5046	< 0.0001
TT	43	13	6.5758	3.0259 to 14.2901	< 0.0001
CT+TT	78	28	7.8506	4.0967 to 15.0444	< 0.0001
Allele specific					
C	0.395(40)	0.7722(77)	5.0217	2.7177 to 9.2791	< 0.0001
T	0.605(60)	0.2278(23)			

MTHFR C677T *gene* polymorphism genotypes in CAD patients with respect to severity:

As inclusion criteria, CAD patients were defined by presence of greater than 50% luminal stenosis in at least one major coronary artery. Patients were segregated into two groups according to the number of involved coronary artery. Group 1 included single vessel disease and group 2 included two and three vessels disease. The frequency of mutant homozygous (TT) and heterozygous (CT) were greater in patients with single vessel disease compared to the patients with two and three vessels disease. (33%, 19% Vs 12,16) and mutant (T) allele frequency was greater in single vessel disease in comparison to two or three vessels disease.(0.6855 vs 0.5299).Table no.3 and Fig 2.

Table no 3: MTHFR C677T *gene* polymorphism genotypes in CAD patients with respect to severity:

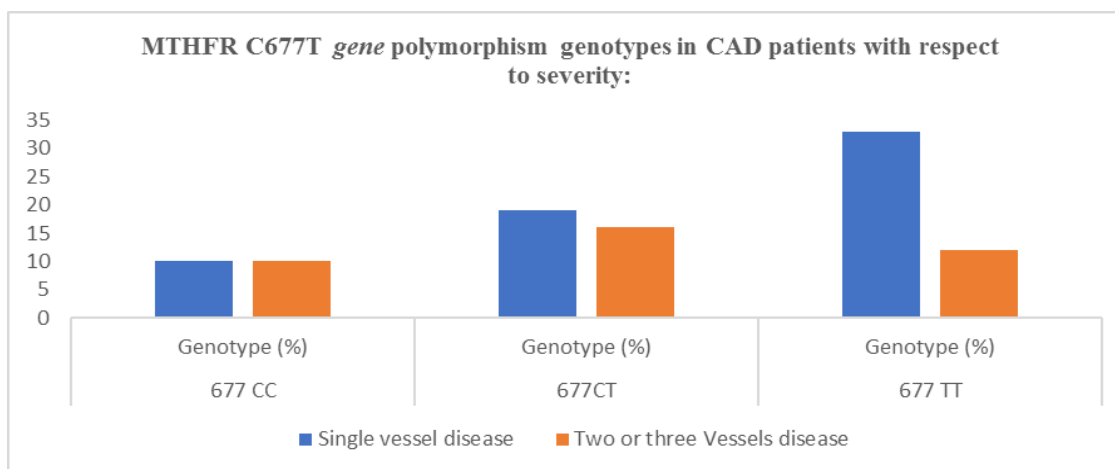| Disease severity	677 CC Genotype (%)	677CT Genotype (%)	677 TT Genotype (%)	(C)Allele frequency	(T) Allele frequency	Chi-square	p-value
Single vessel disease	10	19	33	0.3145	0.6855	4.56	0.1023
Two or three Vessels disease	10	16	12	0.4701	0.5299		

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Fig 2: MTHFR C677T *gene* polymorphism genotypes in CAD patients with respect to severity:

DISCUSSION:

It is a well fact that genetic, epigenetic and environmental may be the causes for the increased risks for the development of congenital heart diseases. A number of studies have implicated the role of MTHFR in folic acid metabolism and cardiovascular diseases. (7) MTHFR is an important and key enzyme in folic acid conversion and development of cardiovascular diseases(8)). It has been reported that C677T polymorphism of MTHFR may cause the enzyme thermolabile and may reduce its activity approximately by 50% and increase plasma homocysteine concentrations. (9)). Hence, the polymorphic variants of MTHFR gene may change the activity of MTHFR and may become a vital determinant of the development of cardiovascular diseases. A number of studies have implicated the potential role of MTHFR gene polymorphisms and CAD, however, the results of these studies have not been consistent. (10) This study is an attempt to evaluate the frequency distribution of MTHFR (C677T) polymorphism among CAD patients and healthy controls in Asian population.

In this study, we have observed that MTHFR gene (C677T) polymorphism distribution differs significantly among CAD patients and healthy controls. It was also analysed that MTHFR (C677T) genotypes provide an increased risk for the development of CAD and this is in agreement with Lu and his team(11) who have found that MTHFR is significantly associated with CAD in Asian population but not in Caucasian population. Furthermore, it was observed

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that MTHFR (C677T) polymorphism mutant genotypes were more frequent in one vessel injury than in more that one vessel injury. To the best of our knowledge, the comparison of this subgroup has not been done and the frequency of this polymorphism has not been done till date. However, to validate the association of the genotypes of this polymorphism with vessel involvement and severity of CAD, we propose to perform this study in a still larger group of patients and healthy controls.

CONCLUSION:

The present study demonstrates that the MTHFR C677T polymorphism plays an important role and provides an increased risk for the development of cardiovascular diseases. However, there are some limitations of the study which are required to be addressed.

Compliance with Ethical Standards: The study was conducted in accordance to GCP and IC MR Guideline and approved by Institutional Ethical Committee of Maulana Azad Medical College, New Delhi, India.

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Conflict of Interest: There is no conflict of interest to conduct the study.

Ethical approval: The study was approved by Institutional Ethical Committee of Maulana Azad Medical College, New Delhi, India.

Informed consent: Written informed consent was obtained from each patient and healthy volunteer.

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