Does the MTHFR C677T gene polymorphism indicate cardiovascular disease risk in type 2 diabetes mellitus patients?

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ABSTRACT

Objective: Diabetes mellitus is a major risk factor for cardiovascular disease (CVD). We investigated the relationship among biochemical and cardiac risk parameters with the methylenetetrahydrofolate reductase (MTHFR) C677T genotype in type 2 diabetes mellitus (T2DM) patients.

Methods: One hundred seven T2DM subjects with severe CVD diagnosed by angiography were included consecutively in this cross-sectional study. Biochemical and clinical parameters were obtained from patients who were not positive for nephropathy and retinopathy. MTHFR C677T genotypes were analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods. Normally and abnormally distributed continuous variables were analyzed using student t- and Mann-Whitney U tests. Categorical variables were analyzed using chi-square test.

Results: In the study, 31 T2DM subjects had the CC (29.0%), 62 had the CT (57.9%), and 14 had the TT (13.1%) genotypes. There were no significant differences between subjects with wild-type (677CC) and with mutant (677CT+677TT) alleles in terms of diabetes duration, visceral fat area, total cholesterol, triglyceride, fasting plasma glucose, systolic blood pressure, diastolic blood pressure, high-sensitivity C-reactive protein, homocysteine (Hcy), and carotid intima-media thickness values.

Conclusion: This study suggests that MTHFR gene polymorphisms can not be used as a marker for the assessment of cardiovascular risk in T2DM patients. *(Anatol J Cardiol 2015; 15: 524-30)*

Keywords: type 2 diabetes mellitus, MTHFR C677T gene polymorphism, carotid intima-media thickness

Introduction

Noninsulin-dependent diabetes mellitus (NIDDM), also known as type 2 diabetes mellitus (T2DM), is a polygenic and multifactorial disease that is considered a major life-threatening health problem throughout the world (1). Diabetes mellitus is a major risk factor for cardiovascular disease and is associated with a high incidence of vascular complications (2). There may also be an interrelationship between factors, like endothelial dysfunction, dyslipidemia, platelet hyperaggregability, impaired hemostasis, and the development of vascular damage (3).

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in the transmethylation pathway, where homocysteine (Hcy) is converted to methionine. A common cytosine-to-thymidine substitution in the MTHFR gene at nucleotide 677 (C677T) changes a highly conserved alanine into valine, resulting in impaired enzymatic activity and leading to hyperhomocysteinemia, an independent risk factor for thrombotic disorders (4). The mutation was reported to be linked to moderate hyperhomocysteinemia in cases where homozygote (677TT) folate levels were low in comparison with heterozygotes or non-carriers (5, 6). Many studies have suggested the contribution of gene variants in relation with the homocysteine metabolism pathway in the susceptibility to obesity, T2DM, or other related traits (7-9). In T2DM patients, mild hyperhomocysteinemia may promote vascular disease through endothelial injury, predisposing the vessels to atherosclerosis (10). It was reported that high homocysteine levels could be an indicator of a close connection between cardiovascular disease and homocysteine in diabetes mellitus patients (11, 12).

Ultrasonographic determination of common carotid intima-medial wall thickness (cIMT) was used as an early marker for atherosclerosis in previous studies. These studies have demonstrated a close correlation between carotid ultrasound mea-

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surement, usually cIMT, and the severity of extracranial carotid atherosclerosis (13, 14). Also, it was noted that cIMT value has been used as a surrogate marker for cardiovascular disease (CVD) in diabetes patients (15). This study was carried out to assess the independent contributions of biochemical and cardiological parameters and the MTHFR C677T genotype to cardiovascular disease in T2DM patients.

Methods

Subjects and identification of diabetes mellitus patients

A total of 107 T2DM subjects (68 females and 39 males) with severe CVD diagnosed by angiography were included consecutively in this cross-sectional study. The patients received a standard questionnaire, including questions regarding the age at the time of T2DM diagnosis, treatment methods, and other related medical issues. Criteria defined by the American Diabetes Association were used to determine whether a subject was positive for T2DM (16). The study was approved by the local ethics committee, and informed consent from each participant was obtained.

Inclusion criteria

In order to determine whether a subject could be included in the study, the following diagnostic criteria were used: symptoms of T2DM plus fasting plasma glucose level ≥126 mg/dL or non-fasting glucose level ≥200 mg/dL, HbA1c value > 6.5%, or ongoing hypoglycemic treatment for at least 1 year. Criteria for CVD were as follows: (1) myocardial infarction (chest pain associated with ECG evidence of myocardial infarction and/or elevated cardiac enzymes) or (2) angiographically proven coronary artery disease (>50% stenosis in 1 or more major epicardial vessels) (3) reported by high systolic (≥140 mm Hg) or diastolic (≥90 mm Hg) blood pressure (17).

Exclusion criteria

Individuals who were unable to provide an adequate blood sample were excluded from the study, as were individuals with liver or kidney disease (including diabetic nephropathy), previous organ transplantation, or retinopathy or those who were receiving steroidal therapy at the time of enrollment for this study. Exclusion criteria for the patients included cardiomyopathy, serious organ disease, systemic illness, chronic alcohol abuse, serious psychiatric illness, and anticonvulsant therapy (17).

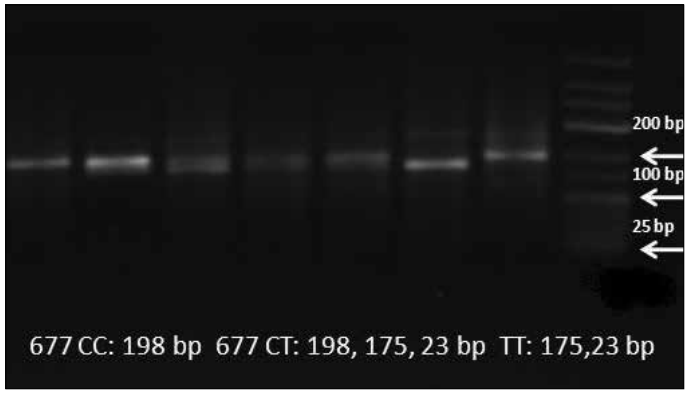

Figure 1. Agarose gel electrophoresis illustrating the MTHFR C677T genotypes

Determination of clinical and biochemical parameters

The clinical and biochemical parameters, as well as cIMT values, were determined using a method described by Aydın et al. (18). Biochemical and cardiological parameters were obtained from venous blood samples of the subjects.

DNA extraction

Genomic DNA was isolated from venous blood samples with ethylenediaminetetraacetic acid (EDTA) using phenol-chloroform extraction methods.

Determination of MTHFR C677T polymorphism

The MTHFR C677T single-nucleotide polymorphism (SNP) was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method using the Hinf I restriction endonuclease enzyme. The DNA segment was amplified from the forward, 5'- TGA AGG AGA AGG TGT CTG CGG GA -3' and reverse, 5'- AGG ACG GTG CGG TGA GAG TG -3' primers. The PCR reaction solution contained 2.5 mL dNTPmix (2 mM), 2.5 mL PCR buffer (10x), 1 mL primer pair (10 pmol), 2.5 mL $MgCl_2$ (16 mM), 2.5 mL Taq DNA polymerase (1U), 1 mL genomic DNA (25-100 ng), and 13 mL sterile dH_2O. The PCR reaction, based on a method described Kowa et al. (19), yielded 198-bp PCR products. The restriction enzyme reaction was performed using 10x Hinf I buffer R (2 mL), 1 mL Hinf I restriction endonuclease (10 U/mL), 10 mL PCR reaction product, and 7 mL sterile dH_2O (total volume of 20 µL). The reaction mixture was incubated at 37°C for 14 hours. The restriction products and genotypes were determined using Hinf I digestion products, yielded by 2.0% agarose gel electrophoresis (Fig. 1).

Statistical analysis

Statistical analyses were carried out using the Statistical Package for the Social Science (SPSS) software program, version 15.0, (SPSS Inc. Chicago, IL, USA). Normally distributed continuous variables were analyzed using student t-test and expressed as mean±SD (standard deviation). Abnormally distributed continuous variables were analyzed using Mann-Whitney U test and expressed as median (min-max). Categorical variables were presented as percentages (%) and analyzed using chi-square test. A $p<0.05$ was considered statistically significant.

Results

Relations between biochemical and cardiological parameters in terms of demographic differences

Thirty-nine male (63.00±12.30 years of age) and 68 female (61.72±10.35 years of age) patients with T2DM were examined in

Table 1. Demographics and clinical parameters of the T2DM patients

Parameters	Male n=39 (36.5%)	Female n=68 (63.5%)	*P*
Age, years	63.00±12.30	61.72±10.35	0.241
Diabetes duration, years	3.00 (0.00-30.00)	7.00 (0.00-35.00)	0.036*
BMI, kg/m^2	30.25±5.10	33.53±6.89	0.006*
cIMT, µm	79.59±21.71	76.47±31.64	0.552
Total cholesterol, mg/dL	170.32±30.62	192.89±43.15	0.003*
HDL-cholesterol, mg/dL	38.00 (22.00-66.00)	43.00 (18.00-107.00)	0.022*
LDL-cholesterol, mg/dL	96.50 (57.00-135.00)	98.00 (55.00-269.00)	0.236
Triglyceride, mg/dL	129.50 (48.00- 584.00)	197.00 (60.00-821.00)	0.028*
Fasting plasma glucose, mg/dL	125.50 (76.00-450.00)	135.00 (63.00-377.00)	0.401
MCV, fL	84.66±4.63	82.12±5.20	0.013*
Serum iron, µg/dL	85.50 (23.00-168.00)	69.00 (19.00-136.00)	0.021*
SBP, mm Hg	142.44±23.34	152.01±20.89	0.038*
DBP, mm Hg	85.38±12.95	87.61±13.74	0.413
hs-CRP, mg/L	1.20 (0.39-53.09)	1.21 (0.14-68.90)	0.833
Folate, ng/mL	6.94 (3.50-17.80)	9.29 (3.73-19.30)	0.009*
Hcy, µmol/L	12.62±2.58	14.83±2.44	0.015*
Vitamin B12, pg/mL	257.00 (150.00-845.00)	240.00 (151.00-647.00)	0.418
HgB, g/dL	13.90±1.06	12.38±1.57	0.000*
Hct, %	41.31±2.97	37.31±4.33	0.000*
HbA1c, %	8.0±1.89	7.8±2.31	0.986
HOMO S, %	2.85 (0.53-8.31)	4.28 (0.57-9.25)	0.201
Visceral fat area, cm^2	13.50 (3.00-25.00)	11.00 (5.00-20.00)	0.001*

*Values in bold indicate statistical significance (*P*<0.05).
The results are shown as mean±SD (Standard deviation) and median (min-max).
BMI - body mass index; cIMT - carotid intima-media thickness; DBP - diastolic blood pressure; Hct - hematocrit; Hcy - homocysteine; HDL - high-density lipoprotein; Hb - hemoglobin; hs-CRP - high-sensitivity C-reactive protein; HOMO S - Homeostasis Model Assessment for Insulin Sensitivity; HbA1c - glycosylated hemoglobin; LDL - low-density lipoprotein; MCV - mean corpuscular volume; N - number of individuals; SBP - systolic blood pressure; T2DM - type 2 diabetes mellitus

Table 2. MTHFR C677T genotypes and alleles in T2DM patients

Genotypes				Alleles	
CC (%)	CT (%)	TT (%)	N (genotypes)	C (%)	T (%)
31 (29.0)	62 (57.9)	14 (13.1)	107	124 (86.9)	90 (13.1)

C - cytosine; MTHFR - methylene tetrahydrofolate reductase gene; T - timin; T2DM - type 2 diabetes mellitus; N - number of genotypes

this study. The mean duration of diabetes was 7.09±8.39 years for males and 8.84±8.07 years for females. Body mass index (BMI) was significantly higher (p=0.006) in females (33.53±6.89) than in males (30.25±5.10). While high-density lipoprotein (HDL) cholesterol (p=0.022), total cholesterol (p=0.003), systolic blood pressure (SBP) (p=0.038), folate (p=0.009) and triglyceride (p=0.028) levels were significantly higher in females than males, homocysteine (Hcy) (p=0.015) and visceral fat area (p=0.001) levels were much lower in females than in males. The gender differences were not meaningful for low-density lipoprotein (LDL, p=0.236) cholesterol, fasting plasma glucose (p=0.401), diastolic blood pressure (DBP) (p=0.413), high-sensitivity CRP (hs-CRP) (p=0.833), vitamin B12 (p=0.418), Homeostasis Model Assessment for Insulin Sensitivity (HOMO S) (p=0.201), HbA1c (p=0.986), and cIMT (p=0.552) in T2DM patients. Also, diabetes duration (years) was significantly (p=0.036) longer in females than in males (Table 1). Additionally, a significant correlation was found between cIMT and age (p<0.001). While the correlation between cIMT and BMI was meaningful (p=0.05), it was not meaningful between cIMT and gender (p=0.330), LDL-cholesterol (p=0.302), HDL-cholesterol (p=0.439), triglyceride (p=0.051), SBP (p=0.296), and DBP (p=0.551) values.

Relations between biochemical and cardiological parameters and cIMT values in terms of MTHFR polymorphism

In the study population, 31 subjects had the CC genotype (29.0%), 62 had the CT genotype (57.9%), and 14 had the TT genotype (13.1%). The frequencies of T and C alleles were 0.131 and 0.869, respectively (Table 2).

As far as the MTFHR C677T genotype is concerned, there were no significant differences between subjects with the wild-type allele (677CC) and mutant allele (677CT+677TT) in terms of diabetes duration in years (p=0.384), visceral fat area (p=0.985),

Table 3. Clinical and biochemical parameters according to MTHFR C677T genotypes in T2DM patients

	MTHFR genotype		
Parameters	**CC (n=31)**	**CT+TT (n=76)**	***P***
cIMT, μm	71.70 (40.00-120.00)	73.30 (40.00-290.00)	0.459
Gender, male/female	12 / 19	27 / 49	0.756
Age, years	62.56±11.84	61.98±11.47	0.319
Diabetes duration, years	2.50 (0.00-35.00)	5.50 (0.00-30.00)	0.384
BMI, kg/m^2	32.17±7.96	32.41±5.81	0.882
Total cholesterol, mg/dL	183.06±33.56	185.53±43.28	0.778
HDL-cholesterol, mg/dL	43.00 (18.00-77.00)	40.50 (22.00-107.00)	0.079
LDL-cholesterol, mg/dL	101.00 (57.00-155.60)	96.50 (55.00-269.00)	0.685
Triglyceride, mg/dL	137.00(48.00-580.00)	182.0 (60.00-821.00)	0.058
Fasting plasma glucose, mg/dL	128.00 (76.00-340.00)	131.00 (63.00-450.00)	0.542
Hct, %	38.96±4.18	38.86±4.29	0.108
SBP, mm Hg	148.17±25.34	148.62±21.02	0.931
DBP, mm Hg	86.17±11.94	87.04±14.05	0.749
hsCRP, mg/L	1.28 (0.14-30.60)	1.20 (0.19-68.90)	0.939
Serum iron, μg/dL	77.0 (26.00-168.00)	76.00 (19.00-149.00)	0.635
Folate, ng/mL	8.47 (3.74-19.30)	8.08 (3.50-17.50)	0.668
Hcy, μmol/L	14.48±3.29	14.64±3.06	0.872
Vitamin B12, pg/mL	238.00 (150.00-507.00)	250.50 (151.00-845.00)	0.814
MCV, fL	83.25±5.00	82.96±5.21	0.792
HgB, g/dL	12.99±1.36	12.90±1.68	0.765
Hct, %	38.96±4.18	38.86±4.29	0.977
HbA1C, %	7.80±1.40	8.10±1.50	0. 691
HOMO S, %	2.99 (0.59-9.25)	3.67 (0.53-9.17)	0.559
Visceral fat area, cm^2	12.00 (3.00-25.00)	12.00 (5.00-23.00)	0.985

The results are shown as mean±SD (Standard deviation) and median (min-max).
BMI - body mass index; cIMT - carotid intimal-medial thickness; DBP - diastolic blood pressure; Hct - hematocrit; Hcy - homocysteine; HDL - high-density lipoprotein; Hb - hemoglobin; hs-CRP - high-sensitivity C-reactive protein; HOMO S - Homeostasis Model Assessment for Insulin Sensitivity; HbA1c - glycosylated hemoglobin; LDL - low-density lipoprotein; MCV - mean corpuscular volume; N - number of individuals; SBP - systolic blood pressure; T2DM - type 2 diabetes mellitus

total cholesterol (p=0.778), triglyceride (p=0.058), fasting plasma glucose (p=0.542), SBP (p=0.931), DBP (p=0.749), hsCRP (p=0.939), folate (p=0.668), Hcy (p=0.872), vitamin B12 (p=0.814), HbA1c (p=0.691), HOMO S (p=0.559), and cIMT (p=0.459) values. In addition, there was no meaningful male/female gender difference between the 677CC (12/19) and 677CT+677TT (27/49) genotype frequencies (p=0.756) (Table 3).

Discussion

In this cross-sectional study, there were no significant differences between subjects with the wild-type allele (677CC) and mutant allele (677CT+677TT) in terms of diabetes duration in years, visceral fat area, total cholesterol, triglyceride, fasting plasma glucose, SBP, DBP, hsCRP, folate, Hcy, vitamin B12, HbA1c, HOMO S, and cIMT values.

Sufficient evidence showing an association between MTHFR 677TT and risk of CVD has not been found (4). However, several studies have shown the link between the MTHFR C677T gene polymorphism and coronary risk (20-26). Arai et al. (20) have pointed out the connection between the MTHFR C677T gene polymorphism and myocardial infarction and carotid wall thickening. In a meta-analysis study, no association was found to exist between the C677T polymorphism and the risk of diabetes mellitus in a Chinese population for diabetic nephropathy (DN) or diabetes mellitus (DM) (21), while other studies claimed that the MTHFR C677T polymorphism might influence DN risk but not for DM (22, 23). It was shown that the MTHFR C677T variant was not an independent risk factor for diabetes or CAD in the population of western Iran (24). In other study, the MTHFR T677T genotype was also concluded not to be a risk factor for the development of CVD in T2DM patients (25). However, Morita et al. (26) reported a significantly higher frequency of the MTHFR C677T allele in Japanese CAD patients. They showed that the association was stronger in homozygotes than in heterozygotes and suggested that the MTHFR C677T gene polymorphism may be a risk factor

for CAD. Also, the prevalence of stroke is markedly higher in the 677CT and 677TT genotypes compared with the 677CC genotype in T2DM patients (27). The same study suggests that the effect of the MTHFR C677T gene polymorphism on stroke may result from T-allele-linked deleterious effects, C-allele-linked protective mechanisms, or both In our 107 T2DM patients, the frequencies of the T and C alleles were 0.131 and 0.869, respectively (Table 2). However, we also did not find a significant association between the C677T gene polymorphism and CVD risk in T2DM patients.

Many studies have reported an association between the MTHFR C677T gene polymorphism and clinical parameters, which is related to vascular complications in T2DM patients (10, 28-33). The 677T allele carriers had significantly elevated BMI and higher SBP than 677CC homozygote carriers in peripheral arterial disease in T2DM patients from an isolated aboriginal Canadian population (10). Also, in this study, 677T carriers and noncarriers were not found to have significant differences in age, hypertension, duration of diabetes, DBP and plasma concentrations of fasting blood glucose, HbA1c, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, and C-reactive protein (10). Yılmaz et al. (29) found no correlation between the risk of coronary artery disease (CAD) and MTHFR genotype in Turkish patients. In an elderly Japanese population, a multivariate analysis revealed that this relationship between homocysteine and MTHFR was independent of other risk factors, involving gender, age, history of hypertension, and LDL and HDL-cholesterol. This analysis also revealed the effects of HDL-cholesterol and MTHFR gene interaction on T2DM patients (34). Our results show that the differences in both biochemical and cardiological parameters between subjects with the wild-type allele (677CC) and mutant allele (677CT+677TT) are not significant (Table 3).

Some studies suggest that hyperhomocysteinemia is a well-known independent risk factor for cardiovascular diseases (35, 36) and is also related with diabetic complications (37-40). Mild hyperhomocysteinemia has been associated with macrovascular complications in diabetic subjects. Araki et al. (11) found higher total Hcy levels in T2DM patients with macroangiopathy than in those without complications. In the other study, high total Hcy levels were also associated with higher CVD risk in diabetic patients than in those with impaired or normal glucose tolerance (12). In this study, plasma Hcy values were not significant (p=0.872) for MTHFR C677T genotypes in Turkish T2DM patients (Table 3).

Many studies have shown that cIMT tends to increase with age and that cIMT values of smokers and patients with dyslipidemia, hypertension, and type 2 diabetes are increased compared with those of control groups (41-46). In addition, in individuals with thicker cIMTs, the risk of stroke and coronary artery disease is known to be higher (1, 47, 48). It was shown that although there was a notable correlation between cIMT and BMI, age, SBP and total cholesterolemia, there was no correlation between cIMT and serum homocysteine and MTHFR gene polymorphisms in nephropathy-free T2DM patients (49). In our study, while cIMT values depended significantly on age (p<0.001) and BMI (p=0.05), there were no meaningful correlations between cIMT values and MTHFR C677T polymorphism, SBP, DBP, LDL and HDL-cholesterol, and triglyceride values.

Consequently, the present study shows that significant differences were not found between subjects with the 677CC allele and 677CT+677TT alleles for cardiac risk factors or for cIMT, hsCRP, and Hcy values in Turkish T2DM patients (Table 3).

Study limitations

Our study has the following limitations. We still could not fully exclude the effect of cardiovascular risk factors in T2DM patients in our results. We did not show lifestyle factors, such as smoking and drinking, and/or the relative basic characteristics of our patients. Another disadvantage was that we had no data on the physical activity and nutrition of the subjects. In order to obtain a clearer picture of the correlation between the MTHFR 677TT gene polymorphism and cardiac risk in T2DM patients, studies should be based on a larger sample size, with the recognition of gene-gene and gene-environment interactions.

Conclusion

We can conclude that the MTHFR 677TT gene polymorphism can not be used as a marker for assessment of cardiac risk in T2DM patients, because we did not observe any significant differences in terms of the MTHFR C677T polymorphism with cIMT values or the other cardiological parameters in 107 Turkish T2DM patients without nephropathy and retinopathy. We can say that geographic heterogeneity and lifestyle factors could affect the relationship between MTHFR genotypes and CVD risk in different populations.

Conflict of interest: None declared.

Peer-review: Externally peer-reviewed.

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İlaçlar çaresiz, yattık masaya
Yatıp da kalmak var, düştük tasaya
Aslında razıyız, haktan yasaya
Yeter ki olmasın tabip elinden.

Dr. Tahir Akman

Drugs are helpless, I am on the operating table.
I can be boxed on the table, I am worried.
In fact, I consent to everything from the God,
Unless from the surgeon.

Tahir Akman, MD