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Association of polymorphism in the thermolabile 5, 10-methylene tetrahydrofolate reductase gene and hyperhomocysteinemia with coronary artery disease

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Abstract *Objective* To determine the incidence of methylene tetrahydrofolate reductase (*MTHFR*) gene 677C→T polymorphism and plasma homocysteine (Hcy) levels in a group of subjects who underwent coronary angiography, in an attempt to establish a correlation between these parameters and the severity of coronary artery disease (CAD) and to investigate the correlation between hyperhomocysteinemia (HHcy) and the presence of 677C→T polymorphism. *Background* Elevated plasma Hcy level is an independent risk factor for CAD. A common mutation (677C→T) in the gene coding for *MTHFR* has been reported to reduce the enzymatic activity and is associated with elevated levels of Hcy, especially in subjects with low folate intake. *Methods* The study group comprised of 84 patients with CAD and 100 age-and-sex matched controls who had no history or clinical evidence of CAD and/or MI. DNA was extracted from peripheral blood and genotypes were determined by polymerase chain reaction, restriction mapping with *Hinf*1, and gel electrophoresis. Conventional risk factors for CAD were prospectively documented. *Results* Allele and genotype frequencies in cases and control subjects were compatible with Hardy–Weinberg equilibrium. The frequencies of TT, CT, and CC genotypes among CAD patients were 4.8, 27.4, and 67.8% and in controls were 1.0, 19.0, and 80%. Hcy levels were higher in patients with triple-vessel disease compared to single and double vessel disease ($P = 0.002$). Multivariate analyses identified HHcy, diabetes mellitus, and hypertension as the independent predictors of CAD. *Conclusions* HHcy appears to have a graded effect on the risk of CAD as well as the severity and extent of coronary atherosclerosis. Our findings support that homozygous genotype of *MTHFR* is a genetic risk factor for CAD. A further study with larger sample size including assessment of vitamin status is needed to better clarify the relationship between *MTHFR* genotypes and CAD.

Keywords Hyperhomocysteinemia · Methylene tetrahydrofolate reductase · Coronary artery disease

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Introduction

Hyperhomocysteinemia (HHcy) is one of the newly recognized risk factors of CAD, MI, stroke, and peripheral arterial and venous thrombosis. Basic and clinical research has established that an increase in plasma Hcy is an independent and graded risk factor for CVD [1]. The magnitude of disease risk is not entirely explained by traditional risk factors, which account ≈50% of all cause mortality [2–4].

HHcy is thought to be responsible for about 10% of the total risk.

The Hcy metabolism represents an interesting model of gene–environment interaction. Elevations of Hcy may be caused by genetic defects in enzymes involved in its metabolism or by deficiencies in cofactor levels. The 5, 10-methylene tetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5, 10-methylene tetrahydrofolate to 5-methyltetrahydrofolate, which is required as a methyl donor for conversion of Hcy to methionine via a reaction that is regulated by the vitamin B_{12}-dependent methionine synthase. A common 677C→T transition in the *MTHFR* gene results in a thermolabile variant with specific decreased enzymic activity and is well established as a genetic determinant of HHcy. The molecular basis of this thermolability is a missense mutation in exon 4 of the *MTHFR* gene, a cytosine to thymine substitution at nucleotide 677, which converts an alanine to a valine codon [5]. The protective effect of folate may be mediated by stabilization of FAD binding. This association of the *MTHFR* genotype with Hcy is well known to be contingent on folate status [6]. Several studies have reported thermolabile *MTHFR* as a risk factor in vascular disease [7–10] with few contrary studies [11, 12]. Nevertheless, due to the high incidence ~40% in the general population and its physiological role, the 677C→T mutation may represent an important genetic risk factor of Hcy associated CAD [13–15].

The prevalence of the 677C→T polymorphism varies greatly in different ethnic groups. This polymorphism among Asians in relation to CAD was studied mainly in Japanese and Chinese [16]. Limited data are available for other Asian populations, especially for Indians. Though, Indians have high prevalence of early CAD [17–19]. During the next decade (2020), the prevalence is likely to increase to 90% for females and 103% for males [20]. We therefore undertook the present study to determine the incidence and correlation of *MTHFR* polymorphism with plasma Hcy levels and CAD.

Materials and methods

Study subjects

The study population comprised 100 healthy controls and 84 patients with CAD. All the subjects were of North Indian origin. For inclusion, cases had to have ≥70% stenosis of any of the major coronary arteries confirmed by cardiac catheterization. Of 84 patients, 41 were single vessel, 31 were double vessel, and 12 were triple vessel disease. Age-and gender-matched clinically healthy controls with background as similar to cases as possible were recruited from hospital-based staff and friends. Controls had to be free of overt vascular disease and a negative family history of CAD. A written informed consent was obtained from every participant after explaining the aims and objectives of the study, which was approved by the local ethics committee.

Blood samples and biochemical investigations

An overnight fasting venous blood sample was collected in EDTA tubes by puncture of cubital vein and a part of sample was kept on ice. It was immediately centrifuged at 2500 rpm for 10 min at 4°C. The plasma fraction was aliquoted and stored at −80°C until Hcy estimation. Lipid profile was done on fresh sample on Beckmann (Beckmann, US) auto-analyzer using enzymatic kits (Randox, UK). Cells were further assessed for genetic polymorphism.

Homocysteine estimation

Homocysteine plasma concentration was assayed by enzyme-linked immunoassay (ELISA) using a standard commercial kit (Diazyme, CA, USA). Briefly, protein-bound Hcy in the serum sample is reduced to free Hcy and converted enzymatically to S-adenosyl Hcy (SAH) before the immunoassay. This is followed by a solid-phase enzyme immunoassay based on competition between SAH in the sample and immobilized SAH bound to the wall of the microtiter plate for binding sites on a monoclonal anti-SAH antibody. After removal of the unbound anti-SAH antibody, a secondary rabbit anti-mouse antibody labeled with the enzyme horseradish peroxidase is added. The absorbance is inversely related to the concentration of total Hcy in the sample. A calibration curve was constructed using a standard (2.0–50.0 μmol/l) provided with the kit.

Genetic analysis

DNA was extracted from peripheral blood leukocytes using standard phenol-chloroform extraction method. The DNA quality was confirmed by electrophoresis using a 0.7% agarose gel and quantity determined using absorbance spectrophotometry. Genetic polymorphism was studied by PCR-RFLP analysis. In order to detect the 677C→T mutation in exon 4, genomic DNA was amplified by PCR using primers as previously reported [5] in a PTC-100 thermal cycler (MJ Research Inc.) followed by restriction enzyme digestion. The PCR mixture (50 μl) contained 100 ng of DNA template, 20 pmol of each primer, 200 mmol each dNTP, 0.5U of Taq polymerase (New England Biolab, UK), and 10× PCR buffer having 25 mmol $MgCl_2$. The cycle

parameters were as follows: 1 cycle of initial denaturation for 3 min at 94°C followed by 35 cycles of denaturation for 50 s at 94°C, annealing for 60 s at 65°C, extension for 50 s at 72°C, and a final extension for 7 min at 72°C. The 198-bp PCR product (10 µl) was digested with the restriction enzyme *Hinf*1 (Roche Diagnostics, USA), at 37°C for 3–4 h in the buffer recommended by the manufacturer. *Hinf*1 can recognize the C-to-T substitution in the fragments. The 198-bp fragment derived from the C allele is not digested by *Hinf*1, whereas the fragments of the same length from the T allele are digested into two fragments of 175, and 23-bp, hence heterozygous subjects showed three fragments of 198, 175 and 23-bp. The *Hinf*I-treated PCR fragments were analyzed by 10% polyacrylamide gel electrophoresis at 90 V for 2.0 h, stained with ethidium bromide and visualized under ultraviolet light.

Statistical analyses

Continuous variables were expressed as mean ± SD and were compared by student's *t*-test or ANOVA for more than two groups. Categorical variables were compared between groups by use of χ^2 test and crude odds ratio. The χ^2 test for homogeneity of proportions was used to test the equality of the prevalence of different genotypes for the healthy control. Multivariate analyses were done with a logistic regression model adjusted for clinically significant variables. Statistical analyses were performed with SPSS 11.0. A probability value of <0.05 was considered statistically significant.

Results

Clinical and biochemical analysis

The clinical and biochemical characteristics of the patients and controls are summarized in Table 1. As expected the patients with CAD had significantly high value of Hcy, apoB, systolic and diastolic blood pressure, fasting sugar, and BMI, whereas low levels of HDL C compared to controls. A significantly higher prevalence of HHcy, diabetes, and hypertension was observed in patients. Mean age of the patients was 51.90 ± 11.36 years while for controls it was 49.83 ± 10.67. Among patients 81.5% were male as compared to 75% male in control group.

Genotype association with phenotype

The prevalence of the *MTHFR* genotypes among subjects from the general population was in the range of Hardy–Weinberg equilibrium. The T and C allele frequency was 0.11 and 0.89 ($\chi 2 = 0.012$, df = 1, P = NS) in the control group. However, T allele frequency was significantly higher in patients and was found to be associated with the increased risk of CAD (OR = 1.93; 95% CI: 1.02–3.66, $P = 0.042$). The frequency distribution of three genotypes was as follows in patients (CC genotype, 67.8%; CT genotype, 27.4%, and TT genotype, 4.8%) and in controls (CC genotype, 80%; CT genotype, 19%, and TT genotype, 1%) ($P = 0.096$). A trend for high prevalence of TT mutant genotype was observed in patients. The *MTHFR* CT genotypes were found to be associated with increased risk of CAD (OR = 1.61; 95% CI: 0.76–3.40) and TT genotypes (OR = 4.95; 95% CI: 0.51–48.65). CT and TT genotypes combined showed a significant association with CAD risk (OR = 1.89; 95% CI: 0.96–3.91, $P = 0.059$). The χ^2 *P*-value for CT genotypes was found to be associated with increased risk of CAD (OR = 1.70; 95% CI: 0.80–3.61, $P = 0.133$) when compared to CC genotype. No significant difference was observed between male and female subjects for either any genotype or allele frequency (Table 1).

Table 1 Clinical and biochemical characteristics of the patients and controls

Variables	Patients ($n = 84$)	Controls ($n = 100$)	*P* value
Age (years)	51.90 ± 11.36	49.83 ± 10.67	0.827
BMI (kg/m^2)	25.76 ± 4.46	22.96 ± 4.78	**0.0001**
Homocysteine (µmol/l)	23.33 ± 11.82	14.57 ± 6.08	**0.0001**
Total cholesterol (mg/dl)	190.00 ± 41.95	184.43 ± 37.79	0.349
LDL cholesterol (mg/dl)	119.61 ± 31.56	113.78 ± 30.46	0.209
HDL cholesterol (mg/dl)	39.38 ± 5.09	42.14 ± 5.75	**0.001**
Triglyceride (mg/dl)	152.73 ± 63.76	138.10 ± 59.65	0.113
SBP (mmHg)	134.42 ± 18.74	122.21 ± 13.04	**0.0001**
DBP (mmHg)	85.68 ± 8.44	82.98 ± 8.84	**0.037**
Sugar (mg/dl)	105.94 ± 35.76	88.75 ± 32.99	**0.001**
Male, *n* (%)	68 (80.9%)	75%	0.144
Smokers, *n* (%)	42 (50%)	40%	0.226
Diabetes, *n* (%)	29 (34.5%)	4%	**0.0001**
Hypertension, *n* (%)	44 (52.4%)	15%	**0.0001**
HHcy, *n* (%)	50 (59.5%)	21%	**0.0001**
MTHFR genotype, n (%)			
CC	57 (67.8%)	80%	0.096
CT	23 (27.4%)	19%	
TT	4 (4.8%)	1%	
Alleles			
C	0.82	0.89	**0.042**
T	0.18	0.11	

BMI, Body mass index; LDL, Low density lipoprotein; HDL, High density lipoprotein; ApoB, Apolipoprotein B, SBP, Systolic blood pressure; DBP, Diastolic blood pressure.

Values are mean ± S.D. *P* values in bold show significance at 1% level

The frequencies of the TT genotype were 0% for single, 50% each for double and triple vessel disease patients, respectively ($P = 0.003$). Hcy levels were significantly higher in patients with multi-vessel disease compared with single and double vessel disease ($P = 0.002$). HHcy was more prevalent among TVD than DVD or SVD patients (Table 2).

The association of the biochemical parameters and other risk factors for CAD was studied in different *MTHFR* genotypes. None of the parameters showed any significant association with *MTHFR* genotypes except Hcy (TT = 33.41 ± 14.89, CT = 23.92 ± 11.09 and CC = 19.93 ± 7.59, $P = 0.0001$). All the four patients with TT genotype had Hcy > 17.5 μmol/l. No significant association was observed between any of the *MTHFR* genotype with diabetes or hypertension (Table 3). Among controls, no significant difference was found for the studied parameters for any of the genotypes.

Multivariate regression analysis

Multiple logistic regression analysis was performed in order to evaluate the risk of HHcy for CAD in the context of other known CAD risk factors such as diabetes mellitus, hypertension, genotypes, age, sex, lipids, and cigarette smoking. Results showed that HHcy was independently associated with the risk of CAD (OR = 5.31, 95% CI: 2.42–11.68; $P = 0.0001$) than others adjusted in the model (Table 4).

Discussion

The *MTHFR*, a gene involved in the metabolism of Hcy, is of particular medical interest as being a younger genetic risk factor for CAD. The relation between elevated plasma Hcy and vascular disease risk is independent of traditional risk factors [21]. Although the association is stronger in case-control studies with established vascular disease [21] than in prospective studies [22, 23], a meta-analysis of prospective studies indicates a robust relation [24]. Further studies suggest that the Hcy level is a strong predictor of cardiovascular events in the early follow-up period and that this risk relation may become attenuated over time [25]. Kluijtmans et al. [8] suggested that risk due to the TT mutant genotype might be confined to those with CAD.

From the analysis of the present study and studies from India and in Indians abroad, the prevalence of T allele is found to be lesser than Caucasians and other Asians [16]. The 'T' allele frequency observed by this study (0.11) is comparable to that of the recent studies conducted in South and North Indian subjects but is higher than Sri Lankans (0.049) [18, 26, 27]. Mexican population has the highest 'T' allele frequency of 0.59. The Japanese and Taiwanese population showed a relatively high 'T' allele frequency of 0.37 and 0.29 [8]. In this study, frequencies of *MTHFR* 677TT genotype in CAD cases and controls were 4.8% and 1% respectively, which is comparable to our previous reports [28] and were slightly higher than recently conducted case–control studies in Asian Indians [18, 26, 29]. The Korean study [30] found 18% TT genotype while Morita et al. [7] reported a 16% TT genotype in Japanese CAD patients, which is 11–13% higher than our results. This polymorphism is reported to have a relatively high frequency throughout the world and can be regarded as a balanced polymorphism that escaped natural selection. Such a polymorphism does not usually imply a serious, lethal disorder. However, it can contribute to the pathogenesis of multifactorial diseases, such as CAD.

The result of present study does support the 677C→T mutation as a risk factor for CAD. The TT *MTHFR* genotype is associated with increased plasma Hcy levels, which could be associated with increased risk of vascular disease. Meta-analyses conducted on individual data from case–control studies [13, 31, 32] have concluded a modest but statistically significant increased risk of CAD in subjects with the 677TT genotype. Accordingly, a recent large meta-analysis of 32 studies ($n = 14{,}870$) showed a graded increase in ischemic stroke risk with increasing 677T allele

Table 2 Distribution of *MTHFR* genotypes and high and low Hcy in CAD patients with different numbers of stenotic coronary artery

MTHFR 677 genotype	SVD ($n = 41$)	DVD ($n = 31$)	TVD ($n = 12$)	*P* value
CC, *n* (%)	34 (59.6%)	20 (35.1%)	3 (5.3%)	
CT, *n* (%)	7 (30.4%)	9 (39.1%)	7 (30.4%)	
TT, *n* (%)	0 (0.0%)	2 (50.0%)	2 (50.0%)	**0.003**
Hcy (μmol/l)	19.93 ± 7.59	23.90 ± 13.08	33.41 ± 14.89	**0.002**
Hcy, (>17.5 μmol/l), *n* (%)	23 (56.1%)	18 (58.1%)	9 (75.0%)	0.492
Hcy, (<17.5 μmol/l), *n* (%)	18 (43.9%)	13 (41.9%)	3 (25.0%)	

SVD = single vessel disease; DVD = double vessel disease; TVD = triple vessel disease.

Values are mean ± S.D. *P* values in bold show significance at 1% level

Table 3 Relationship between *MTHFR* genotypes and biochemical parameters in patients

Parameters	677CC ($n = 57$)	677CT ($n = 23$)	677TT ($n = 4$)	P value
Hcy (μmol/l)	19.93 ± 7.59	23.92 ± 11.09	33.41 ± 14.89	**0.0001**
TC (mg/dl)	182.52 ± 44.73	191.75 ± 47.67	193.67 ± 39.51	0.632
LDL (mg/dl)	113.70 ± 37.18	121.11 ± 35.80	122.67 ± 31.97	0.635
HDL (mg/dl)	40.00 ± 5.9	39.54 ± 4.66	41.33 ± 2.08	0.820
TG (mg/dl)	152.51 ± 57.39	149.86 ± 82.42	129.67 ± 13.18	0.831
ApoB (mg/dl)	99.67 ± 25.01	102.82 ± 29.99	117.33 ± 20.59	0.486
Hhcy, (>17.5 μmol/l), *n* (%)	30 (52.6)	16 (69.6)	4 (100)	0.090
Diabetes, *n* (%)	19 (33.1)	9 (39.1)	1 (25)	0.814
Hypertension, *n* (%)	29 (50.9)	13 (56.5)	2 (50)	0.896

Values are mean ± S.D. *P* values in bold show significance at 1% level

Table 4 Logistic regression analysis of determinants of CAD

Risk factors	Odds ratio (OR)	Confidential interval 95% (CI)		P value
		Lower	Upper	
Hyperhomocysteinemia	5.31	2.42	11.68	**0.0001**
High density lipoprotein	1.11	1.04	1.19	**0.003**
Diabetes	8.48	2.44	29.47	**0.001**
Hypertension	7.50	3.14	17.95	**0.0001**

Values are mean ± S.D. *P* values in bold show significance at 1% level

dose, supporting the causal relationship between the C677T *MTHFR* genotype, elevated Hcy and vascular disease [33]. Mager et al. [34] reported a significantly higher frequency of homozygosity (TT) in patients with early-onset CAD than in patients with later-onset CAD or in control subjects (OR = 2.4; 95% CI: 1.2–4.7). In contrast, a meta-analysis failed to support such hypothesis [35]. One possible reason for these inconsistencies may be associated with differences in the ethnicity of the study subjects.

In current study, TT genotype showed highest concentration of Hcy as reported by others. It shows insignificantly high OR = 4.95, which could be due to small sample size. Further study with larger sample size is required to find out better correlation of TT genotype with CAD.

MTHFR mutation exerts its influence on coronary atherosclerosis through the action of Hcy, which interacts with vascular smooth muscle cells, endothelium function, plasma lipoprotein, coagulation factors, and platelets [36, 37]. However, Hcy concentration is not only influenced by the gene mutation but also by non-genetic factors, such as folate status and/or vitamin B_{12}. Guenther et al. [6] showed that the diminished enzyme activity was attributable to reduce FAD binding. Furthermore, they observed that folate derivatives protected both the wild type and mutant *E. coli* against FAD loss. The effect of this genetic defect may be largely compensated by adequate folate intake [38]. Previous studies reported the TT genotype displaying increased Hcy concentration than the CC counterparts [33, 39]. In our study, it has also been shown that the TT genotype has significantly higher plasma Hcy concentrations. Multivariate regression analysis further strengthens our findings that mild HHcy (OR = 5.31, 95% CI 2.42–11.68, $P = 0.0001$) may be associated with an increased risk of CAD along with diabetes and hypertension. However in univariate analysis, we found that TT genotype was significantly associated with disease severity and elevated level of Hcy.

The current literature offers different suggestions with respect to the role of moderately elevated Hcy and the risk for CAD. Some studies described a linear increase of CAD risk with increasing Hcy [40], others a threshold effect [41] or no risk association at all [42]. Our data are in concordance with recent studies investigating the association of Hcy with coronary disease [43]. A recent prospective nested case–control study from Finland reported no association of Hcy with CAD [44] and the authors argued that the relatively low Hcy levels in the Finnish population might be one reason for this.

The increase in the risk associated with the TT genotype may be seen as small considering that the Hcy concentration is usually 2–4 μmol/l higher in TT than in CC subjects. Some prospective [45] and case–control [4] studies suggest a 20–40% increase in coronary disease risk caused by such an increase in Hcy. Our results show a mean increase of 13.5 μmol/l in Hcy levels in TT genotype than CC genotypes and this may be contributing significantly to the disease risk and severity. As the genetic mutation accounts only for a little variation in plasma Hcy concentration, a study with much larger sample size might

be necessary to demonstrate a true picture of genotype–phenotype association.

Conclusions

A significant association was observed between Hcy levels and TT genotype. Hyperhomocysteinemia and the T allele may be affecting the progression and/or severity of the disease synergistically. Our finding supports an important role of the *MTHFR* gene in CAD and provides evidence of polygenic regulation of Hcy.

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