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ORIGINAL ARTICLE

Association Between Homocysteine Level and Methylenetetrahydrofolate Reductase Gene Polymorphisms in Type 2 Diabetes Accompanied by Dyslipidemia

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Key words: type 2 diabetes mellitus; methylenetetrahydrofolate reductase; polymorphism; homocysteine; hyperlipidemia

Objective To investigate the association between total homocysteine (tHcy) level in plasma and methylenetetrahydrofolate reductase (*MTHFR*) C677T and A1298C genetic polymorphisms in a Chinese Han nationality population with type 2 diabetes mellitus (T2DM) accompanied by dyslipidemia.

Methods This case-control study enrolled T2DM patients with dyslipidemia and without dyslipidemia respectively. Sanger dideoxy-mediated chain-termination method was used to detect the gene polymorphisms of *MTHFR* C677T and A1298C. Plasma tHcy and lipid levels were measured as well. The genotype frequency and allele frequency between the dyslipidemia and non-dyslipidemia groups were compared by using *Chi-square* test. Plasma tHcy level of T2DM patients who carried the different genotypes was compared by Student's *t* test.

Results Finally, 82 T2DM patients with dyslipidemia and 94 ones without dyslipidemia were included in this study. There was a significant correlation between tHcy level and *MTHFR* C677T gene polymorphism in T2DM patients (t=2.27, P=0.02). Moreover, the plasma tHcy level in the dyslipidemia patients who carried *MTHFR* 677 TT genotype was significantly higher than that in those with CT+CC genotype (13.62±6.97 *vs*. 10.95±3.62

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μmol/L, t=2.20, P=0.03); while for patients without dyslipidemia, comparison of the tHcy level between those who carried the above two alleles showed no significantly difference (13.34±6.03 *vs.* 12.04±5.09 μmol/L, t=1.08, P=0.29).

Conclusion *MTHFR* 677TT genotype might associate with higher tHcy level in T2DM patients with dyslipidemia.

TYPE 2 diabetes mellitus (T2DM) is a disorder which has been proved to be associated with dyslipidemia. Dyslipidemia, including increased triglyceride (TG), high total cholesterol (TC), high low-density lipoprotein cholesterol (LDL-C) and decreased high-density lipoprotein cholesterol (HDL-C), which is a risk factor for cardiovascular and cerebrovascular diseases, has a much higher prevalence in T2DM patients than in non-diabetic patients.[1, 2] Therefore, managing blood lipid and identifying risk factors for dyslipidemia happening in diabetic patients is getting more and more important and necessary.

High total homocysteine (tHcy) level in blood could contribute to cytotoxic effects of substances that take part in the oxidative stress process and therefore result in various vascular diseases.[3] In patients with T2DM, vascular disease induced by endothelial injury due to mild hyperhomocysteinemia could thereby increase the probability of angiogenic atherosclerosis.[4] Moreover, it has been reported that hyperhomocysteinemia is associated with the incidence of peripheral arterial diseases, cardiovascular diseases and stroke.[5-7]

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme that participates in homocysteine metabolism.[8] C677T polymorphism in *MTHFR* yields a substitution of alanine to valine and A1298C polymorphism brings a substitution alanine to valine, which induce the functional deficiency of MTHFR enzyme.[9, 10] These deficiencies in MTHFR have been verified to be related with mildly or moderately elevated homocysteine levels in blood.[11] However, the roles of tHcy and genetic polymorphisms of *MTHFR* taking in the occurrence of dyslipidemia in patients with T2DM is still unclear. In the present study, we assess the association between *MTHFR* C677T and A1298C mutations and plasma tHcy level in T2DM patients with dyslipidemia.

PATIENTS AND METHODS

Patients

T2DM patients with or without dyslipidemia who were treated in Shandong Provincial Qianfoshan Hospital from July 2016 to May 2018 were selected. All cases were diagnosed in accordance with T2DM diagnostic criteria issued by the World Health Organization. The dyslipidemia is diagnosed according to the Guidelines for Prevention and Treatment of Dyslipidemia in Adults.[12] The individuals who met the following criteria were included: Chinese Han nationality, ⩾18 years old, having no blood relation, and not taking lipid-lowering drugs and with strictly low-sugar and low-fat diets. Patients with type 1 diabetes, in pregnancy and lactation, having an operation, and with large-area wound and infection were excluded. This study was reviewed and approved by the Medical Ethics Committee of Shandong Provincial Qianfoshan Hospital and informed consents were obtained from all the patients.

Biochemical analysis

All subjects were fasted for 12 hours and 2 ml of venous blood was collected in the early morning. Glycated hemoglobin (HbA1c) was measured by a semi-automatic analyzer (Transasia, Mannheim, Germany). Biochemical parameters including TC, TG, HDL-C, and LDL-C were analyzed using a commercial kit from Roche Diagnostic System Inc. (Switzerland) and the results were read on an automated biochemical analyzer (COBAS 6000/COBAS C 501, Roche Hitachi, Switzerland). The plasma tHcy level was detected by an enzymatic cycle method (Hitachi 7080, Ai Weide Biotechnology, Shandong).

DNA extraction

Genomic DNA was extracted from venous blood using a TIANamp Blood DNA kit (TIANGEN BIOTECH DP348, Beijing) according to the manufacturer's instructions. Theoretically, a centrifugal adsorption column containing silicon matrix material that specifically binds DNA and unique buffer system was used to extract genomic DNA directly from samples in EDTA tubes. The extracted DNA was stored at -80°C for further research.

Genotyping of *MTHFR* C677T and A1298C

Sanger dideoxy-mediated chain-termination meth-

od was used to detect the *MTHFR* C677T and A1298C genotypes. Primer sequences for *MTHFR* C677T amplification were: forward primer 5′-GTGTGGGAGTTTGGAGCAAT-3′, reverse primer 5′-GGGAGCTTATGGGCTCTCCT-3′. Primer sequences for *MTHFR* A1298C amplification were: forward primer 5′-TACCCAGGAGTGGGACGAGT-3, reverse primer 5′-GCACCCTGAGTCCCTCTCAC-3. The reaction was performed in a final volume 50 μl: 2 × Taq Master Mix 25 μl, upstream primer 0.2-1.0 μmol/L (final concentration), downstream primer 0.2-1.0 μmol/L (final concentration), template 1-50 ng (plasmid) and 10 ng-1 μg genome. PCR cycles were as follows: 94°C 90 seconds; 30 cycles of 94°C 20 seconds, 50°C to 60°C 20 seconds, 72°C 60 seconds; 72°C 5 minutes.

Statistical analysis

SPSS 22.0 software was used for data analysis. All continuous data were expressed as mean±standard deviation (SD), and inter-group differences were compared by Student's *t* test. For categorical variables, differences between groups were compared by *Chi-square* test or *Fisher*'s exact test. The gene *Hardy-Weinberg equilibrium* was analyzed using *Chi-square* test. $P<0.05$ was considered statistically significant.

RESULTS

Baseline characteristics of the two groups

A total of 176 T2DM patients were recruited in the study, including 82 with dyslipidemia and 94 without dyslipidemia. The baseline characteristics of the subjects are shown in **Table 1**. There were no significant differences in age, height, disease duration, HbA1c levels and tHcy levels between the two groups ($P>0.05$). However, dyslipidemia patients had higher weight and body mass index values than those without dyslipidemia ($P<0.05$).

Distribution of *MTHFR* gene polymorphisms

MTHFR (C677T and A1298C) genotype peak maps are shown in **Figure 1**. The frequencies of genotype and allele of *MTHFR* C677T and A1298C among T2DM patients with and without dyslipidemia are shown in **Table 2**. The genotype distributions of the included population conformed to the Hardy-Weinberg genetic equilibrium principle. Both the allele and genotype frequencies of *MTHFR* C677T polymorphism showed no significant difference between the patients having dyslipidemia and those with normal blood lipid, so *MTHFR* A1298C polymorphism did ($P>0.05$).

Association between tHcy and *MTHFR* polymorphism

As demonstrated in **Table 3**, there was a significant correlation between tHcy level and genetic polymorphism of *MTHFR* C677T in T2DM patients ($t=2.27$, $P=0.02$). Further analysis showed that in patients with dyslipidemia, plasma tHcy level in *MTHFR* 677TT carriers was significantly higher than that in CT+CC carriers ($t=2.20$, $P=0.03$), while in patients without dyslipidemia, the difference was not statistically significant ($t=1.08$, $P=0.29$).

DISCUSSION

Homocysteinemia, a thiol-containing amino acid mainly derived from dietary intake of methionine, is

Table 1. Comparisons of clinical and biochemical parameters between T2DM patients with and without dyslipidemia§

Groups	*n*	Age (rs)	Height (m)	Weight (kg)	BMI (kg/m^2)	Disease duration(rs)	HbA1c (%)	TC (mmol/L)	TG (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	tHcy (μmol/L)
T2DM without dyslipidemia	94	59.6± 10.34	1.68± 0.08	69.38± 10.58	24.62± 3.27	11.41± 8.21	8.37± 1.83	4.05± 0.64	0.97± 0.33	2.23± 0.56	1.34± 0.24	12.57± 5.50
T2DM with dyslipidemia	82	59.72± 9.94	1.68± 0.07	72.99± 11.10	25.89± 3.21	9.40± 7.60	8.00± 1.57	4.62± 1.31	1.67± 0.95	2.71± 0.92	1.14± 0.35	12.22± 5.60
t value		-0.07	0.31	-2.21	-2.58	1.67	1.43	-3.78	-6.70	-4.27	4.51	0.42
P value		0.94	0.76	0.03	0.01	1.00	0.15	<0.05	<0.05	<0.05	<0.05	0.68

§: Plus-minus values are means±SD.

T2DM: type 2 diabetes mellitus; BMI: body mass index; HbA1c: glycated hemoglobin; TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; tHcy: total homocysteine.

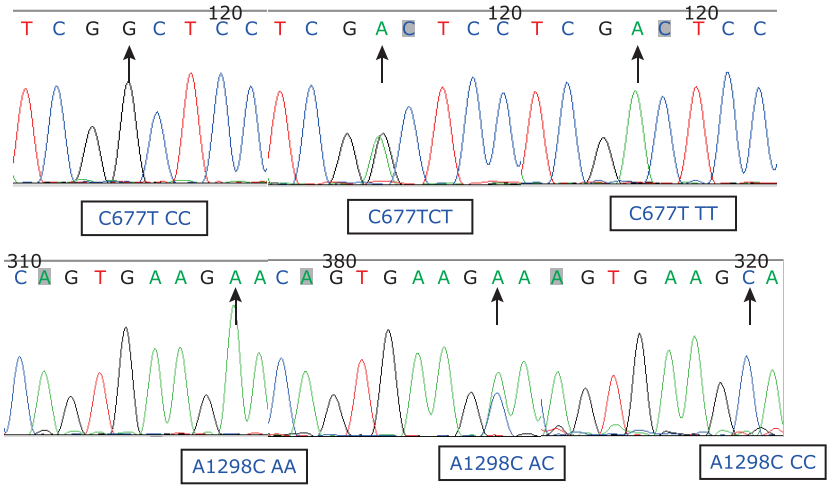

Figure 1. Peak map of direct sequencing of *MTHFR* C677T and A1298C by using dideoxy-mediated chain-termination method.

Figures A, B and C showing the peak map of *MTHFR* C677T CC, CT and TT, respectively, and Figures E, F and G showing the peak map of *MTHFR* A1298C AA, AC and CC, respectively.

MTHFR: methylenetetrahydrofolate redutase.

Table 2. Genotype and allele frequencies of *MTHFR* C677T and A1298C polymorphisms in T2DM subjects with or without dyslipidemia [*n* of cases (%)]

Groups	*n*	*MTHFR* C677T genotype			*MTHFR* C677T allele		*MTHFR* A1298C genotype		*MTHFR* A1298C allele	
		CC	CT	TT	C	T	AA	AC+CC	A	C
T2DM without dyslipidemia	94	12(12.77)	43(45.74)	39(41.49)	67(35.64)	121(64.36)	65(69.15)	29(30.85)	159(84.57)	29(15.43)
T2DM with dyslipidemia	82	6(7.32)	37(45.12)	39(47.56)	49(29.88)	115(70.12)	62(75.61)	20(24.39)	143(87.20)	21(12.80)
χ^2		1.46	0.01	0.65	1.32		0.91		0.49	
P		0.32	1.00	0.45	0.26		0.40		0.54	

Table 3. Comparisons of tHcy levels of T2DM patients who carried the different genotypes§ (μmol/L)

Groups	*n*	*MTHFR* C677T		*t*	*P*	*MTHFR* A1298C		*t*	*P*
		TT	CT+CC			AA	AC+CC		
All the subjects	176	13.48±6.48	11.56±4.52	2.27	0.02	12.47±5.80	12.24±4.86	0.24	0.81
T2DM with dyslipidemia	82	13.62±6.97	10.95±3.62	2.20	0.03	12.34±6.1	11.86±3.51	0.33	0.74
T2DM without dyslipidemia	94	13.34±6.03	12.04±5.09	1.08	0.29	12.60±5.48	12.50±5.65	0.06	0.95

§: Plus-minus values are means±SD.

mainly produced by demethylation of methionine in human body and can be catabolized by methylation, transsulfuration, or methylation substitution to cysteine, which takes an important intermediate in the metabolism of methionine and cysteine.[12-15] Once gene mutations happen in the enzymes involved in the metabolic process of homocysteinemia, tHcy would accumulate in the cells, eventually leading to an elevated concentration of tHcy in blood. Hyperhomocysteinemia has been verified to be one of the most important risk factors for macroangiopathy.[16]

MTHFR is an enzyme that takes a vital role in the metabolism of tHcy and folic acid. *MTHFR* gene, locating on autosome 1P36.3, includes 11 exons and 10 introns.[17, 18] A variety of mutations, such as C677T, A1289C, etc., have been found in *MTHFR* gene, which have been proved to be associated with the occurrence of cardiovascular and cerebrovascular diseases.[19] In

this study, we researched the association of tHcy with *MTHFR* C677T and A1298C polymorphisms in Chinese Han T2DM patients accompanying with or without dyslipidemia. We found that *MTHFR* 677TT genotype was associated with an increased tHcy level in T2DM patients. This finding is consistent with that reported by others.[20, 21]

MTHFR C677T mutation, the most common heat-labile missense mutation of *MTHFR* gene, occurs at the location of catalytic region of MTHFR, which can alter the enzyme activity and heat tolerance of MTHFR. It has been reported that a homozygous mutation of 677TT could reduce the average enzyme activity by about 70%, and a heterozygous mutation of 677CT could reduce the average MTHFR activity by about 35%. The reduced enzyme activity leads to a decrease in the concentration of folic acid in the plasma and an increase in the level of tHcy,[11] thus resulting in hyperhomocysteinemia, hypomethionemia, and other pathological changes. The results of published studies showed that the *MTHFR* C677T polymorphisms and mutation frequency varied significantly in different countries, races, regions or ethnic groups. These factors may influence a person's susceptibility to diseases.[22] It has been demonstrated that the frequency of T allele in the African population is lower than that in other races, and no homozygous mutations have been found, while the T allele frequency in North American and European population is between 5% and 15%.[23] The frequency of *MTHFR* 677TT genotype is 32% and 9%, respectively, in Brazil[24] and Moroccan T2DM patients.[25] In another research on Chinese T2DM patients, the *MTHFR* 677TT genotype frequency is 19%,[26] much lower than our results. In our study, the *MTHFR* 677TT frequency was 47.56% and 41.49%, respectively, in the T2DM patients with or without dislipidemia.

Hyperhomocysteinemia is an independent risk factor for thrombosis and atherosclerosis,[16] which may be associated with impairment of endothelial function and promoting thrombogenic activity in the vessel wall.[27, 28] Previous studies showed that T2DM patients combined with macroangiopathy had higher tHcy level compared with T2DM patients having no complications.[4, 5, 29-31] Due to the ethnic differences in *MTHFR* genotypes and the influence of certain non-genetic factors, the *MTHFR* C677T gene polymorphism in T2DM patients in some ethnics was not associated with dyslipidemia, cardiovascular and cerebrovascular complications.

In conclusion, we found that *MTHFR* 677TT genotype was associated with an increase of tHcy level in T2DM patients, while *MTHFR* A1298C polymorphisms or plasma tHcy levels was not associated with the development of dyslipidemia in T2DM patients. Our study has some limitations. We still could not clarify the risk factors for the dyslipidemia in T2DM patients because of the deficiency of the lifestyle data, including the exact total calories per day, smoking or drinking, and/or conditions of movement. In order to acquire a well-founded correlation between the *MTHFR* gene polymorphism and the prevalence of dyslipidemia among diabetic population, farther studies based on a larger sample size need to be carried out and the interactions between environmental and genetic factors should be further investigated.

Conflict of Interests Statement

The authors declare no conflict of interests.

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