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An MTHFR Variant, Plasma Homocysteine Levels and Late-Onset Coronary Artery Disease in Subjects from Southern Iran

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Abstract: There have been many controversial debates on the role of Hyperhomocysteinaemia (HHcy) as an independent risk factor for Coronary Artery Disease (CAD) during recent years. Furthermore, an alanine/valine (Ala/Val) gene polymorphism at 222nd amino acid of 5,10-methylenetetrahydrofolate reductase (MTHFR) has been considered as a factor that could render this enzyme thermolabile and less active which in turn may yield a subsequent increase in plasma total homocysteine (tHcy) levels. To assess whether this polymorphism is associated with increased risk of CAD and plasma levels of tHcy in a population from southern Iran, a total of 457 patients with angiographically documented multi-vessel CAD were compared with a control group comprised of 371 subjects with <30% stenosis in all major vessels. Nevertheless our results failed to admit a significant difference between CAD individuals and control subjects for Ala/Val polymorphism and plasma Hcy concentrations. However, plasma Hcy concentrations were significantly higher in individuals with Val/Val genotype than subjects with Ala/Ala genotype, but it didn't show a significant association with CAD in our population. Moreover, as the multiple linear regression analysis indicated, smoking habit, folate levels and the MTHFR Val/Val genotype were the only major predictors of tHcy concentrations in the current investigation.

Key words: Coronary artery disease, MTHFR, C677T polymorphism, homocysteine, folate

INTRODUCTION

Coronary artery disease (CAD) is one of the leading causes of morbidity and mortality universally (Lichtenstein *et al*., 2006). In both developing and developed countries CAD is a major public health problem which has induced considerable concerns about its increasing prevalence in the medical community worldwide (He *et al*., 2005). Coronary artery disease is a multi-factorial disease with both environmental and genetic determinants. Thus the etiology of CAD is still not completely understood but it has been demonstrated that individual susceptibility to this disease are associated with variations in some genes (Goldstein *et al*., 1973; Austin *et al*., 2004). Although, numerous meta-analyses have endorsed the involvement of Methylenetetrahydrofolate reductase (MTHFR) in CAD (Wald *et al*., 2002, 2011) but there are also some conflicting results (Lewis *et al*., 2005; Clarke *et al*., 2012).

MTHFR plays an important role in homocysteine metabolism by catalyzing the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which is the major circulatory form of folate and carbon donor for remethylation of homocysteine to methionine in a B12-dependent reaction. A transition of cytosine to thymine at nucleotide 677 in the MTHFR gene converts an alanine to valine at position 222 in the polypeptide which contributes to synthesis of a thermolabile enzyme with a 50% reduction in activity (Frosst *et al*., 1995). This diminished enzyme efficiency has been shown to associate with increased concentrations of plasma total homocysteine (tHcy) particularly in subjects with low folate levels (Kluijtmans and Whitehead, 2001;

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Lievers *et al*., 2001). Hyperhomocysteinaemia (HHcy) has been assumed as an independent risk factor for CAD (Nurk *et al*., 2002; Guo *et al*., 2003) and many clinical and experimental investigations suggest that moderately increased tHcy is also associated with, venous thrombosis, history of myocardial infarction, and peripheral arterial occlusive disease (Rassoul *et al*., 2000; Kluijtmans and Whitehead, 2001). Albeit there are also numerous reports and meta-analyses which have brought somewhat conflicting views about the involvement of tHcy elevation in CAD (Fallon *et al*., 2001; Clarke *et al*., 2012)

In the current investigation, we have evaluated the relationship between the prevalence of C677T MTHFR polymorphism and coronary artery disease and its association to fasting plasma tHcy concentration in 828 individuals who had undergone diagnostic coronary angiography in South Iran.

MATERIALS AND METHODS

Study population: The study population comprised 457 patients (aged 50-79; mean age 67.91±9.86 year) and 371 control subjects (aged 50-83; mean age 68.32±8.72 year) who underwent coronary angiography because of either symptoms of suspected CAD or unrelated conditions such as cardiomyopathy or valvular heart disease. Positive angiography was defined as the presence of greater than 50% coronary diameter reduction as described by an experienced cardiologist, while control subjects were those who had <30% stenosis in all major vessels. All men and women included in this study were over 50 and 55 years of age, respectively. Two hundred eighty three subjects were excluded because of renal dysfunction, liver disease, vitamins supplementation and drug therapies that are known to interfere with homocysteine or lipid metabolism (such as phenytoin, cholestiramine, cyclosporine, methotrexate, trimethoprime, aspirin, statin and etc.). All participants were apprised on the aims of the study and informed written consent was obtained from them. Study was approved by the ethics committee of Shiraz Medical University. Each participant was interviewed by two trained interviewers about the cardiovascular risk factors such as hypertension (defined as systolic/diastolic blood pressures higher than 140/90 mmHg or using antihypertensive medications), cigarette smoking habit (cigarette and/or hookah) and family history of heart disease (defined as incidence of coronary artery disease, coronary artery bypass surgery and myocardial infarction in at least one of the first or second degree relatives). The subjects who smoked at least one cigarette or hookah per day or were ex-smokers were considered as smokers while non-smokers were defined as those who had never smoked. Information about body weight and height were also extracted from the medical files of subjects. All subjects in this study were recruited at three major university hospitals (Saadi, Kowsar and Nemazee) in Shiraz between July 2010 and March 2012.

Biochemical measurements: Ten microlitter of venous bloods were obtained from each subject after a 12 h fasting (5 mL without anticoagulant and 5 mL in EDTA). Measurements of the participant's lipid and sugar profiles included Total Cholesterol (TC), triglyceride (TG), Low-Density Lipoprotein (LDL), High-Density Lipoprotein (HDL) and Fasting Blood Sugar (FBS) were all carried out on blood samples without anticoagulant by standard enzymatic assays (Pars Azmoon, diagnostic kits, Iran). Patients were diagnosed as having diabetes mellitus at enrolment if their fasting glucose level in the serum was higher than 125 mg dL^{-1} or if they were receiving hypoglycemic agents. Meanwhile plasma was separated from EDTA-containing tubes kept on ice in the dark within 90 min. High Performance Liquid Chromatography (HPLC) with fluorescent detection was utilized to tHcy level determination according to Araki and Sako (1987). Plasma folate concentrations were measured by an automated chemiluminescence method (Rozen, 1997).

DNA isolation and polymorphism analysis: DNA was extracted from 5 mL blood samples by salting out method (Miller *et al*., 1988). The region surrounding the supposed mutation site (a 198 bp fragment) was obtained by Polymerase Chain Reaction (PCR) according to protocol conditions and primer sequences published previously (Frosst *et al*., 1995). C677T PCR products were digested with HinfI restriction endonuclease (Fermentas) by overnight incubation at 37°C and then were size-separated by gel electrophoresis using 4% (w/v) agarose. Since C677T mutation brings about in a valine (Val) substitution for an alanine (Ala), resulted three genotypes were as follows: Ala/Ala normal homozygous which was identified as 198 bp fragments; Ala/Val heterozygous characterized with 198, 175 and 23 bp fragments and Val/Val mutant homozygous described by 175 and 23 bp fragments (Frosst *et al*., 1995).

Statistical analysis: Pearson's Chi-square test was used to assess the distribution differences of MTHFR genotypes in patients as compared to controls, as well as Hardy Weinberg proportions of allele distribution. Quantitative data were compared using one way analysis of variance (ANOVA) whereas for qualitative parameters χ^2 test was utilized. Logarithmic transformations were

made on all skewed variables, including total cholesterol, triglycerides, tHcy and Folate. A multiple linear regression analysis was utilized to identify involving variables that have significant effects on tHcy concentrations. SPSS (Statistical Package for Social Sciences) software, version 17.0 (SPSS Inc, Chicago, USA) was used for statistical analyses and a p-value less than 0.05 was considered significant.

RESULTS

The distribution of the MTHFR genotypes in the whole population was compatible with Hardy-Weinberg proportion (p = 0.396), with allele frequencies of 71% and 29% for the Ala and Val alleles, respectively (Table 1). There was no sex-dependent variation in the prevalence of the Val allele frequency or Val/Val genotype. Likewise as Table 2 shows the allele and genotype frequencies between CAD patients and control subjects did not distribute differently (χ^2 = 3.357, df = 2, p = 0.1867).

Demographic and biochemical characteristics of the participants are summarized in Table 3. For some of the conventional cardiovascular risk factors (such as hypertension, familial history of heart disease, diabetes, LDL cholesterol and triglycerides) CAD patients showed a higher prevalence or levels compared with control subjects. Using 15 μmol L^{-1} as the cutoff point of mild hyperhomocysteinaemia-on the basis of a prevailing agreement in the literature (Brattstrom and Wilcken, 2000; Loncar *et al*., 2001; Bennouar *et al*., 2007), -34.14% of patients and 30.19% of control subjects had HHcy (χ^2 = 0.747, p = 0.3874).

Correlation analysis showed a positive and significant but a weak association between tHcy levels and MTHFR gene polymorphism in cases (Spearman's rho = 0.201, p<0.05) and controls (Spearman's rho = 0.171, p<0.05) as well as in whole population (Spearman's rho = 0.186, p<0.05). Bonferroni post hoc multiple comparison method confirmed the existence of a significant (p<0.05) increase in tHcy levels from Ala/Ala to Val/Val genotypes in both patient and control groups. Table 4 summarizes the distributions of the biological parameters in CAD subjects with different MTHFR genotypes. With respect to the conventional risk factors, there was a lower prevalence of smokers in the heterozygous group and higher incidence of familial heart disease in mutant homozygous subjects. Besides, individuals with Val/Val genotype had higher concentrations of homocysteine compared with those who had Ala/Ala genotype. Mutant homozygous

Table 1: Hardy-Weinberg equilibrium test of C677T of MTHFR gene

Genotype	Observed count	Expected count	Genotype frequency (%)	Allele	Observed count	Allele frequency	χ^2	p-value
Ala/Ala	424	418.99	51.21	Ala	1178	0.710	0.72	0.396
Ala/Val	330	340.03	39.86					
Val/Val	74	68.99	8.94	Val	478	0.290		
Total	828		100.00		1656			

Table 2: Different allele and genotype frequencies in control and patient groups

MTHFR	Controls	Patients
Normal Allele Ala	524/742 (70.62%)	654/914 (71.55%)
Mutant Allele Val	218/742 (29.38%)	260/914 (28.44%)
Ala/Ala genotype	182/371 (49.06%)	242/457 (52.95%)
Ala/Val genotype	160/371 (43.13%)	170/457 (37.20%)
Val/Val genotype	29/371 (7.82%)	45/457 (9.85%)

χ^2 = 3.357, df = 2, p-value = 0.1867

Table 3: Biological characteristics of participants in control and patient groups

	Control (n = 371)	CAD (n = 457)
Age (year)	68.32±8.72	67.91±9.86
Sex (Male/Female)	192/179	239/218
BMI (kg m^{-2})	25.04±3.37	25.45±2.94
Smoking (%)	26.42	28.45
Hypertension (%)	54.99	69.15†
Diabetes (%)	56.87	64.33†
Familial history (%)	23.18	33.91†
Total cholesterol[a] (mg dL^{-1})	148.37±66.86	150.71±82.65
LDL cholesterol (mg dL^{-1})	113.41±55.90	121.39±60.05*
HDL cholesterol (mg dL^{-1})	46.65±37.43	47.32±39.11
Triglycerides (mg dL^{-1})	137.75±63.59	145.39±73.02*
Total homocysteine (μmol L^{-1})	15.22±5.43	16.05±5.81
Folate (umol L^{-1})	11.10±3.47	10.79±4.31

a: Despite logarithmic transformations were performed on total cholesterol, triglyceride, tHcy and folate for comparative analyses, but arithmetic means are presented here, *p<0.05 by using the t-test, †p<0.05 by using the χ^2 test

Table 4: Biological parameters in CAD subjects according to different MTHFR genotypes

	Ala/Ala (N = 242)	Ala/Val (N = 170)	Val/Val (N = 45)
Age (year)	66.32±8.13	67.91±7.44	69.96±7.13
BMI (kg m^{-2})	25.35±3.46	26.86±3.09	25.04±2.77
Smoking (%)	33.47	21.18*	28.89
Hypertension (%)	72.31	66.47	62.22
Diabetes (%)	60.74	71.18	57.78
Familial history (%)	28.51	37.06	51.11†
Total cholesterol[a] (mg dL^{-1})	150.61±68.16	151.66±74.45	152.54±79.54
LDL cholesterol (mg dL^{-1})	123.41±57.72	120.53±61.23	118.32±59.15
HDL cholesterol (mg dL^{-1})	46.34±37.22	45.76±41.56	49.82±35.81
Triglycerides (mg dL^{-1})	144.95±67.55	142.86±69.55	148.12±64.23
Total homocysteine (μmol L^{-1})	15.37±5.41	16.14±5.71	18.26±5.46‡
Folate (nmol L^{-1})	11.13±3.78	10.48±3.89	9.69±4.31

a: Despite logarithmic transformations were performed on total cholesterol, triglyceride, tHcy and folate for comparative analyses, but arithmetic means are presented here, *$p<0.05$, Ala/Val vs. Ala/Ala by±square test, †$p<0.05$, Val/Val vs. Ala/Ala by ±square test, ‡$p<0.05$, Val/Val vs. Ala/Ala by Tukey's post-hoc test

subjects showed higher significant levels of tHcy concentrations compared with wild type homozygotes in the control group ($p<0.05$) (data not shown). The same trend was also observed for total population.

Multiple linear regression analysis was performed to identify independent predictors of tHcy levels. Results indicated that only smoking habit ($p<0.03$), folate levels ($p<0.05$) and the MTHFR Val/Val genotype ($p<0.01$) affect plasma tHcy concentrations significantly.

DISCUSSION

CAD is a complex trait that accounts for the leading cause of mortality, morbidity, and disability in Iranian population in a way that almost 50% of all deaths $year^{-1}$ are somehow related with it (Hatmi *et al*., 2007). There are also multiple investigations confirmed that the Iranian population is prone to very high levels of CAD risk factors (Hatmi *et al*., 2007; Karimi *et al*., 2008; Ebrahimi *et al*., 2011). Bearing in mind that several meta-analyses have shown a correlation between hyperhomocysteinaemia and vascular diseases (Boushey *et al*., 1995; Wald *et al*., 2002, 2012) and that the thermolabile form of MTHFR is among the candidate causes for serum Hcy increasing, we conducted current study to appraise the distribution of MTHFR C677T mutation in a relatively high numbers of participants in southern Iran and also to investigate the relative contribution of this polymorphism on plasma tHcy and folate concentrations in our population.

The current study failed to show any significant association between C677T polymorphism and coronary artery disease. This is consistent with some other investigations (Girelli *et al*., 1998; Soriente *et al*., 1998; Gonzalez-Perez *et al*., 2002; Pezzini *et al*., 2002; Kolling *et al*., 2004). However there are also findings confirming a higher prevalence of this mutation in CAD patients compared to controls (Morita *et al*., 1998; Bennouar *et al*., 2007; Dhar *et al*., 2010). But it is noteworthy that tremendous variations could be seen among these studies, such as the selection of control groups (e.g., a true control group who underwent coronary angiography vs. a comparison control group without angiography documents), severity and kind of disease afflicted patient groups (e.g., MI, CAD, premature CAD, etc.), Hcy and folate measurements and assessment of major risk factor interference. It is also possible that the discrepancy observed between various investigations may lie generally on ethnic differences (Lewis *et al*., 2005). To the best of our knowledge, there are also two similar investigations which assessed the relationship between this mutation and coronary atherosclerosis in Iran (Aleyasin *et al*., 2006; Rahimi *et al*., 2009) but they have inconsistent results. Rahimi *et al* (2009) who investigated 176 subjects (117 cases and 59 controls all of whom had objective angiographic information) from western Iran didn't observe a higher prevalence of the mutation in patients. Whereas (Aleyasin *et al*., 2006) came to a significant association between MTHFR C677T mutation and the disease by analyzing 100 patients with angiographically documented CAD and 100 normal volunteers without a history of CAD as the control group. By selecting a control group without objective angiographic information the chance of observing the spurious results in an allelic association study increases. Because there might be some degrees of not clinically evident atherosclerosis in the coronary arteries of those control individuals which in turn could diminish the association intensity between the polymorphism and the disease. Conversely, analyzing a true control group with objective angiographic records supports vigorously the lack of association between MTHFR C677T polymorphisms and CAD observed in our population as well as in the study of Rahimi and colleagues (Rahimi *et al*., 2009).

Moreover, our study suggested that this mutation is associated to higher plasma homocysteine levels in an Iranian sub-population. Results presented here imply that subjects with Val/Val genotype are more susceptible to the hyperhomocysteinaemia which is in agreement with some other studies (Christensen *et al*., 1997; Girelli *et al*., 1998; Morita *et al*., 1998). Actually comparison of mean homocysteine levels between different genotypes in whole population revealed that subjects with the Val/Val genotype had 3.01 μmol L^{-1} higher mean Hcy levels compared with the subjects carrying the wild type homozygous genotype ($p<0.05$) even after adjustment for sex and age. But there is not a significant difference between Val/Val and Ala/Val groups for Hcy levels. In addition to the MTHFR Val/Val genotype, multiple linear regression analysis indicated that smoking habit and folate concentrations were also remained as key determinants of tHcy levels. Smoking is known to be associated with hyperhomocysteinaemia (Nygard *et al*., 1995; Bennouar *et al*., 2007). The pathogenic effects by which smoking leads to coronary heart disease vary from generating hypertension (Fryer *et al*., 1993) and inflammation (Katayama *et al*., 2008) to affecting vascular tree (MacKenzie *et al*., 1994) and increasing the risk of thrombosis (Pomp *et al*., 2008).

However, our data did not show any significant association between tHcy levels and coronary artery disease. Actually, the relationship between plasma tHcy and coronary artery disease is still a matter of argument. While several case control, cross-sectional and even prospective studies confirmed a predictive role for increased level of tHcy in coronary artery events (Nygard *et al*., 1997; Chao *et al*., 1999; Whincup *et al*., 1999; Yoo *et al*., 1999, Chambers *et al*., 2000, Van den Brandhof *et al*., 2001) a few investigations failed to find such relationship (Verhoef *et al*., 1997; Folsom *et al*., 1998; Nikfardjam *et al*., 2001; Sastry *et al*., 2001). Many possible reasons can be presented for these controversial results. For example both the elevated concentrations of tHcy and cardiovascular disease are multifactorial phenomena and parameters like genetic factors, life style and nutrition can affect them (Bozkurt *et al*., 2003). Additionally, dissimilar methodologies such as different inclusion criteria for patient or control groups, various cut off for taking tHcy levels as HHcy and lack of a standard diagnostic method for the definition of CAD in different investigations can also end up to such arguable conclusions (Bozkurt *et al*., 2003). Moreover, homocysteine levels are known to be increased in patients suffering CAD as a consequence of atherosclerosis, probably because of reduced renal clearance due to coexistent of reno-vascular disease (Brattstrom and Wilcken, 2000). Thus in assessing a cause and effect relationship, significant difference in the homocysteine concentrations between Val/Val and Ala/Ala genotypes in a true control group is needed which is absent in some of above studies.

CONCLUSION

We believe that the MTHFR Val/Val genotype has a direct effect on increasing the tHcy levels in plasma but like the majority of studies, our observations are against a role for this SNP as an independent risk factor of coronary artery disease. Besides, while mutant homozygous genotype of MTHFR is associated with increased levels of plasma tHcy in our population, it appears not to be an important determinant of CAD. However the current study suffers from some limitations. First of all this is a retrospective case-control study with a relatively limited population size. Larger sample size and further prospective studies seem necessary to evaluate more accurately the causal influence of a genetic variant on pathogenesis of a disorder. Second, some other key determinants of plasma homocysteine levels such as alcohol consumption and creatinine concentration were not taken into account here. Third, lack of diet data especially describing vegetable or fruits consumption is another weakness of this study. Moreover, we only assessed the association of one SNP in the MTHFR gene. Additional polymorphisms of this gene such as A1298C and its interaction with C677T may have a significant influence on development of coronary atherosclerotic disease (Freitas *et al*., 2008). Thus the role of MTHFR gene as a risk factor for atherosclerosis could not be entirely rule out.

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