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5,10-METHYLENE TETRAHYDROFOLATE REDUCTASE C677T GENE POLYMORPHISM, HOMOCYSTEINE CONCENTRATION AND THE EXTENT OF PREMATURE CORONARY ARTERY DISEASE IN SOUTHERN IRAN

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ABSTRACT

Elevated level of plasma homocysteine (Hcy) has been identified as an independent risk factor for coronary artery disease (CAD). Furthermore, numerous studies have documented the influences of a common polymorphism (C677T) of methylenetetrahydrofolate reductase (MTHFR) on homocysteine levels. However the relationship between this mutation and cardiovascular diseases (CVD) has remained as a controversial issue. The present study was undertaken to investigate the relationship between C677T polymorphism of MTHFR gene, plasma total Hcy levels and the number of affected vessels as a criterion for the extent of CAD. MTHFR genotypes and plasma homocysteine (HCY) concentrations were examined in 231 patients and 300 healthy subjects who underwent diagnostic coronary angiography. A multiple linear regression analysis was performed to identify the predictors of Hcy levels whereas logistic regression model was built to determine the association of Hcy quartiles with the risk of CAD adjusted for risk factors. The prevalence of MTHFR genotypes was similar between CAD patients and non-CAD individuals while the geometric mean of Hcy values was significantly higher in patient group (14.13 ± 4.11 μmol/l) than in control group (10.19 ± 3.52 μmol/l) ($P < 0.001$). Moreover, unlike the MTHFR polymorphism, Hcy concentration increased with increasing number of stenosed vessels and the CAD risk increased about 2 folds in the top two Hcy quartiles (≥ 17.03 and 13.20-17.02 μmol/l) compared with the lowest quartile (≤ 9.92 μmol/l) after controlling for conventional risk factors ($P<0.001$ for both). Our data suggest that hyperhomocysteinaemia (HHcy) is significantly associated to CAD risk increase as well as to the extent of coronary atherosclerosis.

Keywords: Methylene tetrahydrofolate reductase, homocystein, coronary artery disease, vessel score

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INTRODUCTION

Higher plasma homocysteine (Hcy) concentrations are considered as an independent risk factor for coronary artery disease (CAD) (reviewed in De Bree et al., 2002; Ford et al., 2002; Parnetti et al., 2002). Hyperhomocysteinaemia (HHcy) induced atherosclerosis is characterized by endothelial oxidative damage and dysfunction followed by platelet activation, promotion of platelet aggregation, alteration of the normal procoagulant-anticoagulant balance and thrombosis (Durand et al., 1997; Hajjar et al., 1998; Visioli et al., 2002). Elevations of plasma Hcy has been associated with genetic defects in enzymes involved in its metabolism and/or with nutritional deficiencies of vitamin B_6, B_{12}, and folic acid (Brouwer et al., 1999; Kullo et al., 2006).

The 5,10-methylene tetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5,10-methylene tetrahydrofolate to 5-methyltetrahydrofolate. The latter is the methyl donor in the conversion reaction of Hcy to methionine which is catalyzed by the vitamin B_{12}-dependent methionine synthase. MTHFR-encoding gene has been reported to be a genetic determinant of HHcy (Franken et al., 1996). A common substitution in this gene produces a thermolabile variant of MTHFR with specific decreased enzymatic activity which in turn leads to higher total Hcy (tHcy) concentrations in plasma (Frosst et al., 1995). The molecular basis of this thermolability is a substitution of a cytosine to thymine at nucleotide 677 (rs1801133) in exon 4 of the MTHFR gene that converts an alanine to a valine codon (Frosst et al., 1995).

Since the coronary atherosclerotic disease is the leading cause of death and disability among Iranian population (Hatmi et al., 2007), the present study sought to investigate whether there is an association between C667T polymorphism of MTHFR gene and plasma Hcy levels, as well as to evaluate the relationship of this polymorphism and the tHcy concentrations with the extension of the coronary artery disease.

MATERIALS AND METHODS

Study subjects

The study population comprised 300 healthy controls and 231 patients with premature CAD. All subjects in this study were recruited at three major university hospitals (Saadi, Kowsar and Nemazee) in Shiraz between July 2010 and March 2012. Women and men enrolled in the current investigation were all under 55 and 50 years of age respectively. All study participants were informed about the study's objectives, and an informed consent was obtained from those who agreed to participate. The Human Ethics Committee at the Medical University of Shiraz, Iran, approved the study protocol. At the time of subject enrollment, information about the standard risk factors of CAD and relevant data on past medical history such as diabetes mellitus, hypertension, smoking habits, family history and drug therapy was collected from all individuals. Diabetics were recognized as those with FBS levels higher than 126 mg/dl or subjects who were using oral hypoglycemic agents or insulin. Liver dysfunction, renal failure, chronic or acute infectious disease, pernicious anemia, hypothyroidism, use of anti-inflammatory drugs, steroids and vitamins supplementation (which might influence homocysteine levels) were accepted as exclusion criteria. The data of hypertension, smoking habits, diabetes mellitus, and family history of heart disease (defined as the incidence of myocardial infarction (MI), coronary artery disease and coronary artery bypass graft (CABG) in at least one of their first or second degree relatives) were registered as a two level variable. Smoking status was coded as ever and never smokers.

All of the subjects were of Iranian ancestry and none were first- or second-degree relatives.

Premature coronary artery disease

All participants underwent coronary angiography on account of premature CAD as a verified illness or presumptive diagnosis. Coronary angiography was carried out by

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the Judkins technique, with images recorded on compact disks. Subjects with more than 50 % stenosis in one or more of their three major coronary arteries (left anterior descending (LAD), left circumflex (LCx), and right coronary artery (RCA)) or their primary branches were considered as patients. All vessels were assessed from multiple angles by two experienced interventional cardiologists blinded to the patients' MTHFR genotype and tHcy concentration. Based on the number of involved coronary arteries (0-3), participants were subclassified as follows: No vessel disease (NVD) who had no significantly stenosed vessels (< 50 % luminal stenosis); single vessel disease (SVD), who had one significantly stenosed vessel; double vessel disease (DVD), who had two significantly stenosed vessels; and triple vessel disease (TVD), who had severe CAD involving all the three major arteries.

Biochemical analysis

10 mL of venous blood samples (5 mL in EDTA and 5 mL without anticoagulant) were collected from each subject after overnight fasting. Blood samples without anticoagulant were freshly used for measuring the participants' lipid and sugar profiles by standard enzymatic assays (Pars Azmoon, diagnostic kits, Iran). EDTA containing portions which had been kept on ice in the dark were centrifuged within 90 min at 2500 rpm for 10 min at 4 ^{o}C. Resulted plasma fractions were aliquoted and stored at -70 ^{o}C until Hcy and folate estimation. High performance liquid chromatography (HPLC) with a fluorescent detector was used for determining the tHcy levels according to Araki and Sako (1987) whereas the plasma concentrations of folate were obtained by an automated chemiluminescence method (Rozen, 1997). HHcy was defined as a plasma tHcy level ≥15 μmol/L.

Genotype determination

Genomic DNA was extracted from peripheral blood leukocytes using salting out method (Miller et al., 1988). Detection of the C677T polymorphism in the MTHFR gene was performed by PCR-RFLP analysis according to protocol conditions and primer sequences reported previously (Frosst et al., 1995). Briefly, a 198 bp fragment surrounding the supposed mutation site was obtained by the following primers:
forward:
5'-TGAAGGAGAAGGTGTCTGCGGGA-3';
reverse:
5'-AGGCGGTGCGGTGAGAGTG-3'.

The PCR mixture (20 ml) contained 50 ng of genomic DNA, 10 pmol of each primer, 1.5 mM $MgCl_2$, 2.5 mM of each dNTP, 1X PCR buffer and 1 U of Taq polymerase (Fermentas). The cycle parameters were 3 min initial denaturation at 94 °C, followed by 35 subsequent cycles of amplification at 94 °C for 45 sec, at 65 °C for 45 sec, and 72 °C for 45 sec. Final extension step was performed at 72 °C for 7 min. The PCR products were then subjected to *HinfI* (Fermentas) digestion by overnight incubation at 37 °C. Fragments were size-separated by gel electrophoresis using 4 % (w/v) agarose. Allele C was not digested and remained 198 bp after digestion 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.

Statistical analysis

Continuous variables were presented as mean ± standard deviation (SD) and were compared by Student's t-test or ANOVA for more than two groups. Scheffe's post-hoc test was utilized to discriminate the significant differences among a group of means because it is fairly conservative and therefore very robust to the F-test outcome (Snedecor and Cochran, 1980). Kolmogorov–Smirnov test was used to assess the normality of distribution of continuous variables. Because of the right-skewed distribution of tHcy and folate, analyses were performed using log-transformed data for these variables to reduce kurtosis. Thus geometric means of the mentioned variable are presented. Chi-square test was em-

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ployed to compare categorical variables as well as to assess the Hardy-Weinberg equilibrium. We used univariate analyses to estimate the association of Hcy values with other variables. For this purpose, Pearson correlation coefficients were computed to evaluate the relationships between continuous variables and Hcy concentrations whereas Student's t-test was used to compare the mean values of Hcy in dichotomous variables (Hypertension, familial history of heart disease, smoking habit, diabetes and sex). Subsequently, significantly associated variables were further analyzed by a multiple linear regression analysis to determine independent predictors of plasma tHcy levels. A logistic regression model was fitted to examine the independent impact of different clinical and biochemical factors on premature CAD. Furthermore, to evaluate the graded effect of Hcy concentrations on the risk of CAD, another logistic regression analysis - with Hcy quartiles as independent variables and the lowest quartile as reference - was carried out. Respective odds ratios (OR) were calculated for an unadjusted analysis as well as for a adjusted model which had been controlled for parameters that may contribute to the risk for CAD such as MTHFR genotype, diabetes, sex, hypertension, age, lipid profile, familial history and smoking status. Statistical analyses were all performed by the software package SPSS 17.0 (Statistical Package for the Social Science, SPSS, Inc., Chicago, Illinois) and a probability value ≤ 0.05 was used to establish statistical significance.

RESULTS

Clinical and biochemical parameters

Baseline characteristics and clinical biochemistry parameters of the patients and controls are summarized in Table 1. Altogether 273 men (aged 21-50 years; mean 47.44) and 258 women (aged 27-55 years; mean 51.13) were successfully genotyped in this study. As expected, the premature CAD group showed higher concentrations of LDL, triglyceride and Hcy, and lower concentrations of HDL compared with the control group. A significantly higher prevalence of diabetes, HHcy, familial history of heart disease and hypertension was also observed in patients. Plasma folate concentration did not differ significantly between premature CADs and healthy subjects. The two groups were matched for age (P=0.439) and sex (p=0.088). The relative frequencies of males in the patient and control groups were 54.98 % and 48.67 % respectively.

C677T polymorphism, premature CAD and homocysteine levels

The prevalence of the MTHFR genotypes was in the range of Hardy–Weinberg equilibrium both in the control (χ^2=2.61, df=2, P=0.106) and patient (χ^2=1.9, df=2, P=0.168) groups. C677T mutation frequency has been also presented in Table 1 for the patient and control groups. There was no significant difference between the two groups regarding genotype (χ^2 = 2.874, df = 2, P=238) and allele (χ^2=3.132, df=1, P=082) frequencies. The TT genotype was observed in 8 % of the controls and 10.8 % of the patient group. No significant difference was observed in the prevalence of either any genotype or allele frequencies between male and female subjects (P>0.05).

The premature CAD group consisted of 231 patients (aged 29-55 years; mean 50.31 years), including 78 (33.77 %) with one-, 62 (26.84 %) with two-, and 91 (39.40 %) with three-vessel disease while no vessel disease group (aged 21-55 years; mean 52.09 years) comprised of 300 subjects. Although a trend for higher prevalence of TT genotype was observed across the four subgroups of involved vessels, C677T polymorphism did not show a significant correlation with the extent of premature CAD (P=0.641) (Table 2). On the other hand, as Table 2 displays, tHcy concentrations were significantly higher in patients with TVD (vs. SVD and NVD) and DVD (vs. NVD) (P<0.05 for both). In addition, the prevalence of HHcy was significantly lower in NVD subjects compared to DVD (P=0.027) and TVD (P=0.001) groups. Other investi-

gated parameters had statistically similar distributions among different patient subgroups (i.e. SVD, DVD and TVD) (data not shown). Chi-square analysis also failed to find any significant difference in the number of diseased vessels between males and females (P>0.05).

Table 3 presents the distribution of biochemical and clinical factors according to the MTHFR genotypes in the patient group. No significant association was observed between investigated factors and MTHFR genotypes except for the prevalence of HHcy and mean tHcy concentration. Patients with TT genotype had significantly higher concentrations of tHcy compared to the wild type homozygotes (P<0.001) and heterozygotes (P<0.05). A same trend was observed for HHcy (Table 3) as patients with thermolabile homozygous genotype (TT) were 1.894 times more likely to have HHcy than patients with C allele carriers (recessive genetic model: TT vs. CT+CC; OR=1.894, 95 % CI: 1.198-2.994, P=0.006).

Table 1: Biological characteristics of participants in patient and control groups

Variables	Patients (SVD+DVD+TVD) (n=231)	Controls (NVD) (n=300)	P value
Age (years)	50.31 ± 6.11	52.09 ± 7.03	0.439
Gender (male/female)	127/104	146/154	0.088
BMI (kg/m^2)	25.74 ± 3.22	25.11 ± 3.87	0.542
Total cholesterol (mg/dl)	159.26 ± 65.32	155.23 ± 67.28	0.382
HDL cholesterol (mg/dl)	35.13 ± 8.43	38.16 ± 9.61	0.041
LDL cholesterol (mg/dl)	117.71 ± 59.43	109.52 ± 57.39	0.014
Triglycerides (mg/dl)	155.32 ± 63.21	140.25 ±61.65	0.039
GM Folic acid (nmol/l)	9.87 ± 3.04	11.15 ± 3.21	0.433
GM tHcy (µmol/l)	14.13 ± 4.11	10.19 ± 3.52	<0.001
HHcy, n (%)	104 (45.02)	91 (30.33)	0.001
Diabetes, n (%)	57 (24.67)	42 (14)	0.002
Hypertension, n (%)	83 (35.93)	71 (23.67)	0.003
Smokers, n (%)	65 (28.14)	77 (25.67)	0.554
Familial history, n (%)	70 (30.30)	59 (19.67)	0.006
***MTHFR* genotype, n (%)** **CC** **CT** **TT**	 118 (51.1) 88 (38.1) 25 (10.8)	 174 (58) 102 (34) 24 (8)	 0.238
Alleles **C** **T**	 324 (70.13) 138 (29.87)	 450 (75) 150 (25)	 0.082

Abbreviation: GM: Geometric Means; Continuous data are presented as mean ± SD

Table 2: Distribution of MTHFR genotypes, HHcy and Hcy concentrations in studied population with different numbers of stenotic coronary artery

	NVD (n=300)	SVD (n=78)	DVD (n=62)	TVD (n=91)
***MTHFR* genotypes** **CC, n (%)** **CT, n (%)** **TT, n (%)**	 174 (58) 102 (34) 24 (8)	 40 (51.28) 31 (39.74) 7 (8.97)	 34 (54.84) 22 (35.48) 6 (9.68)	 44 (48.35) 35 (38.46) 12 (13.19)
GM tHcy (µmol/l)	10.19 ± 3.52	11.71 ± 2.87	13.12 ± 3.54†	14.54 ± 4.11*
HHcy, n (%)	91 (30.33)‡	31 (39.74)	28 (45.16)	45 (49.45)

* Significantly different from NVD and SVD by ANOVA and Scheffe's post-hoc multiple comparison of the means of log-transformed data (P<0.001)
† Significantly different from NVD by ANOVA and Scheffe's post-hoc multiple comparison of the means of log-transformed data (P<0.01)
‡ Significantly different from DVD (χ^2=5.120, df=1, P=0.027) and TVD (χ^2=11.249, df=1, P=0.001)

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Table 3: Biochemical and clinical parameters in premature CAD subjects (SVD+DVD+TVD) with different MTHFR genotypes

Variables	677CC (n=118)	677CT (n=88)	677TT (n=25)
Age (years)	50.24 ± 6.07	49.32 ± 6.01	51.43 ± 7.13
BMI (kg/m^2)	24.89 ± 3.92	25.53 ± 3.60	26.11 ± 3.86
Total cholesterol (mg/dl)	156.43 ± 59.11	158.73 ± 62.02	160.03 ± 66.04
HDL cholesterol (mg/dl)	36.44 ± 9.12	37.53 ± 8.76	34.82 ± 8.28
LDL cholesterol (mg/dl)	114.83 ± 54.82	120.47 ± 61.60	117.33 ± 57.64
Triglycerides (mg/dl)	153.75 ± 59.73	154.76 ± 57.33	156.86 ± 62.22
GM Folic acid (nmol/l)	10.88 ± 4.05	9.66 ± 3.67	9.34 ± 3.11
GM tHcy (µmol/l)	11.56 ± 3.89	13.89 ±3.83	15.82 ± 4.05*†
HHcy, n (%)	46 (39.98)	40 (45.45)	18 (72)‡
Diabetes, n (%)	31 (26.27)	20 (22.73)	6 (24)
Hypertension, n (%)	41 (34.75)	34 (38.64)	8 (32)
Smokers, n (%)	33 (27.97)	25 (28.41)	7 (28)
Familial history, n (%)	35 (29.66)	29 (32.95)	6 (24)

† Significantly different from CC by ANOVA and Scheffe's post-hoc multiple comparison of the means of log-transformed data (P<0.001)
* Significantly different from CT by ANOVA and Scheffe's post-hoc multiple comparison of the means of log-transformed data (P<0.05)
‡ Significantly different from CC (χ^2=9.095, df=1, P=0.004) and CT (χ^2=5.491, df=1, P=0.024)

Determinants of tHcy concentrations

In univariate analysis plasma tHcy levels were correlated with folate level (r=-0.183, P=0.002). Likewise, the mean tHcy level was found to be higher in smokers and those with thermolabile homozygous genotype (recessive model; TT vs. CC+CT). Finally, the stepwise multiple linear regression analysis which was adjusted for folate and smoking status (adjusted R^2=0.38, P<0.001), showed that rs1801133 in the recessive model was a significantly independent predictor of tHcy levels (P < 0.001; Table 4).

Predictors of premature coronary artery disease

In order to identify effective factors predisposing to premature CAD, a logistic regression analysis with backward selection strategy was conducted. Categorical variables (hypertension, gender, diabetes, MTHFR genotypes, familial history of heart disease and tobacco use) and continuous factors (tHcy, folate, TC, TG, HDL, LDL, BMI and age) were included in the model as independent variables. Results showed an independent association between tHcy and the risk of premature CAD (OR=9.04, 95% CI: 2.64-26.11; P =0.001). Hypertension, diabetes, familial history of heart disease and LDL cholesterol were also independently associated with premature CAD (Table 5).

Additionally, to evaluate the graded impact of tHcy levels on the premature CAD, a logistic regression analysis was performed with the independent variable being Hcy quartiles. In our population 25th, 50th and 75th percentiles of Hcy (ranged from min=3.50 to max=69 µmol/l) were 9.92, 13.19 and 17.02 µmol/l respectively. Table 6 presents association results between homocysteine quartiles and risk of premature CAD in two models: unadjusted model and a model adjusted for conventional risk factors of coronary atherosclerotic disease such as smoking, hypertension, lipids, age, diabetes, positive familial history of heart disease, MTHFR and sex. Apparently the odds ratio (OR) for premature CAD increased with increasing quartiles of Hcy (Ref=lowest quartile) both before and after adjustment. The adjusted model showed that subjects in the top two homocysteine quartiles (≥17.03 and 13.20-17.02 µmol/l) are 2.04 (95 % CI, 1.65-3.07) and 2.13 (95 % CI, 1.81-3.13) times more likely to have premature CAD than those in the first quartile (≤9.92 µmol/L) (P<0.001 for both) (Table 6).

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Table 4: Multivariate regression analysis for tHcy levels

	Univariate analysis		Multivariate analysis	
	R*	P value	β	P value
rs1801133, TT vs. CC+CT	-	<0.001	0.221	<0.001
Folate	-0.183	0.002	0.192	0.011
Smokers	-	0.012	0.093	0.038
Diabetes	-	0.123		
Familial history	-	0.168		
Sex	-	0.766		
Age	0.067	0.284		
BMI	0.046	0.342		
Total cholesterol	0.022	0.643		
HDL cholesterol	0.112	0.085		
LDL cholesterol	0.073	0.114		
Triglycerides	0.033	0.532		

*Calculated for log-transformed data of tHcy

Table 5: Risk factors for premature CAD with logistic regression

Risk factors	β-coefficient	Standard error	Adjusted OR (95 % CI)	P value
tHcy	2.011	0.916	9.04 (2.64-26.11)	0.001
Hypertension	0.885	0.332	2.31 (1.39-3.77)	0.011
Diabetes	0.753	0.265	2.09 (1.17-3.43)	0.026
Familial history	0.722	0.301	2.21 (1.24-3.69)	0.033
LDL cholesterol	0.901	0.442	2.29 (1.32-3.87)	0.020

Table 6: Association between tHcy quartiles and risk of premature CAD

tHcy quartile (µmol/l)	OR (unadjusted)	OR (adjusted)
≤ 9.92 (reference)	-	-
9.93-13.19	1.32 (0.72-2.33)	1.19 (0.67-2.09)
13.20-17.02	3.44 (1.87-7.02)*	2.04 (1.65-3.07)*
≥ 17.03	5.11 (2.97-10.02)*	2.13 (1.81-3.13)*

*P < 0.001 compared with the reference group (first quartile)

DISCUSSION

Homocysteine hypothesis indicates that mild to moderate hyperhomocysteinemia is an independent predictor for the development of cardiovascular disease (CVD). However, there are also studies which have shed doubt on the role of this thiol-containing amino acid in the pathogenesis of atherosclerotic vascular disease (reviewed in Ueland et al., 2000; Lentz and Haynes, 2004). These controversial results brought up a homocysteine controversy instead of the homocysteine hypothesis (reviewed in Smulders and Blom, 2011). In addition, several numbers of studies, from the early 1990s, have introduced various evidences for the genetic basis of elevated levels of Hcy (reviewed in Trabetti, 2008). Among the candidate genes, the MTHFR is of particular medical interest as being a risk factor for hyperhomocysteinemia as well as for CVD. Although some meta-analyses have found an association between the MTHFR 677T allele and atherosclerosis, vascular disease and myocardial infarction

(Wald et al., 2002; Cronin et al., 2005; Laraqui et al., 2007), others failed to detect such a relationship (Brattstrom et al., 1998; Klerk et al., 2002). Ethnicity differences could be a responsible parameter for at least some of these controversial results.

The result of this investigation did not support an independent association of the MTHFR C677T polymorphism either with the presence of premature CAD or with its extent. However, a positive relationship was observed between the TT MTHFR genotype and elevated plasma tHcy levels. Folic acid and smoking habits were also among the significant predictors of tHcy levels in the current study. Folate status is the most important determinant of plasma homocysteine levels in the general population (Nygard et al., 1998; Selhub et al., 1999), and its low intake or blood concentrations increase the risk of CVD (Morrison et al., 1996; Rimm et al., 1998). Several mechanisms such as lipid peroxidation, platelet activation and aggregation, reduced VonWillebrand factor, inflammation, hypertension, endothelial damage and vasoconstriction (Harker et al., 1976; Fryer et al., 1993; Bartecchi et al., 1994; MacKenzie et al., 1994) have been suggested to explain the causal role of smoking on coronary atherosclerosis so far. Due to these results, the presence of a direct and consistent association between the gene variant and plasma tHcy levels is admitted in the studied population.

On the other hand, in the current study tHcy level was positively associated not only with the presence of premature CAD but also with the number of involved vessels which is in agreement with some previous reports regarding the correlation between tHcy levels and number of atherosclerotic vessels (von Eckardstein et al., 1994; Montalescot et al., 1997; Chao et al., 1999; Tokgozoglu et al., 1999; Yoo et al., 1999; Kerkeni et al., 2006; Alam et al., 2008). There are also a few case-control studies in the literature which were unable to verify such a relationship (Bozkurt et al., 2003; Guerzoni et al., 2009). There is still a study in which tHcy levels showed a significant correlation with the extent of coronary atherosclerosis in patients with low cardiovascular risk profiles unlike in patients with high risk profiles (Tsai et al., 2000). These controversial results involving the relation between plasma tHcy levels and the extent of CAD could be partly attributed to the variable stenosis criteria used in defining CAD by different studies. Some investigators have considered ≥ 50 % stenosis as the cut off point for CAD diagnosis (von Eckardstein et al., 1994; Tokgozoglu et al., 1999; Kerkeni et al., 2006; Guerzoni et al., 2009), whereas in other studies CAD criterion has been taken to be $\geq$ 70-75 % stenosis (Montalescot et al., 1997; Chao et al., 1999; Yoo et al. 1999; Alam et al., 2008). Moreover homocysteine circulating levels are affected by some various factors that differ in each population such as genetic factors, dietary habits, geographic and demographic differences and life style (Bayer et al., 2002; Bozkurt et al., 2003). Additionally independent risk factors predisposing individuals to atherosclerosis may also vary among various populations.

Furthermore, like some previous reports (Verhoef et al., 1997; Chao et al., 1999; Selhub et al., 2000; Pajunen et al., 2002; Panayiotou et al., 2009) we found evidence of a graded effect of Hcy quartiles on the risk of premature CAD. Significant odds ratios of top two Hcy quartiles in comparison to the lowest quartile even after controlling for other conventional risk factors of CVD together with the robust association of tHcy levels with TT genotypes of MTHFR gene makes it reasonable to suggest that MTHFR mutation mediates its influence on atherosclerosis by means of HHcy.

In conclusion, these data suggest that an elevated plasma Hcy level, but not MTHFR mutation, is associated with the extent of premature coronary artery disease. In addition, our findings admit the role of the MTHFR T allele as an independent determinant of plasma tHcy concentration. However, the present study has some limitations. Pathologic studies have shown that

arterial thrombosis and plaque rupture are episodic events which could significantly affect the severity of stenosis at the time of angiography (Sullivan et al., 1990). On the other hand, it has been revealed that more than 50 % of the coronary surface is usually covered by raised plaque (Solberg and Strong, 1983) and considerable portion of coronary events could be resulted from their progression (Azen et al., 1996). Thus, estimating the extent of CAD by the number of involved vessels would contribute to underestimate the risk of atherosclerosis and may not provide the best reflection of this process. There are multiple scoring systems (Leaman et al., 1981; Gensini, 1983; Sullivan et al., 1990; Montalescot et al., 1994; Birnie et al., 1998) which can evaluate the severity or extent of CAD much better than traditional vessel score approach used here. Besides, some other key determinants of plasma tHcy levels such as vitamins B6 and B12, caffeine consumption, creatinine concentration and exercise were not taken into account in the present investigation. Since TT genotype, smoking status and folic acid levels can only predict 38 % of tHcy changes in our population (Table 4), assessment of other Hcy predictors seem indispensible.

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