

ORIGINAL ARTICLE

Methylenetetrahydrofolate Reductase Gene Polymorphism C677T is Associated with Increased Risk of Coronary Heart Disease in Chinese Type 2 Diabetic Patients

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Objective Chronic cardiovascular diseases induced by long-term poor blood glucose control are the main cause of death in patients with type 2 diabetes mellitus (T2DM). Previous researches report that methylenetetrahydrofolate reductase gene (*MTHFR*) polymorphisms might influence the occurrence of coronary heart disease (CHD) in T2DM patients. The purpose of this study was to evaluate whether *MTHFR* C677T and A1298C mutations are associated with the risk of CHD in T2DM patients.

Methods A total of 197 subjects with T2DM were studied, of which 95 patients with CHD. The genotypes of *MTHFR* C677T and A1298C were analyzed by using dideoxy chain-termination method, and compared between patients with CHD and those without CHD.

Results We found that the frequency of the 677T allele was significantly higher in T2DM patients with CHD than those without CHD ($P=0.011$). However, there was no significant difference in any of the examined haplotypes between T2DM patients with and without CHD. Furthermore, the 677T allele was associated with a higher risk of CHD development in diabetic patients with lower homocysteine (Hcy) levels ($\leq 15 \mu\text{mol/L}$)

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($P=0.006$), while no effect of *MTHFR* gene polymorphism on the incidence of CHD was found in patients with higher Hcy levels ($>15 \mu\text{mol/L}$) ($P=0.491$).

Conclusion The *MTHFR* C677T gene polymorphism is associated with the risk of CHD of diabetic patients and could be used as an effective marker for CHD in Chinese diabetic populations with normal Hcy levels.

AT present, the prevalence of type 2 diabetes mellitus (T2DM) has been rapidly increasing worldwide.^[1] According to the latest statistics of the International Diabetes Federation (IDF), there were approximately 415 million diabetics worldwide in 2015, of which about 109.6 million were Chinese.^[2] The complications of T2DM mainly include cardiovascular diseases, cerebrovascular diseases, peripheral vascular diseases, nephropathy, neuropathy and retinopathy.^[3-5] As one of the major macrovascular complications of T2DM, coronary heart disease (CHD) is the main cause of morbidity and mortality in T2DM.^[6]

Methylenetetrahydrofolate reductase (*MTHFR*) encoded by the *MTHFR* gene is a key enzyme in the homocysteine (Hcy) metabolic pathway, which re-methylates Hcy to methionine.^[7] *MTHFR* polymorphisms had been reported to be associated with elevated plasma Hcy.^[6, 8] There are two common polymorphisms of the *MTHFR* gene: one is a Cytosine (C) to Thymine (T) mutation at nucleotide C677T, which results in an alanine to valine substitution, and another is an Adenine (A) to Cytosine (C) mutation at nucleotide A1298C, resulting in an alanine to valine substitution.^[9, 10] The dysfunctional mutations in C677T and A1298C are related to a decrease of *MTHFR* activity, leading to a relatively insufficient methylation process and consequently increasing the plasma concentration of Hcy.^[8, 10, 11] Elevated plasma Hcy level has been identified as a potential risk factor for the development of vascular diseases.^[12, 13] Hyperhomocysteinemia (Hhcy) is associated with endothelial dysfunction, prethrombotic state and coronary atherosclerosis in patients with T2DM.^[6, 14-17]

However, the frequency of the *MTHFR* mutation varies by ethnicity, and the conclusions whether the polymorphism of *MTHFR* could be a predictor for the CHD in T2DM patients are not consistent.^[6, 18-23] The main purpose of this study was to evaluate the frequency of C677T and A1298C mutations in the Chinese population with T2DM and to assess whether their polymorphisms are associated with the risk of CHD in T2DM.

MATERIALS AND METHODS

Study subjects and data collection

A total of 197 subjects with T2DM were included in this study, of which 95 patients were combined with CHD. All of the study subjects were older than 30 years old. Basic information including gender, age, height and weight was collected by questionnaire.

Diabetes was diagnosed according to the diabetes diagnostic criteria published by the World Health Organization (WHO).^[24] CHD was diagnosed according to the patient's physical examination, supplementary examination, and medical history. All of the diagnoses of CHD were consistent with the diagnostic criteria issued by International Society of Cardiology and WHO.^[25] Exclusion criteria: pregnancy and lactating women; patients with primary heart diseases; severe kidney diseases with serum creatinine (Scr) levels $>132 \mu\text{mol/L}$ (serious kidney diseases may be an important factor leading to atherosclerosis); patients taking folic acid or vitamin B₆ or B₁₂. The study was approved by the Medical Ethics Committee of The First Affiliated Hospital of Shandong First Medical University and complied with the principles of the Helsinki Declaration of 1975. Informed consent was obtained from all subjects prior to enrollment.

Biochemical assay

After overnight fasting, 2 ml of venous blood of the study subjects were collected into an EDTA-tube. Plasma was immediately separated at 4°C and kept at -80°C. The remaining blood was dispensed into 1.5 ml of enzyme-free EP tubes and kept at -80°C for DNA extraction. The plasma concentration of Hcy was measured using an enzyme-linked immunosorbent assay (ELISA) kit. Total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), hemoglobin A1c (HbA1c) and Scr concentrations were measured via enzymatic method using an automatic biochemical analyzer (7600, Hitachi Inc, Japan). Fasting plasma glucose (FPG) was assessed by the glucose oxidase method.

DNA extraction and genotyping

Genomic DNA was extracted from the peripheral venous blood using the Tiangen Blood DNA Kit (Tiangen, Beijing, China). The genotype of *MTHFR* C677T and A1298C was analyzed by dideoxy chain-termination method. A 25 µl reaction mixture for Polymerase Chain Reaction (PCR) system contained 0.25 µl 2×Taq DNA polymerase, 1 µl forward primer (3.2 pmol/µl), 1 µl reverse primer (3.2 pmol/µl), 1 µl DNA, 0.5 µl dNTP mixture, and added ddH₂O to 25 µl finally. The PCR reaction conditions included pre-denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. The sequences of primers for the *MTHFR* C677T and A1298C amplification were as follows: *MTHFR* C677T (forward 5'GTGTGGGAGTTTGAGCAAT3', reverse 5'GGGAGCTTATGGGCTCTCCT3'), *MTHFR* A1298C (forward 5'TACCCAGGAGTGGGACGAGT3', reverse 5'GCACCCTGAGTCCCTCTCAC3'). The PCR product was electrophoresed by an ABI3730XL (ABI, Carlsbad, California, USA) sequencer after purified.

Statistical analysis

All statistical analyses were performed using the SPSS 22.0 statistical analysis software (IBM, Armonk, New York, USA). The continuous variables were expressed as mean±standard deviation, and the categorical variables were expressed as percentages. Two-sided *t* test and *Chi-square* test were used to analyze continuous and categorical variables, respectively. Haplotypes analysis was performed using SHEsis online haplotype analysis software (<http://analysis.bio-x.cn/>). Results were considered statistically significant for the value of $P < 0.05$. *Chi-square* test was used to validate the Hardy-Weinberg equilibrium. The value $P > 0.05$ indicated that the genotype frequencies of the samples were consistent with Hardy-Weinberg equilibrium.

RESULTS

Clinical characteristics of the study patients

The clinical characteristics of the participants are shown in **Table 1**. There were no significant differences in the variables including gender, age, FPG, lipid, blood pressure, or Scr among T2DM subjects with or without CHD ($P > 0.05$).

Table 1. Characteristics of T2DM patients with and without CHD

Items	T2DM patients without CHD	T2DM patients with CHD	<i>P</i> value
<i>n</i>	102	95	
Male [<i>n</i> (%)]	67 (65.69)	62 (65.26)	0.950
Age (yrs)	63.14±6.78	64.34±8.11	0.263
Current smoker [<i>n</i> (%)]	26 (25.49)	27 (28.42)	0.547
Age at T2DM diagnosis (yrs)	50.23±7.93	51.67±9.80	0.257
Diabetes duration (yrs)	12.91±5.97	12.66±6.66	0.642
BMI (kg/m ²)	25.07±2.99	25.18±2.79	0.787
FPG (mmol/L)	7.95±2.08	8.10±2.33	0.733
HbA1c (%)	8.57±1.67	8.33±2.17	0.115
TC (mmol/L)	4.59±1.02	4.44±1.05	0.337
TG (mmol/L)	1.60±0.96	1.42±0.67	0.553
HDL-C (mmol/L)	1.21±0.28	1.20±0.39	0.523
LDL-C (mmol/L)	2.64±0.83	2.52±0.75	0.436
SBP (mm Hg)	133.26±12.42	131.01±13.62	0.228
DBP (mm Hg)	75.85±7.34	74.89±9.17	0.420
Scr (µmol/L)	66.21±18.40	70.25±22.48	0.128

T2DM: type 2 diabetes mellitus; CHD: coronary heart disease; BMI: body mass index; FPG: fasting plasma glucose; HbA1c: hemoglobin A1c; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure; Scr: serum creatinine.

Association between *MTHFR* C677T and A1298T polymorphisms and CHD development in T2DM patients

MTHFR C677T and A1298C were genotyped by dideoxy chain-termination method, with the sequencing map shown in **Fig. 1**. All 197 subjects with T2DM were genotyped at *MTHFR* C677T, and only 167 subjects (100 with CHD and 67 without CHD) were genotyped at A1298C.

The distributions of *MTHFR* C677T and A1298C genotypes in T2DM patients with or without CHD are shown in **Table 2** and **Table 3**. The frequencies of *MTHFR* 677CT+TT in T2DM patients with and without CHD were 90.53% and 78.43%, respectively. The frequencies of the CT+TT genotype and T allele were more common in T2DM patients with CHD than in those without CHD ($P = 0.020$, 0.011). However, the frequencies of *MTHFR* 1298AC+CC genotype or alleles showed no significant difference between these two

groups ($P>0.05$).

There were three haplotypes at the two loci of *MTHFR* (C677T and A1298C) when the haplotype frequency is $>3\%$. The haplotypes of the *MTHFR* (C677T and A1298C) are showed in **Table 4**. No significant difference was found in any of the examined haplotypes between T2DM patients with CHD and those

without CHD.

Interaction between *MTHFR* (C677T and A1298C) gene polymorphism and Hcy levels

The Hcy levels in T2DM patients with CHD were significantly higher than those without CHD (14.26 ± 6.09 vs. 11.76 ± 4.13 $\mu\text{mol/L}$; $P=0.001$). We divided the

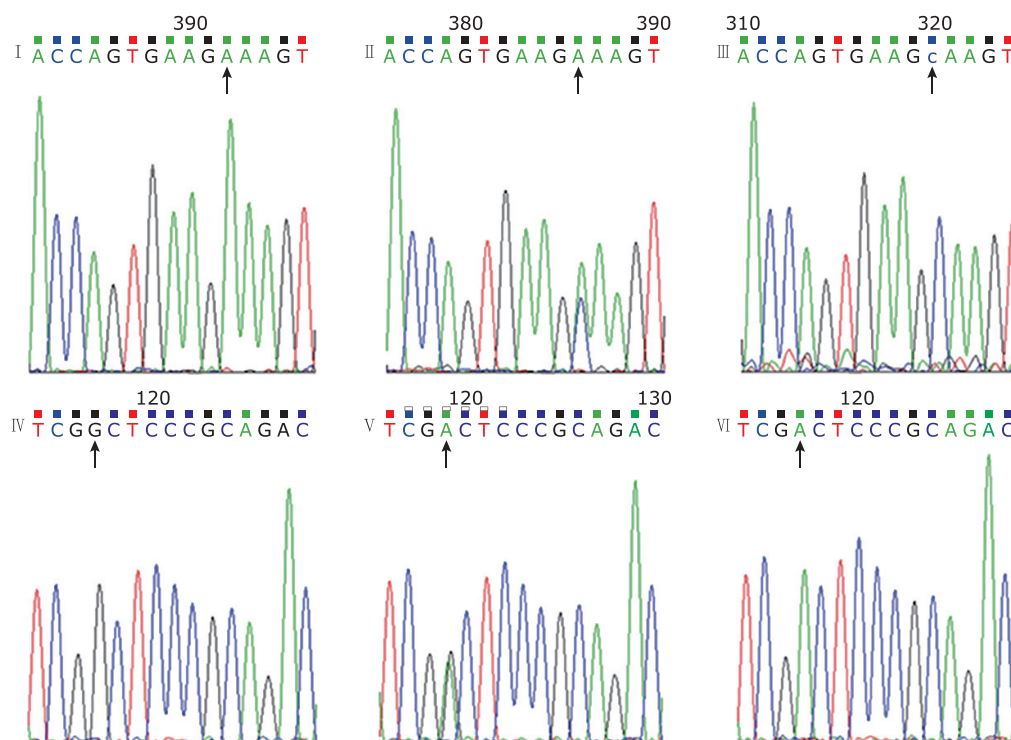


Figure 1. The sequencing maps of *MTHFR* A1298C genotypes AA (I), AC (II) and CC (III) as well as C677T genotypes CC (IV), CT (V) and TT (VI). Arrows indicate the mutation sites.

MTHFR: methylenetetrahydrofolate reductase gene.

Table 2. Distribution of *MTHFR* C677T genotype in T2DM patients with and without CHD

Genotypes and alleles	T2DM patients [n (%)]		OR (95%CI)	P value
	Without CHD (n=102)	With CHD (n=95)		
CC	22 (21.57)	9 (9.47)		
CT+TT	80 (78.43)	86 (90.53)	2.63 (1.14-6.05)	0.020
C allele	92 (45.10)	62 (32.63)		
T allele	112 (54.90)	128 (67.37)	1.70 (1.13-2.56)	0.011

OR: odds ratio; CI: confidence interval.

Table 3. Distribution of *MTHFR* A1298C genotype in T2DM patients with and without CHD

Genotypes and alleles	T2DM patients [n (%)]		OR (95%CI)	P value
	Without CHD (n=100)	With CHD (n=67)		
AA	64 (69.07)	48 (74.60)		
AC+CC	36 (30.93)	19 (25.40)	0.70 (0.36-1.38)	0.303
A allele	162 (83.51)	115 (87.30)		
C allele	38 (16.49)	19 (12.70)	0.70 (0.39-1.28)	0.251

diabetic patients into two groups according to Hcy levels (lower levels: ≤ 15.0 $\mu\text{mol/L}$; higher levels: >15 $\mu\text{mol/L}$) and assessed the relationship between *MTHFR* polymorphism and CHD development. Data showed that the frequency of the *MTHFR* 677CT+TT genotype was significantly higher in patients with CHD than that in those without CHD in the lower Hcy levels group ($P=0.006$), while no significant difference was observed in the higher Hcy level group ($P>0.05$). There was no significant difference in the distribution of A1298C genotypes (AA vs. AC+CC) between patients with and without CHD, neither in lower nor in higher Hcy level groups ($P>0.05$) (**Table 5**).

DISCUSSION

In the pathway of Hcy metabolism *MTHFR* is a key enzyme that is able to re-methylate Hcy into methionine.^[7] Dysfunctional mutations of the *MTHFR* gene are related to a decrease in *MTHFR* activity, resulting in an increase in the plasma concentration of Hcy.^[8, 10, 11] Several studies have confirmed the important role of Hhcy in vascular complications.^[12, 13] An elevated plasma level of Hcy is associated with endothelial dysfunction, prethrombotic state and coronary atherosclerosis in patients with T2DM.^[6, 14-17] These effects of Hcy could be explanatory of a close correlation between the mu-

tations of *MTHFR* gene that cause Hhcy and the presence of CHD in patients with T2DM.

In the present study, the data showed that the 677T allele was significantly associated with an increased risk of CHD in patients with T2DM, while no association was found between A1298C polymorphism and CHD development. These results are in accordance with previous research results conducted in studies including Czech, Polish and Chinese participants.^[20, 26-28] Although *MTHFR* 677T has been verified to be a risk allele for CHD in the past. However, opposite conclusions still exist. Several studies have reported no association between CHD and the *MTHFR* 677T allele in patients with T2DM.^[29-31] The contradictory results exist in various populations, suggesting that further studies including a larger sample size and different populations are required before confirming the role of the *MTHFR* 677T allele in CHD development. Our results showed that there was no significant difference in the haplotypes of *MTHFR* (C677T and A1298C) gene between T2DM patients with CHD and those without CHD. These findings suggested that the haplotypes of the *MTHFR* (C677T and A1298C) gene are not associated with susceptibility of CHD in patients with T2DM.

On the other hand, studies have confirmed that an elevated plasma level of Hcy has been identified as a potential risk factor for the development of CHD in

Table 4. Association of haplotypes in the *MTHFR* gene with risk of CHD

Haplotypes*	Frequency		OR (95%CI)	P value
	T2DM without CHD (n=67)	T2DM with CHD (n=67)		
C-A	0.267	0.216	0.75 (0.45-1.26)	0.275
C-C	0.183	0.142	0.73 (0.40-1.34)	0.310
T-A	0.543	0.642	1.49 (0.95-2.33)	0.084

*The order of polymorphism in haplotypes: C677T and A1298C.

Table 5. Association of *MTHFR* (C677T and A1298C) gene polymorphisms with the risk of CHD in T2DM patients among different Hcy level groups

Hcy levels ($\mu\text{mol/L}$)	<i>MTHFR</i> genotypes	Patients without CHD (n)	Patients with CHD (n)	OR (95%CI)	P value
5-15.0	677CC	21	4	4.39 (1.42-13.54)	0.006
	677CT+TT	67	56		
	1298AA	55	31		
>15.0	1298AC+CC	31	10	0.57 (0.25-1.32)	0.189
	677CC	1	5	0.46 (0.05-4.35)	0.491
	677CT+TT	13	30		
	1298AA	9	17		
	1298AC+CC	5	9	0.95 (0.25-3.71)	0.945

Hcy: homocysteine.

patients with T2DM.^[6, 32-34] Our findings also showed that plasma Hcy values were significantly higher in T2DM patients with CHD than those without CHD. Clinically, Hcy could be a useful predictive biomarker for CHD risk in type 2 diabetic patients. However, for the patients with normal Hcy levels, it is necessary to find another reliable biomarker to predict CHD. In our study, *MTHFR* C677T mutation showed great value in predicting CHD in T2DM patients with lower Hcy levels. We have found that the *MTHFR* 667T allele could be used as a biomarker of CHD risk in T2DM patients with "normal" Hcy levels. However, for patients with higher Hcy levels, it seems that *MTHFR* gene polymorphism could not be a valuable marker to predict CHD progression.

The small sample size is one of the main limitations of this study. Our study was conducted with 197 T2DM patients. Therefore, there was insufficient statistical power to detect correlation. In particular, the sample size of the present study was limited when stratified by Hcy levels. The limitation of the sample size may cause deviations in the statistical results. Furthermore, this study was performed with a Chinese population, and the results need further investigation to confirm whether they could be generalized to other ethnic groups.

In conclusion, the *MTHFR* C677T gene polymorphism is associated with the risk of CHD and can be used as an effective marker for CHD prediction in diabetic patients with normal Hcy levels.

Conflicts of Interest Statement

The authors declare no conflict interests.

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中文摘要

亚甲基四氢叶酸还原酶基因 C677T 多态性与 中国 2 型糖尿病患者冠心病风险增加相关

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摘要

目的 长期血糖控制不佳引起的慢性心血管疾病是 2 型糖尿病患者主要的死亡原因。研究表明: 亚甲基四氢叶酸还原酶基因 (methylenetetrahydrofolate reductase gene, *MTHFR*) 多态性可能会影响 2 型糖尿病患者冠心病的发生。本研究旨在评估 *MTHFR* C677T 和 A1298C 基因突变是否与 2 型糖尿病患者冠心病的发生风险相关。

方法 本研究一共纳入 197 例 2 型糖尿病受试者, 其中 95 例受试者合并冠心病。采用双脱氧链终止法对 *MTHFR* C677T 和 A1298C 进行基因分型, 并分析比较合并冠心病与不合并冠心病受试者等位基因频率的差异。

结果 合并冠心病 2 型糖尿病受试者的 677T 等位基因频率明显高于不合并冠心病的受试者 ($P=0.011$)。单倍型频率在合并冠心病与不合并冠心病的 2 型糖尿病受试者之间无显著的差异。在同型半胱氨酸水平较低的糖尿病受试者中 ($\leq 15\mu\text{mol/L}$), 携带 677T 等位基因的受试者发生冠心病风险更高 ($P=0.006$), 而在同型半胱氨酸水平较高的糖尿病受试者中 ($>15\mu\text{mol/L}$), *MTHFR* 基因多态性对冠心病的发生风险无影响 ($P=0.491$)。

结论 *MTHFR* C677T 基因多态性与糖尿病患者发生冠心病的风险相关, 对于具有正常同型半胱氨酸水平的中国糖尿病人群, 其可作为预测冠心病风险的有效标志物。

关键词: 亚甲基四氢叶酸还原酶; 基因多态性; 2 型糖尿病; 冠心病; 同型半胱氨酸