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Original Article

Association of MTHFR C677T and A1298C polymorphisms with the development of type 2 diabetic nephropathy and their interaction with environmental factors

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Abstract: Type 2 diabetic nephropathy is a major cause of end-stage renal disease. MTHFR plays a vital role in folate metabolism, DNA methylation, and RNA synthesis. The aim of this study was to investigate the association between *MTHFR* C677T and A1298C genomic polymorphisms and development of type 2 diabetic nephropathy in a Chinese population. A hospital-based case-control study was performed. A total of 162 patients with type 2 diabetic nephropathy and 302 controls were recruited from the First Affiliated Hospital of Xinxiang Medical University between January 2013 and February 2015. Genotyping of the *MTHFR* C677T and A1298C polymorphisms was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods. By the chi-square test, a statistically significant difference was observed between the patients and controls in regards to the genetic distributions of *MTHFR* C677T (χ^2=13.51, *P*=0.001), whereas no significant difference was observed in the genetic distributions of *MTHFR* A1298C. Individuals carrying with the TT genotype of *MTHFR* C677T was associated with a significant increase in type 2 diabetic nephropathy risk compared to the CC genotype, and the adjusted OR was 3.79 (1.69-8.70). In addition, the T allele of *MTHFR* C677T significantly elevated type 2 diabetic nephropathy risk in comparison to the C allele (OR=1.60, OR=1.18-2.17). In conclusion, we found that the *MTHFR* C677T genomic polymorphism can influence the development of type 2 diabetic nephropathy in a Chinese population.

Keywords: *MTHFR,* C677T, A1298C, diabetic nephropathy, Chinese population

Introduction

Type 2 diabetic nephropathy is a major cause of end-stage renal disease, and it shows high mortality in diabetic patients [1]. Type 2 diabetic nephropathy was increasing rapidly worldwide. Although improvements in early detection and treatments have decreased the mortality rate of type 2 diabetic nephropathy in recent years, the lack of effective preventative measures for type 2 diabetic nephropathy remains a major public health problem. The development of type 2 diabetic nephropathy occurs over a long period of time, involves multifactorial processes, and has many associated risk factors, including high blood pressure, high glomerular filtration rate, glycemic control, and race were reported to be associated with type 2 diabetic nephropathy development [2]. Besides, genetic susceptibility may also be an important determinant of both the incidence and severity of type 2 diabetic nephropathy [3]. Previous studies have reported that many genetic factors, such as angiotensin II type 1 receptor (*AT1R*) gene, plasminogen activator inhibitor-1 (PAI-1) gene, hypoxia-inducible factor-1α (*HIF-1α*) gene, MicroRNA-125, Interleukin-6R, peroxisome proliferators-activated receptor γ (*PPARγ*) gene, matrix metalloproteinase 9 (*MMP9*) gene and transcription factor 7-like 2 gene (*TCF7L2*), play important roles in the development of type 2 diabetic nephropathy [4-10].

Methylenetetrahydrofolate reductase (*MTHFR*) locates in 1p36.3. MTHFR plays a vital role in folate metabolism, DNA methylation, and RNA synthesis. MTHFR irreversibly catalyzes the

conversion of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, which is the main form of folic acid in plasma and tissues, and is involved in the conversion of homocysteine into S2 adenosine methionine. S2 adenosine methionine plays an important role in DNA methylation, nucleic acid synthesis, and metabolism. A common C677T mutation (rs1801133) in the *MTHFR* gene has been widely studies, and it causes the conversion of the amino acid alanine to valine at position 226 in the protein.

This genomic polymorphism is correlated with a 50% reduction of *MTHFR* enzyme activity, an increased risk in plasma homocysteine concentration, a decreased risk in plasma folic acid concentration and a high homocysteine that causes the vascular injury [11, 12]. Genomic polymorphism of A1298C (rs1801131) is another common mutation and locates in exon 7, and this genomic variation could cause a change from glutamate to alanine with decreased enzyme activity in vitro. Genetic polymorphisms of *MTHFR* C677T and A1298C result in decreased gene transcription, and their associated amino acid substitutions may influence the function of the *MTHFR* protein. Previous studies have reported *MTHFR* C677T and A1298C genomic polymorphisms are along with increased plasma homocysteines are correlated with development of complication of type 2 diabetes, such as diabetic retinopathy and diabetic nephropathy [13-17], but the results are conflicting. The aim of this study was to investigate the association between *MTHFR* C677T and A1298C genomic polymorphisms and development of type 2 diabetic nephropathy in a Chinese population, and their interaction with environmental characteristics.

Material and methods

Subjects

A hospital-based case-control design was performed in this study. A total of 162 patients diagnosed with type 2 diabetic nephropathy were recruited from the First Affiliated Hospital of Xinxiang Medical University between January 2013 and February 2015. Patients with type 2 diabetes mellitus were confirmed according to the criteria from WHO in 1999 [18]. Nephropathy in diabetic patients was defined as the proteinuria of at least 500 mg/24 h and glomerular filtration rates less than 25 mL/min. The exclusion criteria were as follows: those with a history of type I diabetes mellitus, other tumors, other endocrine diseases except for type 2 diabetes mellitus and liver diseases, and intake of folate, Vitamin B_6 and Vitamin B_{12}.

A total of 302 control subjects were selected from individuals who received health examinations in the First Affiliated Hospital of Xinxiang Medical University between May 2014 and February 2015. All control subjects were confirmed to be free of a history of type 2 diabetes mellitus, nephropathy or endocrine diseases, as well as serious liver diseases.

The demographic and lifestyle characteristics of all study subjects were collected from a structured questionnaire, including age, sex, diabetic duration, hypertension and body mass index (BMI). Clinical information was collected from medical records, including systolic and diastolic blood pressure and the levels of total cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein, and creatinine. In type 2 diabetic nephropathy patients, the mean ages were 57.54±8.50 years, and there were 56 males and 106 females. The BMI of patients was 25.83±3.11 kg/m^2, and the duration of diabetes was 12.53±4.35 years. In controls, the mean age of type 2 diabetic nephropathy patients were 55.95±9.11 years, and there were 125 males and 177 females. The BMI of diabetic controls was 24.13±2.72 kg/m^2.

Blood sample (5 mL) was taken from each patient and control subject for analysis and stored in EDTA-containing tubes at -20°C until using. All the investigated subjects signed an informed consent before enrollment. The protocol was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University, and performance of this study was according to the Helsinki Declaration of 1964.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). Genotyping of the *MTHFR* C677T and A1298C polymorphisms was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods. The primers for the *MTHFR* C677T were 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' (forward) and 5'-AGGACGGTGCGGTGAGAGTG-3' (reverse). The primers for

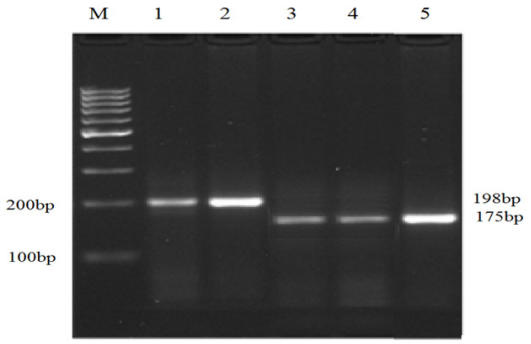

Figure 1. Polymorphism determination of *MTHFR* C667T gene by PCR-RFLP: Lane 1 and 2 were the CC genotype; lane 3 and 4 were the CT genotype; lane 5 was the TT genotype.

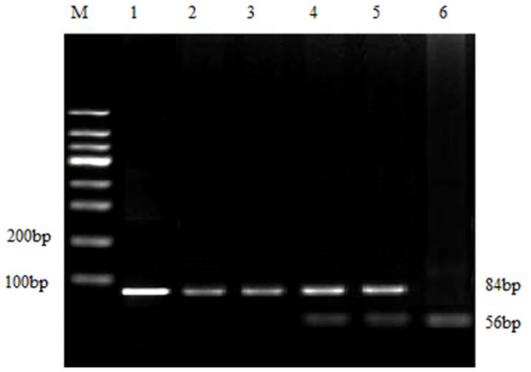

Figure 2. Polymorphism determination of *MTHFR* A1298C gene by PCR-RFLP: Lane 1, 2 and 3 were the CC genotype; lane 4 and 5 were the AC genotype; lane 6 was the AA genotype.

MTHFR A1298C were 5'-TCCTCTTCCCCTGCC-TTTG-3' (forward) and 5'-CCACTCCAGCATCAC-TCACTTT-3' (reverse). The restriction enzymes for digestion of *MTHFR* C677T and A1298C were HinfI and MboII, respectively. The *MTHFR* C677T and A1298C polymorphisms result in the digestion of the 198 bp and 163 bp, respectively. PCR was performed in a 25 μl reaction mixture containing 2.0 μl of DNA, 1.0 μl of each primer, 2.0 μl of dNTP mixtures, 2.0 μl of $MgCl_2$ solution, 2.0 μl of Taq DNA polymerase and 2.5 μl of 10× PCR Buffer. The PCR condition was set at: 94°C for 5 minutes, and then followed by 36 cycles of 94°C for 55 s, 55°C for 55 s and 72°C for 60 s, and a final elongation of 7 minutes at 72°C.

For *MTHFR* C677T, the CC genotype was digested into 198 bp fragments, the CT genotype was digested into 175 and 23 bp fragments, and the TT genotype was digested into 175 and 23 bp fragments (**Figure 1**). For A1298C, the CC genotype was digested into 84, 31, 30 and 18 bp fragments, the AC genotype was digested into 84, 56, 31, 30, 28, 28 and 18 bp fragments, and the AA genotype was digested into 56, 31, 30, 28, 28 and 18 bp fragments (**Figure 2**). The PCR products were analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The DNA bands were visualized under UV light.

Statistical analysis

The demographic and clinical characteristics, as well as *MTHFR* C677T and A1298C genotype frequencies in type 2 diabetic nephropathy patients and control subjects were compared using chi-squared (χ^2) tests or Student's *t*-test. The goodness-of-fit χ^2-test was performed to determine whether the genotype frequencies at *MTHFR* C677T and A1298C were in agreement with the Hardy-Weinberg equilibrium (HWE). The minor allele frequencies of *MTHFR* C677T and A1298C were compared with those in National Center for Biotechnology Information SNP database. The association between *MTHFR* C677T and A1298C genotype polymorphisms and susceptibility to type 2 diabetic nephropathy was analyzed by multiple logistic regression analysis, and the results was expressed by Odd's ratio (OR) along with 95% Confidence Interval (CI). Moreover, Spearman interaction analysis was taken to estimate the correlation between *MTHFR* C677T and A1298C genotype polymorphisms and demographic, lifestyle and clinical characteristics in the risk of diabetic nephropathy. The above analyses were completed by Stata software version 12.0 for Windows (StataCorp, College Station, Texas, USA). All statistical tests were two-sided with a statistical significance level of $P<0.05$.

Results

The demographic, lifestyle and clinical data of type 2 diabetic nephropathy patients and control subjects are shown in **Table 1**. Using Chi-square test, we observed that type 2 diabetic nephropathy patients were more likely to have higher age (t=1.83, *P*=0.03), BMI (t=6.10, *P*<0.05), glucose (*t*=26.93, *P*<0.05), HbA1c (t=17.61, *P*<0.05), triglyceride (t=8.52, *P*<0.05), total cholesterol (t=8.08, *P*<0.05) and low-den-

Table 1. Demographic, lifestyle and clinical data of study subjects

Variables	Patients N=162	%	Controls N=302	%	χ^2 test or *t* test	*P* value
Age, years	57.54±8.50		55.95±9.11		1.83	0.03
Gender						
Female	56	34.57	125	41.39		
Male	106	65.43	177	58.61	2.06	0.15
BMI, kg/m^2	25.83±3.11		24.13±2.72		6.10	<0.05
Hypertension						
No	95	58.64	219	72.52		
Yes	67	41.36	83	27.48	9.28	0.002
Glucose, mml/L	12.65±4.80		5.02±0.82		26.93	<0.05
HbA1c, %	9.32±3.65		4.85±1.82		17.61	<0.05
Triglyceride, mmol/L	1.72±0.23		1.55±0.19		8.52	<0.05
Total cholesterol, mmol/L	5.26±1.06		4.52±0.87		8.08	<0.05
High-density lipoprotein, mmol/L	1.04±0.42		1.45±0.35		11.20	<0.05
Low-density lipoprotein, mmol/L	4.36±1.46		2.82±1.53		10.50	<0.05
Creatinine, mmol/L	154.60±11.42		67.42±18.45		54.76	<0.05
Duration of diabetes, years	12.53±4.35					

Table 2. Genotype frequencies of *MTHFR* C677T and A1298C between type 2 diabetic nephropathy patients and controls

MTHFR	Patients	%	Controls	%	χ^2 test	*P* value	P for HWE	MAF	
								Controls	Database
C677T									
CC	69	42.59	162	53.64					
CT	72	44.44	127	42.05					
TT	21	12.96	13	4.30	13.51	0.001	0.05	0.2533	0.2454
A1298C									
AA	81	50.00	163	53.97					
AC	69	42.59	123	40.73					
CC	12	7.41	16	5.30	1.18	0.55	0.24	0.2566	0.2494

sity lipoprotein (t=54.76, *P*<0.05), have lower high-density lipoprotein (t=11.20, *P*<0.05) and suffer from hypertension (χ^2=9.28, *P*<0.05) in comparison to the control subjects.

The genotype frequencies of *MTHFR* C677T and A1298C polymorphisms in type 2 diabetic nephropathy patients and controls are shown in **Table 2**. According to the goodness-of-fit chi-squared test, the genotype distributions of *MTHFR* C677T (*P*<0.05) and A1298C (*P*=0.24) polymorphisms were agreement with Hardy-Weinberg equilibrium in the control group. By the chi-square test, a statistically significant difference was observed between the patients and controls in regards to the genetic distributions of *MTHFR* C677T (χ^2=13.51, *P*=0.001), whereas no significant difference was observed in the genetic distributions of *MTHFR* A1298C. The minor allele frequencies of *MTHFR* C677T and A1298C in controls were similar to those reported by the National Center for Biotechnology Information (NCBI) SNP database.

As determined by multiple logistic regression analysis, individuals carrying with the TT genotype of *MTHFR* C677T were associated with a significant increase in type 2 diabetic nephropathy risk compared to the CC genotype, and the adjusted OR was 3.79 (1.69-8.70) (**Table 3**). In addition, the T allele of *MTHFR* C677T significantly elevated type 2 diabetic nephropathy risk when compared with the C allele (OR=1.60,

Table 3. Relationship between *MTHFR* C677T and A1298C genomic polymorphisms and susceptibility to type 2 diabetic nephropathy

MTHFR	Patients	%	Controls	%	OR (95% CI)[1]	*P* value
C677T						
CC	69	42.59	162	53.64	1.0 (Ref.)	-
CT	72	44.44	127	42.05	1.33 (0.87-2.03)	0.16
TT	21	12.96	13	4.30	3.79 (1.69-8.70)	<0.05
Allele						
C	210	64.82	451	74.67	1.0 (Ref.)	-
T	114	35.19	153	25.33	1.60 (1.18-2.17)	0.002
A1298C						
AA	81	50.00	163	53.97	1.0 (Ref.)	-
AC	69	42.59	123	40.73	1.13 (0.74-1.71)	0.55
CC	12	7.41	16	5.30	1.51 (0.62-3.58)	0.31
Allele						
A	231	71.30	449	74.34	1.0 (Ref.)	-
C	93	28.70	155	25.66	1.17 (0.85-1.59)	0.32

[1]Adjusted for age, gender, BMI, hypertension, glucose, HbA1c, triglyceride, total cholesterol, high-density lipoprotein, low-density lipoprotein and creatinine.

Table 4. Interaction *MTHFR* C677T genetic polymorphism with Demographic, lifestyle and clinical variables in the risk of type 2 diabetic nephropathy

Variables	Correlation coefficient value	*P* value
Age	0.013	0.53
Male	0.015	0.47
BMI	0.011	0.62
Suffering from hypertension	0.024	0.31
Glucose	0.059	0.02
HbA1c	0.026	0.28
Triglyceride	0.027	0.27
Total cholesterol	0.025	0.29
High-density lipoprotein	0.034	0.21
Low-density lipoprotein	0.032	0.23
Creatinine	0.026	0.28
Duration of diabetes	0.031	0.22

OR=1.18-2.17). However, no significant relationship was observed between *MTHFR* A1298C polymorphism and susceptibility to diabetic nephropathy.

Moreover, we conducted interaction between *MTHFR* C677T and demographic, lifestyle and clinical characteristics in the risk of diabetic nephropathy, such as age, gender, BMI, hypertension, glucose, HbA1c, triglyceride, total cholesterol, high-density lipoprotein, low-density lipoprotein and creatinine (**Table 4**). Moreover, we found *MTHFR* C677T polymorphism had interaction with the glucose value in the risk of type 2 diabetic nephropathy (Correlation coefficient value=0.059, P= 0.02).

Discussion

Single nucleotide polymorphisms (SNPs) are DNA sequence polymorphisms caused by a single nucleotide variation, and the frequency of genetic polymorphisms is at least 1% in a population. The mutations include the transformation of a single base by transversion, insertion, or deletion, and SNPs are thought to affect susceptibility to human diseases. In recent years, genomic susceptibility to diseases has attracted a growing attention to research the genetic polymorphisms involving in pathogenesis of diseases. One of these genes that might be associated with type 2 diabetic nephropathy is *MTHFR* gene. In the present study, we firstly evaluated the relationship between *MTHFR* C677T and A1298C genetic polymorphisms and type 2 diabetic nephropathy risk in a Chinese population, and we observed that the *MTHFR* C677T genomic variation did contribute to the development of type 2 diabetic nephropathy in a Chinese population.

MTHFR is an important enzyme in the metabolic process of homocysteine, which could catalyze 5,10-methylene four hydrogen folic acid into 5,10-methylenetetrahydrofolate. During the process of metabolism process, the produced methyl promotes homocysteine into methionine, and thus reduces the plasma level of homocysteine. The mutation of *MTHFR* C677T and A1298C could influence the activity and thermal stability. Previous studies have indicated that low activity of *MTHFR* could promote the level of plasma homocysteine that is the independent risk factor for the atherosclerosis and arterial thrombosis related diseases [19, 20]. The homocysteine could dam-

age vascular endothelial cells through oxidative stress, stimulate the diary of vascular smooth muscle proliferation and collagen synthesis, increase platelet adhesion and coordinate glycosylation, and then contribute to the pathogenesis of type 2 diabetic nephropathy through changing selective filtration function and the aperture size of glomerulus and increasing glomerular filtration rate.

Polymorphisms contribute to the regulation the expression of protein and play an important role in the discrepancies in the susceptibility and severity to a disease in human. Previous studies have shown that the *MTHFR* C677T and A1298C genomic polymorphisms are correlated with several kinds of endocrine diseases, such as obesity, Graves' disease, rheumatoid arthritis, osteoporosis, polycystic ovary syndrome and hypertension [21-25]. Lewis et al. conducted a population-based study in a Caucasian population, and reported that the TT genotype of *MTHFR* C677T was associated with an increased risk of obesity BMI≥30 [21]. Mao et al. carried out a study with 199 Graves' disease patients and 235 healthy controls, and reported that the CT+TT genotypes of *MTHFR* C677T were associated with an approximately 42% reduction in the risk of this disease in women [22]. Brambila-Tapia et al. carried out a study in 71 rheumatoid arthritis patients, and reported that *MTHFR* C677T polymorphism conferred a risk of developing osteoporosis in patients with rheumatoid arthritis [23]. Jain et al. carried out a study to investigate the relationship between *MTHFR* C677T polymorphism and polycystic ovary syndrome, and found that the CT genotype of *MTHFR* C677T was correlated with the susceptibility to hyperlipidemia in women with polycystic ovary syndrome [25]. Alghasham et al. carried out a study with 123 hypertensive cases and 250 healthy controls, and showed that *MTHFR* C677T and A1298C genomic variations were associated with risk of hypertension in patients with obesity and diabetes [24]. These results have indicated that *MTHFR* C677T polymorphism is correlated with risk of developing endocrine diseases.

In regards to the role of *MTHFR* C677T and A1298C polymorphisms in type 2 diabetic nephropathy risk, several previous studies have shown conflicting results [26-31]. Zhou et al. reported that the TT genotype and T allele of *MTHFR* C677T might be a significant genetic molecular marker for the risk of type 2 diabetic nephropathy in patients [26]. El-Baz et al. carried out a study in a Egyptian population, and reported that the *MTHFR* C677T and A1298C were genetic risk factors for type 2 diabetic nephropathy in patients with type 2 diabetes [29]. Sibireva carried out a study in a Chinese population with 90 patients with diabetic nephropathy, and reported that *MTHFR* C677T mutation was associated with increased blood coagulation potential and platelet hyperactivation [31]. Cui et al. conducted a meta-analysis with 12 studies in a Chinese population, and reported that *MTHFR* 677T allele showed significant association with diabetic nephropathy, but not for diabetes mellitus [30]. In our study, we found that only TT and T allele of *MTHFR* C677T could modify the development of type 2 diabetic nephropathy in a Chinese population, but no such relationship was established in regard to *MTHFR* A1298C variant. Therefore, additional studies with larger sample sizes are needed to validate our findings.

Some limitations should be considered in this study. First, the selection bias could not be avoided, since the patients and controls were selected from one hospital. However, the genotype distributions of *MTHFR* C677T and A1298C are in agreement with the Hardy-Weinberg equilibrium in controls and are similar with the MAF in NCBI SNP database, suggested that the study population could represent the general population. Second, our analysis might overlook the possibility of gene-gene or SNP-SNP interactions, or linkage disequilibrium between polymorphisms. Further investigations with more sample sizes are expected to confirm our results.

In conclusion, we found that the *MTHFR* C677T genomic polymorphism can influence the development of type 2 diabetic nephropathy in a Chinese population, and further studies using larger sample sizes and employing either similar or different analytic strategies may help to elucidate the impact of *MTHFR* C677T and A1298C polymorphisms on risk of diabetic nephropathy.

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Disclosure of conflict of interest

None.

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