Original Research Article

Study of association of MTHFR 677 C→T gene polymorphism in coronary artery disease

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Abstract

Coronary Artery Disease (CAD) continues to be a major cause of mortality, globally for the last few decades. Hyperhomocysteinemia (HCA) resulting from MTHFR 677 C→T polymorphism as a reason for CAD in India has been debatable. The purpose of this study was to analyze the possible association between MTHFR 677C→T polymorphism with CAD by performing a case control study in subjects from Navi Mumbai. Subjects with CAD (n=49) below the age of 65 were compared with a similar number of age and gender matched controls without CAD. According to genotypic analysis, 46 and 48 individuals with CC genotype; 3 and 1 individual with CT genotype were found in cases and controls respectively. No individuals with TT genotype were found in either group. Statistical analysis of biochemical parameters revealed significantly higher levels of plasma homocysteine (Homocysteine) in cases than in controls (p= 0.00), which was statistically significant. However the analysis did not show a significant difference in the Homocysteine levels of CC and CT genotypes within the cases (or in controls). The T allele presents a higher relative risk (OR= 3.0632) in susceptibility to CAD, however, this is not statistically significant (p = 0.3361). Although our study failed to find any association between the existing polymorphisms of MTHFR gene and CAD, it did find an association between HCA and CAD.

Key Words: C677T MTHFR, CAD, homocysteine, hyperhomocysteinemia.

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INTRODUCTION

Coronary Artery Disease (CAD) is one of the biggest challenges in healthcare the world is facing today. Globally, an estimated 7.4 million people lost their lives due to CAD in the year 2015[1]. In recent years, the prevalence and mortality due to CAD is declining in the developed countries, but sadly that is not the scenario in the developing ones[2]. According to the *Second Indo-US Healthcare Summit*, the prevalence of CAD in India, a developing country, has increased 300%, in the last 30 years, with the rate of increase in CAD accelerating to 6-8% per year[3].Reports from the Registrar General of India show that in 2001-2003, CAD accounted for 17% of the total deaths, which further rose to 23% in 2010-2013[4-5].CAD is a multi factorial disorder. Obesity, Type 2 diabetes are its co morbidities. Inflammation plays an important role in developing CAD in obese and diabetic patients[6,7]. Some routine biomolecules also play an important role as cardiac markers[8] and homocysteine is one of it[9]. In the year 1969, Dr. Kilmer S. McCully, M.D., checked the relationship between the death due to heart attack of an 8 week old infant with a cobalamine metabolism defect and the death due to stroke of an 8 year old patient having homocysteinuria[10,11]. He

concluded that elevated homocysteine concentrations in the blood lead to atherosclerosis. Homocysteine is an endothelial toxin which can cause inflammation of the endothelial lining of blood vessels, causing atherosclerosis, myocardial infarction, heart failure and stroke[12]. Normal levels of homocysteine in the blood range from 4 – 15 µmol/L. Hyperhomocysteinemia (HCA), a condition characterized by levels of homocysteine greater than 15 µmol/L, results from a diet high in animal protein and low in fruits and vegetables since these vegetables are the source of folic acid and other B vitamins required for metabolizing homocysteine[33]. HCA can cause arterial stiffness as it may bring about remodelling of the arterial wall causing arterial damage[12]. This condition also predisposes individuals to an increased platelet adhesion to vascular walls and increases thrombotic tendency[12]. Apart from diet, another etiological culprit incriminated in causing HCA leading to CAD is the genetic factor. Mutations in several genes may be culpable in the development of HCA and CAD. One such gene is the Methylene tetrahydrofolate reductase (MTHFR) gene, incumbent at chromosome 1p 36.3, having several mutations of which C677T is the most notorious one. C677T is implicated in several disorders viz. Down's syndrome, Acute Lymphoblastic Leukemia, Colorectal cancer, Ischemic stroke and also in atherosclerosis leading to CAD[12]. The enzyme MTHFR as the name suggests, reduces 5,10-methylenetetrahydrofolate to the predominant circulatory form of folate, namely 5-methyltetrahydrofolate. The product so formed acts as a single carbon donor for remethylation of homocysteine to methionine [13] [Figure 1]. Methionine itself serves as a precursor for synthesis of S-adenosyl methionine (SAM), which serves as a methyl donor in most methylation reactions viz. the methylation of DNA, proteins, neurotransmitters and phospholipids[12]. A mutation at the 677th position of the gene, replacing a C with a T, leads to substitution of Valine in place of Alanine in the translated enzyme[14].This substitution gives rise to a thermo-labile enzyme with reduced or no activity at all[15,16], ultimately leading to HCA and hypomethylation of the before mentioned biomolecules (because of a paucity of SAM) both of which have far reaching consequences. The objective of this study is to examine the association between MTHFR 677C→T gene polymorphism and CAD in the subjects from Navi Mumbai by performing a case control study; and to evaluate the possibility of using MTHFR C/T alleles as markers for screening and identification of patients predisposed to hyper homocysteinemia and at risk for developing Coronary Artery Disease.

MATERIAL AND METHODS

Subject selection: This Case-control study was conducted on 98 patients attending the medical OPD at the Dr. D. Y. Patil Hospital and Research Centre, of which half the subjects, 49, were diagnosed with CAD while the remaining half were healthy subjects tested negative for CAD. The subjects included in the study were of either gender in the ratio of 3:1 (male: female) in both the study groups (Case and Control) with their age as or below 65 years, diagnosed with (Cases) or without (Controls) CAD. A signed informed consent was collected from each subject. Approval for the study was obtained from the ethics committee of the hospital prior to its initiation.

Biochemical Analysis: Fasting blood samples anticoagulated with Ethylene diamine tetracetic acid (EDTA) was collected from subjects after 12 hours of fasting for genotypic studies and separately in a plain container with no-anticoagulant to measure the biochemical parameters. *In vitro* quantitative estimation of the following biochemical parameters: homocysteine, total cholesterol (TC), triglycerides (TG), very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) were carried out at the D. Y. Patil School of Biochemistry and D. Y. Patil Medical College and Research Centre. LDL to HDL ratio was also determined from the above.

DNA extraction and Characterization: Extraction of DNA from whole blood of the subjects was performed using a commercial DNA extraction kit for whole blood (QIAamp DNA Blood Mini Kit, Qiagen, India). The integrity of the extracted DNA was checked by electrophoresing the nucleic acid using 1% agarose gel.

Genotyping: Genotyping of MTHFR gene was performed using the primers reported from prior study by Matam *et al* [25] [Table 1]. *In silico* verification of primers was performed using Primer Blast[17]. PCR reactions were performed in a thermal cycler (Bio-Rad, USA) programmed with an initial denaturation at 94 for 4 minutes, followed by 35 cycles of denaturation at 94 for 30 seconds, annealing at 54 for 45 seconds and extension at 72 for 45 seconds and terminating the process with a final extension at 72 for 12 minutes. Amplification of the 198bp MTHFR C677T SNP containing segment was confirmed by subjecting the PCR product to electrophoresis using 2% agarose gel. The PCR products were digested at 37 for 2 hours with 2 units of Hinf1 enzyme in a total reaction volume of 20µL. The Hinf1 enzyme for Restriction Enzyme Digestion was validated using the online software NEB cutter. Hinf1 digested the 677C→T mutated MTHFR to 175bp and 23bp products which was visualized on a 3% agarose gel. The genotypes observed were either CC (with a 198bp band); or CT

(with 198bp, 175bp and 23bp bands); whereas no TT: (with 175bp and 23bp bands) were observed.

Statistical Analysis: Data analysis was performed using the statistical software for windows IBM SPSS STATISTICS (Version 20). T-test was used for comparing the lipid profile and homocysteine levels between cases and controls and also to compare the same between the genotypes in the patient group. The allelic relative risk of developing disease for allele T relative to allele C was determined using the online software MedCalc Online Calculator [18]. Cut Offs for lipid profiles as per ATPIII guidelines were considered to determine the association between the risk profile and CAD[19].The certainty that the population follows Hardy-Weinberg equilibrium was authenticated by calculating Chi Square (χ^2) manually.

RESULTS

Table 1 Information on primers and PCR product

Forward primer	TGAAGGAGAAGGTGTCTGCGGGA
Reverse primer	AGGACGGTGCGGTGAGAGTG
Product length	198 bps

Table 2: Comaprison of lipid profile and plasma homocysteine levels between cases and controls

Profile	Cases (n=49) (Mean ± SD)	Controls (n=49) (Mean ± SD)	P value
TC (mg/dL)	206.0163 ± 52.54342	178.9653 ± 38.50107	0.005
TG (mg/dL)	144.692 ± 61.6358	128.604 ± 60.7465	0.196
VLDL (mg/dL)	29.504 ± 14.3839	25.522 ± 12.3016	0.144
LDL (mg/dL)	130.482 ± 39.2502	110.271 ± 37.1802	0.010
HDL (mg/dL)	42.651 ± 13.8357	43.167 ± 14.4094	0.857
L/H	3.3541 ± 1.55247	2.7978 ± 1.24746	0.053
HOMOCYSTEINE(µmol/L)	13.18 ± 3.751	9.43 ± 2.752	0.000

Table 3: The genotypic and allelic frequencies in cases and controls

Groups	Genotypic Frequency CC	CT	TT	Allelic Frequency C	T
CAD Patients (Cases)	0.94 (46)	0.06 (03)	0.00 (00)	0.97	0.03
Non CAD patients (Controls)	0.98 (48)	0.02 (01)	0.00 (00)	0.99	0.01
Total Population	**0.96 (94)**	**0.04 (04)**	**0.00 (00)**	**0.99**	**0.02**

Table 4: Calculation of relative risk (Odds Ratio)

Profile	C	T
Odds Ratio (95% CI)	0.3265 (0.0334, 3.1943)	3.0632 (0.3131, 29.9723)
Z Statistics	0.962	
Significance Level	p = 0.3361	

Table 5: Comaprison of lipid profile and plasma homocysteine levels between CC and CT genotypes in cases

Profile	Genotype (Cases) CC (n=46) (Mean ± SD)	CT (n=3) (Mean ± SD)	P value
TC (mg/dL)	207.926 ± 48.67	176.733 ± 107.6664	0.324
TG (mg/dL)	147.122 ± 60.2067	107.433 ± 86.0119	0.285
VLDL (mg/dL)	30.059 ± 14.2670	21.00 ± 16.4353	0.295
LDL (mg/dL)	133.678 ± 37.7965	81.467 ± 31.4999	0.024
HDL (mg/dL)	42.659 ± 13.8326	42.533 ± 17.0016	0.988
L/H	3.4493 ± 1.55404	1.8933 ± 0.30925	0.093
HOMOCYSTEINE(µmol/L)	12.93 ± 3.660	17.00 ± 3.606	0.068

Table 6: Analysis of lipid profile as per ATPIII guidelines for cases and controls

Profile (Cut Off values)	Cases	Controls	Stastical Analysis
TC (mg/dL)			P=0.0008 OR = 4.31
Risky (≥ 200)	31	14	95% Confidence Interval
Optimal (< 200)	18	35	(CI) of OR = 1.84-10.07
TG (mg/dL)			P=0.14 OR = 1.88
Risky (≥ 150)	21	14	95% CI of OR = 0.81-4.34
Optimal (< 150)	28	35	
VLDL (mg/dL)			P=0.0027 OR = 3.63
Risky (≥ 30)	29	14	95% CI of OR = 1.56-8.41
Optimal (< 30)	20	35	
LDL (mg/dL)			P=0.0671 OR = 2.15
Risky (≥ 130)	25	16	95% CI of OR = 0.95-4.87
Optimal (< 130)	24	33	
HDL (mg/dL)			P=0.4076 OR = 0.71
Risky (≤ 40)	17	21	95% CI of OR = 0.31-1.6
Optimal (> 40)	32	28	
Homocysteine (µmol/L)			P=0.0016 OR = 27.87
Risky (≥ 15)	18	1	95% CI of OR = 3.54-219.49
Optimal (< 15)	31	48	

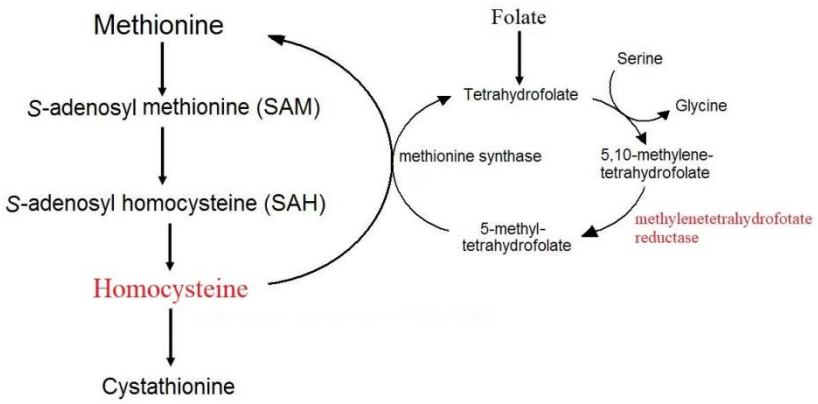

Figure 1: The pathway for the metabolism of Homocysteine(in red) in humans showing the role of the enzyme(in red) in the pathway.

Figure 2: Shows the percentage of people above and below the cut-off values for each profile, with the outer circle showing the cases and the inner showing controls; RED for subjects with risky values and BLUE for subjects with optimal values[31].

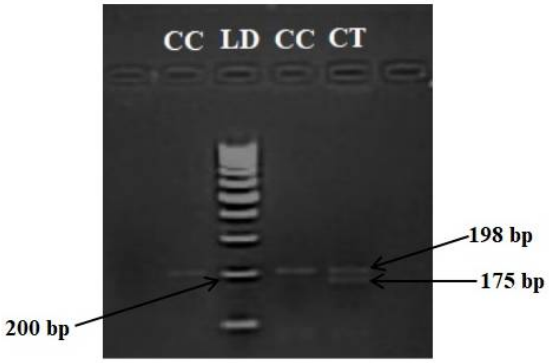

Figure 3: Agarose gel picture with two genotypes of MTHFR C677T polymorphism LD = 100 bp DNA molecular ladder

Cases and controls were age matched and ratio of females to males was approximately 2:3 in both the groups. Table 2 shows the statistical analysis of lipid profile. There was a significant difference ($p < 0.05$) recorded between TC, LDL and homocysteine levels of cases and controls. The mean fasting levels of plasma homocysteine in the cases was found to be 13.1 µmol/L and the geometric mean 12.59 µmol/L with the values ranging from 5 to 21 µmol/L Using 15 µmol/L as cut off for plasma homocysteine levels, we got 18 subjects (36.73%) in the case group and 1 in (2.04%) the control group with >15 µmol/L. From the PCR – RFLP analysis, individuals with homozygous CC genotype were determined to be 46 (93.88%) of cases and 48 (97.96%) among controls. Individuals with the heterozygous CT genotype in the cases and controls were determined to be 3 and 1 respectively. None of the subjects in either group had the homozygous TT genotype. The genotypic and allelic frequencies are shown in Table 3. We found the prevalence of CT genotype to be 6.12% among the cases and 2.04% in the controls. The T allele frequency in controls was found to be 1.02% and that of the patient group was 3.06%. On calculating odds ratio, it was seen that T allele presents a higher relative risk (OR=3.0632, 95% CI: (0.3131- 29.9723, but $p> 0.05$) in susceptibility to CAD as shown in Table 4. Chi Square (X^2) was found out to be 0.046, ie. $X^2< 3.84$, thus proving that the population is in Hardy-Weinberg equilibrium. Homocysteine levels was not found to be significantly different in subjects with CC and CT genotypes among the cases, despite the mean Homocysteine of CT genotype (17 µmol/L) being more than that of CC genotype (12.93 µmol/L). Among all the biochemical parameters, only LDL levels showed a significant difference between CC and CT genotype in the case group ($p < 0.05$). [Table 5] Cut-offs as per ATPIII guidelines for lipid parameters were taken for calculating relative risk of high risk profile over optimal [Table 6][19].TC, VLDL and homocysteine levels could be significant independent predictors of CAD.

DISCUSSION

In the present study, the frequency of homozygous wild type (CC) genotype was more frequently detected than the other two genotypes with no TT genotype in either of the groups. The T allele presents a higher relative risk (OR = 3.0632) for susceptibility to CAD but the risk was not statistically significant. This could be related to the small sample size which was studied. Also, our study failed to show a significant association between the CT genotype and HCA. The present study showed significantly higher levels of plasma homocysteine concentrations in the case group as compared to the control group, highlighting the probability of it being an independent risk factor for CAD. The same was observed with serum cholesterol and LDL levels. Like in Gupta *et al.,* (2012)[20], even we introduced the cut offs as per ATPIII for lipid parameters. The statistics with the cut offs showed a significant difference between the risky and optimal values of lipid parameters establishing a positive association of TC, VLDL and Homocysteine levels with CAD. MTHFR 677C→T polymorphism has received a lot of attention with a few studies around the world showing positive association[14,21,22], with CAD and same is the case with a few Indian studies as well [23,25,27]. A study on a North Indian population reported a significant association between Homocysteine levels and TT genotype, and also stated that the synergy between HCA and the T allele could be affecting the severity of CAD[25]. A study on a small sample of the Fars province, Iran reported 83.3% prevalence of polymorphic homozygous TT genotype in their cases, further proving MTHFR C677T polymorphism to be an important risk factor for myocardial infarction[22]. Another study with the East Indian population also reported a positive association of the MTHFR C677T polymorphism with CAD[23]. A study in the population of southern part of India noted the association of T allele with the increased risk of CAD[26]. A meta-analysis by Chao Xuan *et al.,* (2014)[27], which included 35 studies on 9,329 cases and 15,076 controls, demonstrated the positive association of the T allele with myocardial infarction[27]. However, there were also several studies around the world showing negative associations[13,28-30]. A study in a North Indian population reported that the T allele was not associated with the risk of CAD[28]. A recent study in a South Indian population also failed to show any association between the polymorphism and the disease[13]. After additional serum folate adjustments, a study in the population of Taiwan reported the association of MTHFR C677T polymorphism with increased plasma Homocysteine but its association with CAD was negative[29]. Similar results were reported in a study with subjects from Southern Iran, where the polymorphism was associated with the increased Homocysteine levels, but not with CAD[30]. Our results were concurrent with the results of Eftychiou *et al.,* (2012)[31] and Gupta *et al.,* (2012)[20,] where no association was found between MTHFR 677C→T polymorphism and CAD but a positive association was found between high levels of Homocysteine and CAD. The lack of an association may be due to the multifactorial etiology, in coronary artery disease including factors such as diet, exercise, obesity and folate/ cobalamine levels. Some studies suggested that homocysteine causes the inhibition of the hepatic

synthesis of the main HDL apolipoprotein, ApoA1, thus reducing the concentration of HDL[32, 33].

CONCLUSION

In conclusion, the current observation did not support the association between MTHFR 677C→T polymorphism and CAD, however, high levels of homocysteine and CAD did show some links.

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