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**Original Research Article**

# Association of genetic and epigenetic modification in MTHFR gene with coronary artery disease patients in north Indian population

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## ABSTRACT

**Background:** Methylene tetra hydro folate reductase (MTHFR) gene polymorphism C677T (rs180113) and DNA methylation in promoter region of MTHFR gene may contribute to the development of coronary artery disease however the results have been inconsistent across studies with different populations, so the aim of our study is to explore the association of polymorphism in MTHFR gene and methylation in promoter region with coronary artery disease (CAD) and other risk factor (lipid profile, homocysteine, vitamin $B_{12}$ and folic acid levels) leading to CAD in of north Indian population.
**Methods:** Total 100 CAD patients and 100 healthy controls were enrolled in the study. Genotyping of rs1801133 SNP (C677T) is done by PCR-RFLP and DNA methylation study in promoter region by methylation specific PCR. Lipid profile analysis by automated chemistry analyzers, serum homocysteine, folic acid and vitamin $B_{12}$ was assayed by ELISA.
**Results:** As per our finding the T allele (OR=3.03, 95% CI=1.74-5.27) and hyper methylation in promoter region of MTHFR increases the odds of coronary artery disease, (OR=3.05, 95% CI=1.7-5.6). Study participants with CT and TT genotype had significantly higher homocysteine (Hcy) (p=0.001), lower folic acid level (p=0.0), and HDL levels (p<0.0001) than those with CC genotype. The study subjects with hyper methylated promoter region have a significantly high homocystenemia levels (p=0.001).
**Conclusions:** The TT genotype of the MTHFR C677T gene polymorphism and hyper methylation in promoter region of MTHFR, is associated with CAD and can be useful in identification of new biomarkers, development of preventive and therapeutic strategies for CAD.

**Keywords:** CAD, Hyper homocysteinemia, Methylation, MTHFR gene, Polymorphism

## INTRODUCTION

Worldwide, cardiovascular disorders are thought to be the third leading cause of death.[1] The most prevalent cardiovascular disease is coronary artery disease (CAD), which affects 126 million individuals globally (1,655 per 100,000), or around 1.72% of the world's population, and will reach to 1,845 cases by the year 2030.[2-4] Reduced blood circulation to the cardiac muscle leads CAD, which is usually brought on by atherosclerosis, or plaque buildup on the coronary arterial walls. Up to this point, a number of risk factors have been identified but hyper homocysteinemia (HHcy) and abnormal blood lipids have been widely accepted as major risk factor.[5-7]

Homocysteine, a sulfhydryl-containing amino acid that does not form proteins undergoes re methylation to form methionine, universal methyl group donor. Vitamin $B_{12}$ (cobalamin) and folate act as cofactor and methyl group donor respectively in the re-methylation of homocysteine to methionine.[8,9]

The enzyme 5, 10-methylene tetra hydro folate reductase (MTHFR), which catalyzes the conversion of 5, 10-methylene tetra hydro folate to 5-methyltetrahydrofolate, is a crucial regulator of the re methylation pathway of homocysteine to methionine. The activity of the enzymes can be impacted by polymorphism that is C to T missense mutation (677CT) in exon 4 of the MTHFR gene and epigenetic alteration that is methylation in the promoter region of MTHFR gene which can lead to hyperhomocysteinemia (HHcy). Although some studies have shown association between polymorphism and methylation of MTHFR gene's promoter region with coronary artery disease but the results are inconsistent.[10-14] In addition, association between alteration in MTHFR gene and other risk factors like serum lipid profiles, folic acid and $B_{12}$ is still unclear. Therefore, the goal of this investigation was to determine whether the MTHFR C677T gene polymorphism and hyper methylation in the MTHFR promoter region increases risk for development of coronary artery disease in north Indian population and to evaluate their association with serum folate, vitamin $B_{12}$ and parameters of lipid profile which will help us in prevention, early detection, and treatment.

## METHODS

The department of biochemistry, the department of cardiology, the ICMR-National Institute of Pathology, and the VMMC and Safdarjung Hospital in New Delhi, India, jointly participated in a case-control study. Clearance from ethical committee of Vardhman Mahavir Medical College and Safdarjung Hospital was obtained before proceeding for the study. Informed written consent was obtained from each subject before enrolling them in the study. The sample size was calculated using the software EpiInfo3.5, and the minimum calculated sample size came out to be 103 in each group using the prevalence of MTHFR C667T polymorphism as 25% in coronary artery disease patients and 9.5% in controls, 95% CI, 80% power.[15]

### *Inclusion criteria*

Case- known cases of coronary artery disease. Control-healthy individuals with no evidence of CAD and no family history of CAD.

### *Exclusion criteria*

Patients suffering from renal insufficiency, liver insufficiency, history of gastrectomy, gastric bypass surgery, malabsorption syndromes, patients with diseases of the malignant tumors, psoriasis and thrombophilia, recent supplementation of vitamin $B_{12}$ and folic acid (within last 3 months), consumption of drugs that might affect serum vitamin $B_{12}$ levels and folic acid levels, smokers and alcoholic.

The study used random sampling to select 100 healthy participants (controls) who did not have any chronic illnesses and 100 patients (both genders) who had been diagnosed with CAD by angiography for each patient, a thorough history and general physical examination were recorded.

The lipid profile was analyzed on automated biochemistry analyzers in NABL (National Accreditation Board for Testing and Calibration Laboratories) accredited biochemistry department of central clinical laboratory of our hospital. Serum folic acid, vitamin $B_{12}$ and serum homocysteine was analyzed using commercially available ELISA kit. Krishgen DNA blood kit was used to extract genomic DNA from peripheral blood leukocytes. The Indian company "Eurofins Scientific" created the primers. The EpiTect Bisulfite Kit was used for bisulfite converted methylation specific PCR. A spectrophotometer was used to assess the purity of the DNA. Polymerase chain reaction-restriction fragment length polymorphism was used to study genotype rs1801133 (PCR-RFLP).

To create a 198 bp PCR product, the following primers are used: forward: 5'TGAAGGAGAAGGTGTCTGCGGGA3'; reverse: 5'AGGCGGTGCGGTGAGAGTG-3'. DNA template (3 µl), forward and reverse primers (.25 µl each), PCR master mix (10 µl), and nuclease-free water (6.5 µl) were used in the DNA amplification process. Initial denaturation at 95°C for 5 minutes was followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 56.2°C for extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes. Using NEB cutter 2.0, the restriction enzyme endonuclease HinfI has been selected to be employed for the digestion of amplicons. In order to perform restricted digestion, we used 5 µl of PCR amplicon, 1 µl of HinfI, enzyme buffer 1 µl of enzyme and 8 µl of nuclease-free water were thoroughly mixed and left at 37°C overnight. Fragments were then separated according to their size by gel electrophoresis using 2% agarose. Allele C was not digested and remained 198 bp whereas allele T was cut into two fragments of 175 bp and 23 bp, thus heterozygous subjects showed three fragments of 198, 175 and 23 bp as shown in Figure 1.

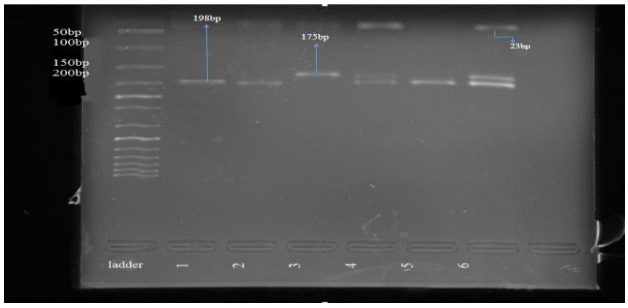


**Figure 1: Electrophoresis gel picture showing the restriction digestion of MTHFR PCR product with HinfI enzyme. Lane no. 1- PCR product, lanes 2, 5- homozygous wild, lanes 4 and 6- heterozygous, lane 3- homozygous polymorphic.**

### *Methylation specific PCR (MSP)*

Utilizing the EpiTect Bisulfite Kit and a thermal cycler, sodium bisulfite was used to transform the previously isolated leukocyte DNA. Unmethylated cytosine residues in the target DNA are converted to uracil when it is exposed to sodium bisulfite, whereas the methylated cytosine remains unaltered. As a result, we employed two sets of primers for methylation specific PCR since bisulfite treatment results in different DNA sequences for methylated and un methylated DNA. The primers used for methylation of the MTHFR gene promoter region include primers. Forward: 5′TAGATTTAGGTACGTGAAGTAGGGTAGAC3. Reverse: 5′GAAAAACTAATAAAAACCAGACAGA3′. For Unmethylated: forward: 5′TTTAGGTATGTGAAGTAGGGTAGATGT3′ reverse: 5′CAAAAAACTAATAAAAAACCAACAAA-3′.[16]

The PCR conditions used are as follows: 5 minutes of initial denaturation at 95°C, followed by 40 cycles of, 30 seconds of denaturation at 95°C, 30 seconds of annealing at 58°C, 30 seconds of extension at 72°C, 5 minutes of final extension at 72°C, and 4°C for short-term storage. The final result was electrophoresed after being loaded (7 µl) onto a 2% agarose gel with ethidium bromide. The DNA bands were then seen using a gel documentation system. If the 180 bp product was present in the set of un methylated primers, methylation in the promoter region is not present. If the 180 bp product was present in the set of methylated primers the 180 bp product, methylation in the promoter region is present as shown in (Figure 2).

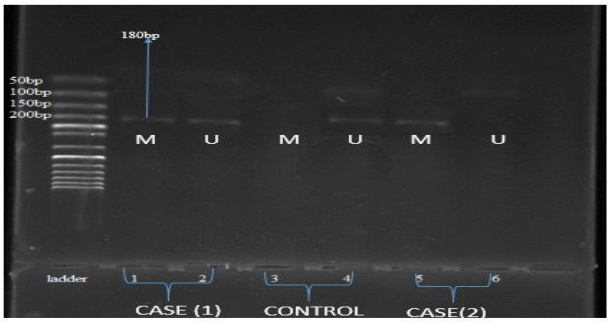


**Figure 2: Agarose gel electrophoresis of MTHFR gene methylation-specific polymerase chain reaction. M represents methylation-specific primers; U represents unmethylated-specific primers. Lanes (1-2) and (5-6) = cad case, lanes 3 and 4= normal control**

### *Statistical analysis*

With the help of SPSS version 21, statistical analysis was carried out. The unpaired t test and one way ANOVA was used to assess differences between mean values for the biochemical data in study subjects. The Chi-square test was used to determine if the data conformed to the Hardy-Weinberg equilibrium. To determine the correlation between groups for genotypes, alleles, and MTHFR gene promoter methylation, odds ratios (ORs) were performed. Results are presented as mean±SD, and when necessary, the 95% confidence interval (CI) was indicated.

## RESULTS

In the study, 100 angiographically confirmed cases of CAD patients, and 100 healthy individuals with age and gender matched were included as shown in Table 1. The mean of serum homocysteine levels in cases (24±19 µmol/l) were significant, higher than in controls 12±10 µmol/l (p value <0.0001). However no significant difference has been observed in serum folic acid level and Vitamin $B_{12}$ in case as compare to controls. We have also found that serum HDL levels are significantly low in cases as compare to controls (p=0.01). Cholesterol levels were high in cases as compare to control (p=0.001), shown in Table 2.

**Table 1: Demographic data of study subject.**

| Parameters | Case | Control | P value |
|---|---|---|---|
| **Mean age (years)** | 53.9±9.3 | 51.9±8.07 | 0.1 |
| **% male** | 79 | 75 | 0.6 |
| **% female** | 21 | 25 | |

**Table 2: Comparison of cardiovascular risk variables in study subject.**

| Parameters | Case (mean±SD) | Control (mean±SD) | P value |
|---|---|---|---|
| **Cholesterol (mg/dl)** | 196.64±50 | 169.1±45.9 | 0.001 |
| **HDL (mg/dl)** | 33.8±7.8 | 36.6±8.1 | 0.01 |
| **LDL (mg/dl)** | 149.2±27.8 | 139.9±41.09 | 0.06 |
| **S.TG (mg/dl)** | 122.75±35.5 | 122.5±32.2 | 0.9 |
| **Homocysteine (µmol/l)** | 24±19 | 12±10 | <0.0001 |
| **FA (ng/ml)** | 13.5±4.1 | 14±3.3 | 0.8 |
| **Vitamin $B_{12}$ (pg/ml)** | 396±90.6 | 419.7±92.7 | 0.07 |

Using Pearson's correlation coefficient test, we observed that serum homocysteine levels were negatively correlated with both serum folate and serum vitamin $B_{12}$ levels for cases (folic acid, r=-0.4, p<0.001), (vitamin $B_{12}$, r=-0.3, p=0.02) as well as controls (folic acid, r=-0.2, p=0.04), (vitamin $B_{12}$, r=-0.32, p=0.02) which was statistically significant. Negative correlation of serum homocysteine with serum folate and vitamin $B_{12}$ signifies the importance role of both folate and vitamin $B_{12}$ in homocysteine metabolism.

### *Genotype frequencies*

The distribution of genotypes within each group was in Hardy-Weinberg equilibrium. Allele and genotype frequencies in both patient and control group are described in Table 3.

**Table 3: Comparison of genotype frequency in study subject.**

| | | Cases (n=100) | Controls (n=100) | P value | $\chi^2$ | Odds ratio (95% CI) |
|---|---|---|---|---|---|---|
| **Genotype** | CC | 57 | 82 | | | |
| | CT | 32 | 15 | <0.001 | 15.02 | |
| | TT | 10 | 3 | | | |
| | HWE | $\chi^2$=2.7, p=0.2 | $\chi^2$=4.3, p=0.1 | | | |
| **Allele** | C | 146 | 179 | | | |
| | T | 52 | 21 | <0.001 | 15.47 | 3.0359 (3.0359-1.1105) |

**Table 4: Relationships between serum homocystein, folic acid, vitamin $B_{12}$ and lipid profiles with MTHFR C677T polymorphism among CAD Cases and control participants.**

| Factors | Case | | | | Control | | | |
|---|---|---|---|---|---|---|---|---|
| | CC | CT | TT | P value | CC | CT | TT | P value |
| **HCY (µmol/l)** | 18.6±8.5 | 30.02±14.2 | 36.9±13.2 | <0.0001* | 10.5±5.1 | 13.4±6.6 | 25±1 | <0.0001* |
| **Total cholesterol (mg/dl)** | 171.0±40.9 | 187.8±51.2 | 201.6±68.3 | 0.09 | 168.7±46.9 | 172.4±42.1 | 165.8±55.1 | 0.95 |
| **HDL (mg/dl)** | 35.1±8.0 | 30.6±8.3 | 23.6±4.9 | <0.0001* | 36.9±8.0 | 35.8±9.1 | 27.1±3.1 | 0.1 |
| **LDL (mg/dl)** | 145.8±29.9 | 154.2±25.5 | 152.4±20.8 | 0.3 | 139.3±41.6 | 144.4±43.3 | 133.4±8.8 | 0.8 |
| **TG (mg/dl)** | 118.1±34.6 | 131.9±36.8 | 118.6±31.6 | 0.18 | 124.4±34.0 | 114.1±23.1 | 114±9.5 | 0.4 |
| **FA (ng/ml)** | 14.2±3.9 | 11.3± 3.7 | 11.0±4.1 | 0.01 | 14.0335±2.9 | 12.0±3.7 | 10.5±2.16 | 0.01 |
| **Vitamin $B_{12}$ (pg/ml)** | 410.5±90.8 | 390.9± 99.9 | 350.7±78.3 | 0.15 | 419.5±96.5 | 421±81.2 | 420±43.5 | 0.1 |

**Table 5: Comparison of MTHFR gene methylation profile in study group.**

| Group | Methylated | Partially methylated | OR (95%CI) | P value | Chi-square value |
|---|---|---|---|---|---|
| **Case** | 39 | 61 | 2.9126 (1.5-5.5) | 0.0017 | 9.81 |
| **Control** | 18 | 82 | | | |

**Table 6: Relationships between serum homocystein, folic acid, vitamin $B_{12}$ and lipid profiles with methylation profile of MTHFR among CAD cases and control participants.**

| Factor | Cases | | | Controls | | |
|---|---|---|---|---|---|---|
| | Methylated (N=39) | Partially methylated (N=61) | P value | Methylated (N=39) | Partially methylated (N=61) | P value |
| **HCY (µmol/l)** | 29.5±12.3 | 20.8±20.5 | 0.0008 | 18.2±4.2 | 9.8012±4.9 | <0.0001 |
| **Total cholesterol (mg/dl)** | 190.1±49.6 | 200.7±51.7 | 0.3 | 180.2±47.7 | 166.5±45.4 | 0.2 |
| **HDL (mg/dl)** | 30.9±8.6 | 33.9±8.2 | <0.0001 | 34.3±7.7 | 36.9±8.3 | 0.2 |
| **LDL (mg/dl)** | 147.1±35.8 | 150.5±21.4 | 0.5 | 144.5±48.9 | 138.8±39.2 | 0.5 |
| **TG (mg/dl)** | 117.5±35.5 | 126.1±35.1 | 0.2 | 119.4±36.7 | 123.2±31.3 | 0.6 |
| **FA (ng/ml)** | 12.6±3.8 | 13.9±3.9 | 0.1 | 13.8±3.1 | 13.5±3.2 | 0.6 |
| **Vitamin $B_{12}$ (pg/ml)** | 373.641±86.2 | 400.6±98.5 | 0.16 | 422.6±111.07 | 419.01±88.6 | 0.6 |

We have observed T allele frequency was significantly higher in the patient group (26%) than the control group (10%) (p<0.001, OR=3.03, 95% CI (1.74-5.27). When we compared the mean value of serum homocysteine level, folic acid and vitamin $B_{12}$ with MTHFR 677 C/T (rs1801133) polymorphism, we found CT and TT genotyping has higher mean concentration of serum homocysteine (p<0.0001) and low-level folic acid (p=0.01) in both cases and controls and this association was statistically significant for both cases and control but no significant association has been observed for vitamin $B_{12}$ levels. We have also compared serum lipid profile with MTHFR 677 C/T (rs1801133) genotyping in our study subjects. Our data showed that patients with CT and TT Genotype have lower serum HDL level p<0.0001 in cases, although the serum HDL levels are low in TT and CT genotype but the difference was not significant in control group, also no significant differences had been found between the genotypes and other blood lipid profile parameters.as shown in Table 4.

***Methylation levels in cad and healthy subjects***

In our study patients with CAD had a higher frequency of hyper methylation in promoter region as compare to the individuals in Control (OR=3.05, CI=1.7-5.6, p value=0.01), as shown in Table 5. When we compared the mean of serum homocysteine level, folic acid levels and vitamin $B_{12}$ and methylation profile in study subject, we found that, mean serum homocysteine level was significantly higher in study subjects with hyper methylated promoter region both for cases and control group as compare to study subjects with partial methylated promoter region ($p<0.008$), however no significant association has been observed for vitamin $B_{12}$ levels and folic acid level. When we compared lipid profile parameters with methylation profile of promoter region, no significant association had found as shown in Table 6.

## DISCUSSION

Hyperhomocysteinemia is defined as homocysteine levels above 15 µmol/l (normal range: 5-15 µmol/l). According to some observational studies for every 5 µmol/l increase in serum homocysteine levels, risk of CAD is increased by32%.[17] In our study we have found that serum homocysteine levels are higher in CAD patients as compare to controls which supports the conclusion of earlier studies that homocysteine is a risk factor for CAD.[17] Folate and vitamin $B_{12}$ are involve in re-methylation pathway of homocysteine so in our study we compared average serum folate and $B_{12}$ levels but no significant difference had been found between cases and controls (Table 1) but when we correlated serum homocysteine levels with serum folate and vitamin $B_{12}$, negative association was found between them both in cases as well as controls which is statistically significant. The findings of our study were consistent with studies done by Zhang et al, Chambers et al and Zhou et al.[18-20]

***C677T polymorphism and CAD***

In our, study, we found higher frequency of T allele in cases as compared to controls OR=3.03, $p<0.01$. In some studies done by Dhar et al, Matam et al, Raina et al they have also found the association of the C677T polymorphism with CAD.[21-23] Despite the increased frequency of the T allele in patients compared to controls, Pandey et al and Vashist et al found no significant connection between T allele frequency and CAD.[24,25] On the other hand some researchers' findings indicated a negative correlation between CAD and MTHFR gene polymorphism.[25-27] In our study we have also observed a statistically significant association between the MTHFR (C677-T) polymorphism with hyperhomocysteinemia $p<0.0001$.which is in accordance with the studies done by Lakhdar et al and Long et al however according to the study done by Rassoul et al relationship of serum hyperhomocysteinemia seemed to be independent of MTHFR C677T polymorphism.[28-30] When we compared serum folic acid and $B_{12}$ levels with C677T polymorphism we have found that CT and TT genotype had lower level of serum folic acid and vitamin $B_{12}$ levels but this association was significant only for folic acid in case as well as in controls, similar finding has been observed by Tsang et al and Shivkar et al in their studies.[31,32]

Dyslipidemia which is one of the independent risk factor for CAD is associate with both genetic and environmental factors but at present, the correlation between MTHFR gene polymorphism and serum lipid profiles is still controversial in diverse ethnic groups.[33] In the studies done by Jiang et al and Wang et al they have found that MTHFR C667T gene polymorphism is correlated with dyslipidemia.[34,35] Zhang et al showed that MTHFR 677T carriers exhibit significantly increased serum levels of TC and LDL-C.[36] However, Liang et al have found that there is no correlation between MTHFR C667T gene polymorphism and dyslipidemia.[37] Our results showed that, TT and CT genotype had lower levels of HDL-C than CC genotype, however significant difference have been observed only foe CAD patients, similar finding have been observed in study done by Guan et al on Chinese population.[38]

***DNA methylation and CAD***

In the current investigation, we have found that the hyper methylation profile in the promoter region of MTHFR gene is associated with CAD. The subjects with CAD showed a higher frequency of individuals with a hyper methylated profile then individuals in control group. These differences are statistically significant ($p=0.001$, OR=2.91, 95%CI: 1.5-5.5). We have also found a statistically significant association between hyper homocysteinemia in the study group and hyper methylation of the MTHFR gene promoter region ($p$ value $<0.008$). In the study done by Kulkarni et al they had examined the relationship between pre-eclampsia and MTHFR gene promoter region methylation in the placental tissue of 30 healthy pregnant women and 57 pre-eclampsia patients in India.[39] Their findings also imply that plasma Hcy levels is associated with MTHFR gene promoter regions hyper methylation. Dos Santos Nunes et al discovered a similar link in patient with type 2 diabetes mellitus and hyper methylation in the MTHFR gene promoter region.[16]

In contrary to that the study done by Xu et al on ischemic stroke patient they have found that hypermethylation in the promoter region of MTHFR gene have a protective nature and decreases the serum homocysteine level in study subjects.[40] When we compare vitamin $B_{12}$, folic acid level and parameters of lipid profile no significant association has be observation in our study. In the study done Bezerra et al they have found that hyper methylated promoter region of MTHFR gene to be associated with increased level of LDL and total cholesterol levels which is contrary to our results.[41]

There are some limitations of the study. As genetic and epigenetic modification in MTHFR gene can be due to race and geographical origin and our study was hospital-based, the associations and the underlying mechanisms still need further studies with large-scale (population-based) samples and modified designs.

## CONCLUSION

From the present study it was concluded that, the CT, and TT genotype of MTHFR C677T gene polymorphism and promoter region hypermethylation increases the risk of CAD and the association of T allele carrier with CAD is being partly mediated by blood lipids and potentially a mediated by non-lipid pathways. Folic acid supplementation can help patient with MTHFR C677T gene polymorphism but not with promoter region hypermethylation. In further scope, longitudinal follow up of participants could be done for predicting development of future cardiac events.

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