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Methylene Tetrahydrofolate Reductase C677T Mutation and Left Ventricular Hypertrophy in Turkish Patients with Type II Diabetes Mellitus

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This study was designed to investigate, in the Turkish population, the association of methylene tetrahydrofolate reductase (MTHFR) C677T polymorphism and left ventricular hypertrophy (LVH) in patients with type II diabetes mellitus. Our study included 249 patients with type II diabetes mellitus (102 men, 147 women) and 214 healthy volunteers as controls (91 men, 123 women). MTHFR C677T genotypes were determined by polymerase chain reaction, restriction fragment length polymorphism techniques. No differences were observed in the distribution of MTHFR genotypes or allele frequencies in the cases versus the controls. The frequency of the MTHFR-mutated allele (T) was 31.7% in the type II diabetes mellitus versus 31.1% of the controls. The homozygous mutation (T/T) in the MTHFR gene was identified in 12% of the type II diabetes mellitus versus 9.3% of the controls. Patients with the TT genotype showed a higher prevalence of LVH when compared to patients with the CC and CT genotypes (p = 0.01). The MTHFR gene C677T mutation may be a possible risk factor for the development of LVH in the type II diabetic patients

Keywords: Diabetes mellitus, Left ventricular hypertrophy, Methylene tetrahydrofolate reductase gene, Turkish

Introduction

Diabetes mellitus (DM) is a major risk factor for cardiovascular disease (Wilson *et al.*, 1998; Grundy *et al.*, 1999). The increased coronary heart disease (CHD) risk in subjects with type 2 diabetes mellitus is partially explained by an association with the established risk factors, such as hypertension, hyperlipidemia, and obesity (Audelin and Genest, 2001). In addition, an independent association between homocysteine (Hcy) and cardiovascular disease has been shown in retrospective studies for patients with DM (Chico *et al.*, 1998; Hoogeveen *et al.*, 1998).

The concentration of homocysteine (Hcy) in the plasma is regulated by several factors, genetic and acquired (Bostom *et al.*, 1995; Kang and Wong, 1996). Elevated concentrations of Hcy, both under fasting conditions and postmethionine load conditions, were correlated with several atherothrombotic and cardiovascular disorders (Roes and Rodgers, 1993; Spence *et al.*, 1999; Guangsen and Chongwen, 2001; Haraki *et al.*, 2001; Passaro *et al.*, 2001). Previous studies discussed the potential interaction between Hcy and glucose intolerance as a risk factor for atherosclerosis (Hultberg *et al.*, 1991; Robillon *et al.*, 1994).

Methylene tetrahydrofolate reductase (MTHFR) is an enzyme in the transmethylation pathway where Hcy is converted to methionine. A common cytosine to the thymidine substitution of the MTHFR gene at nucleotide 677 (C677T) alters a highly-conserved amino acid (alanine to valine), which results in impaired enzyme activity and hyperhomocysteinemia (Ueland *et al.*, 2001).

Several studies were designed to show the relationship between homocysteinemia and diabetic complications. Their results differed (Neugebauer, 1998; Fujita *et al.*, 1999; Abdella, 2000; Shpichinetsky *et al.*, 2000; Ambrosch *et al.*, 2001; Agullo-Ortuno *et al.*, 2002; de Luis *et al.*, 2002). In the previous study, Agullo-Ortuno *et al.* (2002) studied the concentration of Hcy in the plasma of a group of type 1 and type 2 DM patients and took into account whether hyperhomocysteinemia was related to complications of the disease, such as macroangiopathy, nephropathy, retinopathy, and neuropathy. They found that the relationship between the Hcy levels and prevalence of macroangiopathy, retinopathy, and nephropathy in type 1 DM patients while were not found in the type 2 DM patients.

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In this investigation, we studied the C677T mutation of the MTHFR gene with respect to its effect on the left ventricular hypertrophy in patients with type 2 DM.

Materials and Methods

Patient selection and clinical investigation We included 463 unrelated individuals in this study. This included 249 type 2 diabetic patients [126 female (59%), mean age 58 ± 12; 74 male (41%), mean age 57 ± 12] and 214 non-diabetic controls. The patients were selected from the Taksim State Hospital, Istanbul. During ascertainment, the WHO definitions and criteria for diabetes were used (Report of a WHO Consultation, 1999) The patients received a standard questionnaire containing questions regarding the age at the type 2 diabetes mellitus diagnosis, family history, treatment method, and other medical issues. Only patients with a clinical diagnosis of type 2 diabetes mellitus and a history of at least 2 years of treatment without insulin use were recruited.

Blood pressure was measured as recommended by the American Medical Association (Kirkendall *et al*., 1980). The subjects laid on their spines for 10 min, after which their blood pressure was measured with a mercury sphygimomanometer. The readings were taken from the left and right arms and recorded to the nearest 2 mmHg. The mean was then calculated. Weight and height were recorded, and the body mass index was calculated using the formula BMI, weight/height^2 (kg/m^2). A detailed medical history and physical examination, as well as an estimation of the left ventricular hypertrophy (LVH) by echocardiography, were performed for all of the studys patients. Height and weight were measured and the body mass index was calculated (weight in kilograms divided by the square of height in meters); obesity was considered present if the body mass index exceeded 30. There were 28 (11.2%) left ventricular hypertrophy, 90 (36.1%) hypertension, and 56 (22.5%) obese patients with type 2 DM.

The control group [91 male (43%), 123 female (57%), and mean age 54 ± 10] contained only individuals with normal fasting glucose and a negative family history of type 2 diabetes mellitus among first-degree relatives. This group primarily included the spouses of type 2 diabetic patients and volunteers.

DNA isolation Blood specimens were collected in tubes containing EDTA. DNA samples were extracted from whole blood with salting-out procedure (Miller *et al*., 1988).

Analysis of MTHFR C677T Mutation The DNA samples were analyzed for the C677T missense mutation by polymerase chain reaction with locus-specific primers and a subsequent analysis of a restriction fragment length polymorphism that was created by the mutation (Frosst *et al*., 1995). The primers for PCR amplification of the region spanning the 677 locus were 677F (5'- TGAAGGAGAA GGTGTCTGCGGGA-3') and 677R (5'-AGGACGGTGCGGTGA GAGTG-3'). The PCR reactions were conducted in a 50 µl reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol 677F, 50 pmol 677R, 2.5 units DNA Taq Polymerase (MBI Fermentas, Hanover, USA), and 0.5-1.0 µg of genomic DNA. The 677 C → T substitution created a

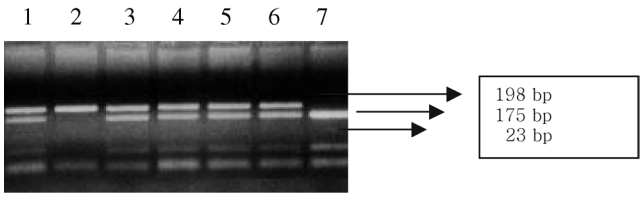

Fig. 1. Genotyping for the C677T polymorphism in the MTHFR gene. The presence of a T nucleotide at position 677 in the MTHFR gene creates a restriction site for the *Hin*fI enzyme. A 198 bp fragment of the gene encompassing the polymorphism was amplified by PCR and subjected to digestion with the *Hin*fI enzyme. The homozygous individuals for the C allele (CC genotype) were identified by the presence of a single 198 bp product. The homozygous for the T allele (TT genotype) were identified by the presence of two products of 175 bp and 23 bp. The heterozygous individuals (CT genotype) were identified by the presence of all products of 198 bp, 175 bp, and 23 bp. Lane 1, CT heterozygous; lane 2, C homozygous; lanes 3-6, CT heterozous; lane 7, TT homozygous.

*Hin*fI recognition sequence, which digested the initial polymerase chain recognition product of the 198 base-pair (bp) into 175 and 23 bp fragments. Presence of the mutation was determined by enzymatic digestion of the initial polymerase chain reaction product with *Hin*fI (MBI Fermentas) at 37°C for 24 h. The digested DNAs were separated on 3% nusieve agarose gel in 1x Tris borate EDTA buffer, followed by staining by an ethidium bromide solution. The MTHFR C677T genotypes were typed by visualization under ultraviolet light (Frosst *et al*., 1995) (Fig. 1).

Biochemical analyses After the subjects had fasted overnight, blood samples were drawn in plain tubes as well as with EDTA. The samples were centrifuged for 10 min at 1,500 × *g* at room temperature, then the plasma was removed. Plasma glucose levels were determined using the automated glucose oxidase method. Immunoturbidimetry (Hitachi 902) with Tina-quant HB1AC II (Roche Diagnostics, Basel, Switzland) was used to assay the HbA1c level.

Statistical analyses Statistical analyses, using the SPSS version 10.0, included the X^2 test for genotype and allele frequency comparison. Clinical characteristics were compared by a Students t-test. The C677T allele frequencies were estimated by gene counting methods. A p value of less than 0.05 was regarded as being statistically significant.

Results

Clinical Investigation The diabetic patients and controls had similar distributions of sex and age. The diabetic patient groups had a significantly higher level of fasting glucose (95 ± 20 vs. 213 ± 108 mg/dl), HbA1c (5.7 ± 1.5 vs. 8.7 ± 3.7%) ($p < 0.01$), body mass index, as well as systolic and diastolic blood pressures ($p < 0.000$) when compared to the healthy controls (Table 1).

Table 1. Clinical characteristics in patients with type II diabetes mellitus

	Patient (n=249)
Gender (Male/Female) (n)	102/147
Body Mass Index (kg/m^2)	27,8 ± 4,7
Systolic pressure (mmHg)	139 ± 27
Diastolic pressure (mmHg)	83 ± 15
Diabetes duration (years)	9,5 ± 7,7
Smoking (%) (Yes/No)	61.6/38.4
Alcohol consumers/nonconsumers (%)	25/75
LVH (%)	11,2
Obesity (%)	22,5

n, number of individuals.

Table 2. Allele and genotype distribution of MTHFR C677T in T2DM patients and controls

Group	Control (n=214)	Type II DM (n=249)
Genotypes		
CC	47,2% (101)	48,6% (121)
TT	9,3% (20)	12,0% (30)
CT	43,5% (93)	39,4% (98)
Alleles		
C	68,9% (295)	68,3% (340)
T	31,1% (133)	31,7% (158)

Number of individuals in parentheses.

As shown in Table 2, the genotype distribution and allele frequencies for the MTHFR gene were not significantly different between the type 2 diabetic patients and the controls. The distribution of MTHFR genotypes in the patients and controls did not significantly deviate from the Hardy-Weinberg equilibrium when the in-patient and control groups frequencies of CC, CT, and TT genotypes were 0.48, 0.39, 0.12; and 047, 0.44, 0.09, respectively ($X^2 = 1.29$, $p > 0.05$). The frequency of the MTHFR mutated allele (T) was 31.7% in the type 2 diabetes mellitus versus 31.1% of the controls. The homozygous mutation (T/T) in the MTHFR gene was identified in 12% of the type 2 diabetes mellitus versus 9.3% of the controls. The heterozygous mutation (C/T) was observed in 39.4% of the type 2 diabetes mellitus versus 43.5% of the controls.

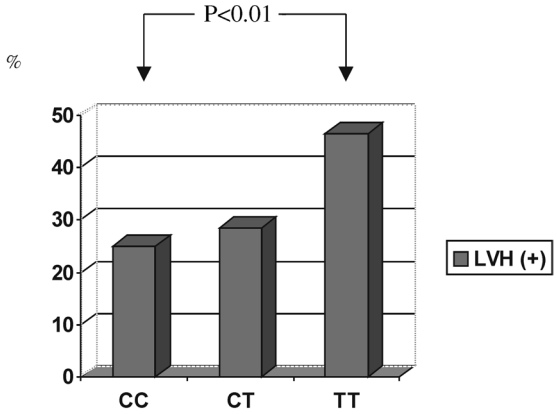

Fig. 2. Distrubution of MTHFR genotypes in respect to presence of LVH in type II diabetic patients.

We found higher blood pressure in the patients with type 2 diabetes mellitus with left ventricular hypertrophy than with left ventricular hypertrophy, but it was not statistically significant (systolic pressure, 140 ± 29 vs. 134 ± 25; diastolic pressure, 86 ± 16 vs. 81 ± 16) (data not shown). Also, we observed no significant influence of MTHFR genotypes and alleles on the blood pressure in the patient and control groups ($p > 0.05$) (Table 3). However, patients with the TT genotype showed a higher prevalence of left ventricular hypertrophy (LVH) when compared to the patients with CC and CT genotypes ($p = 0.01$, $X^2 = 6.49$, odds ratio: 3.76, 95% CI: 1.28-11.00). In the patient group, the number of left ventricular hypertrophy was 7, 8, and 13 in the CC, CT, and TT genotypes, respectively (Fig. 2).

Discussion

An independent association between the homocysteine level, cardiovascular disease (CVD), and diabetic complications was shown in retrospective studies for patients with DM. Both

Table 3. Effects of MTHFR alleles on serum homosysteine in type II diabetic patients

MTHFR C677T	Type II Diabetics		Controls	
Alleles	SBP (mmHg)	DBP (mmHg)	SBP (mmHg)	DBP (mmHg)
C+	139 ± 27	84 ± 15	121 ± 15	74 ± 12
T+	137 ± 29	82 ± 15	119 ± 13	74 ± 12
Genotypes				
CC	140 ± 26	85 ± 15	124 ± 15	75 ± 11
TT	136 ± 29	83 ± 14	121 ± 11	75 ± 8
CT	138 ± 31	82 ± 16	119 ± 14	74 ± 12

C(-), Absence of C677T C allele; C(+), Presence of C677T C allele; T(+), Presence of C677 T allele.

genetic and environmental (e.g., dietary) factors affected the homocysteine level (Bostom *et al*., 1995; Kang and Wong, 1996). One of the most common genetic defects of homocysteine metabolism is a mutation in the enzyme methylene tetrahydrofolate reductase (MTHFR). Homozygosity for the C677T MTHFR mutation has been associated with intermediate and mild hyperhomocysteinemia (Ueland *et al*., 2001). C677T homozygosity is correlated with a 3-fold increased risk for premature cardiovascular disease in patients with mild hyperhomocysteinemia, even without other known risk factors, such as hypertension, hyperlipidemia, or diabetes (Yoo and Park, 2000; Mager *et al.,* 2002; Spence *et al*., 2002). Patients with this mutation responded well to the folic acid treatment, which lowered the plasma homocysteine level (Ma *et al*., 1996; Herman *et al.*, 1999). In the present study of the Turkish population, the frequencies of MTHFR genotypes are similar to the population. There is also a strong association between the MTHFR genotype and LVH development in the type 2 diabetic patients.

The highest CVD score results were found in DM patients with hyperhomocysteinemia. Several cross-sectional, case-control, and prospective studies showed the independent relationship between hyperhomocysteinemia and the increased risk of CVD in patients with DM (Audelin and Genest, 2001). In a previous study, hypertensive patients with T allele had increased carotid artery size, as demonstrated by intima plus media thickness (TT, 0.79 +/- 0.05 mm *vs.* CT + CC, 0.67 +/- 0.02 mm; $P < .02$), relative wall thickness (TT, 0.23 +/- 0.01 mm *vs.* CT + CC, 0.20 +/- 0.005 mm; $P < .02$), and surface area (TT, 19 +/- 1.9 mm^2 *vs.* CT + CC, 15 +/- 0.55 mm^2; $P < .05$). Also, it was demonstrated that the MTHFR genotype and systolic blood pressure independently influence the intima-media thickness and together account for about 11% of its variations ($r^2 = 0.11$, $F = 9.7$, $dF = 1\text{-}205$, $P < .0001$). Homozygosity for the T allele of the MTHFR gene is an independent risk factor for the development of early atherosclerotic organ damage in hypertensive patients (Ravera *et al.*, 2001).

In the present study, patients with the TT genotype had approximately a 2.5- fold increase of LVH risk. We think that the TT genotype contributes to LVH because of its link to hyperhomocysteinemia.

Recently, renal patients with the TT genotype were found to be more susceptible to hyperhomocysteinemia than those with the CC genotype. Some researchers have shown an association between C677T and diabetic nephropathy for both type 1 and type 2 DM (Neugebauer *et al*., 1998; Shcherbak *et al.*, 1999; Noiri *et al.*, 2000). However, not all researchers found this association (Fujita *et al.*, 1999; Smyth *et al*., 1999). Our results do not support a role for the 677T allele in the diabetic nephropathy in the type 2 DM patients. In this study, we found no higher frequency of the T allele of the MTHFR gene in type 2 DM group with nephropathy than the C allele. Because these studies were made in different populations, it may be that there are ethnic differences in terms of this relationship.

In conclusion, we suggest that the MTHFR C677T mutation may be an important genetic determinant of the development of the left ventricular hypertrophy, independent of blood pressure, in Turkish type 2 diabetic patients.

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