ARTICLE

Interaction of *MTHFR* C677T polymorphism with smoking in susceptibility to diabetic nephropathy in Chinese men with type 2 diabetes

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Abstract

We investigated the interaction of *MTHFR* C677T polymorphism (rs1801133) with smoking in susceptibility to diabetic nephropathy (DN) in Chinese men with type 2 diabetes mellitus (T2DM). We studied 655 Chinese men with T2DM, who were divided into two groups (321 with DN and 334 without DN). The genotype of *MTHFR* C677T polymorphism was detected by real-time polymerase chain reaction. *MTHFR* TT genotype carried a higher risk of DN compared with the CC genotype (OR = 2.05; $P = 0.002$). The T allele showed marked association with DN development in patients who smoked, using additive, recessive, and dominant models (OR = 1.60, 1.83, and 1.88, respectively; $P = 0.006$, 0.002, and 0.04, respectively), which was not observed in the nonsmoking group. Patients with TT and CT genotypes, who smoked had a higher risk of DN compared with the control group (non-smoking with CC genotype; OR = 3.73 and 2.28, respectively; $P < 0.001$ and $P = 0.004$, respectively), whereas the other groups were not observed. In conclusion, the T allele of rs1801133 may be a risk factor for DN in Chinese men with T2DM, and synergy appears to exist between the *MTHFR* rs1801133 and smoking in susceptibility to DN.

Introduction

Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes mellitus (DM) [1]. Type 2 DM (T2DM) is becoming increasingly prevalent worldwide. Among the Chinese adult population, the estimated overall prevalence of diabetes was 10.9% in 2013 [2]. Approximately 30–40% of T2DM patients develop DN. The determinants of DN are complex, and it includes genetic and environmental factors. There appears to be an inherited predisposition for DN, and many investigations on the familial clustering of DN in T2DM and the heritability of DN provide compelling evidence that genetic factors contribute to DN susceptibility [3, 4]. Furthermore, a few candidate genes have been reproducibly associated with DN. Genetic studies may provide valuable information regarding the pathobiology of DN and potential targets for its treatment.

It is reported that the level of plasma homocysteine (Hcy) might be closely linked with progression of DN [5]. Hcy metabolism is regulated by methylenetetrahydrofolate reductase (MTHFR). *MTHFR* C677T polymorphism (rs1801133) changes amino acid 222 from alanine to valine (A222V), which causes reduced MTHFR activity and elevated Hcy level, and *MTHFR* C677T polymorphism has been studied as a candidate genetic risk factor for DN. Compared with *MTHFR* CT or CC genotype, individuals with TT genotype have higher plasma Hcy levels [6]. Many studies about the association between *MTHFR* C677T polymorphism and DN have shown heterogeneous results [7–9], which might have been influenced by different methods and insufficient statistical power.

In recent years, greater emphasis has been laid on the joint effects of genetic and environmental factors on

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complex disease traits. Prospective and retrospective studies have found that smoking is closely related to DN progression [10–13]. The influence of smoking on association between the *MTHFR* C677T polymorphism and the risk of DN has received little attention.

Based on these observations, we explored the association of smoking and *MTHFR* C677T polymorphism with DN in a cohort of men with T2DM in Beijing, China, to evaluate the combined effect of *MTHFR* C677T and smoking on DN progression.

Materials and methods

Patients

This was a case–control study of 655 male patients with T2DM, diagnosed according to the World Health Organization 1999 criteria [14]. They were all hospitalized at the China–Japan Friendship Hospital, Beijing, China between August 2016 and December 2017. This study was approved by the hospital Institutional Ethics Committee. Written informed consent was obtained from all patients.

DN patients

Individuals who had a history of DN were defined as the DN group ($n = 321$). Inclusion criteria for the DN group were: diagnosis of T2DM; 24-h proteinuria > 500 mg/24 h, or albumin creatinine ratio (ACR) > 30 mg/g. Exclusion criteria were: known proteinuria before onset of diabetes; other primary or secondary renal diseases (e.g., IgA nephropathy, membranous nephropathy, lupus nephritis, obstructive renal disease, renal stone disease, and acute urinary tract infection); or malignancy.

T2DM patients

Individuals who were diagnosed with T2DM for ≥ 7 years and without DN were defined as controls ($n = 334$). Controls were unlikely to have DN in the future, which increased the sensitivity in detecting the association between *MTHFR* C677T and DN, and had ACR < 30 mg/g. We excluded patients with other primary or secondary renal diseases (e.g., IgA nephropathy, membranous nephropathy, lupus nephritis, obstructive renal disease, renal stone disease, and acute urinary tract infection), or malignancy.

Smokers consisted of individuals who smoked at least one cigarette/day for a minimum of 2 years. As most of the women were non-smokers, only men were included in the present study. The method of data collection was the same as in our previous study [15].

Genomic DNA extraction and genotyping

Genomic DNA was extracted from EDTA-treated whole blood using QIAamp DNA Blood Mini Kit (Qiagen). The quality and concentration of DNA was determined using the NanoDrop 1000 spectrophotometer (ThermoScientific). Genotyping of *MTHFR* C677T was performed by polymerase chain reaction (PCR) using TaqMan SNP Genotyping Assay (Applied Biosystems). The *MTHFR* primer sequences were forward: 5′-GGCTGACCTGAAGCA CTTGAA-3′, reverse: 5′-AGAAAAGCTGCGTGAT GATGAA-3′. The probe sequences were FAM-5′-TCTGCGGGAGTCG-3′-MGB; VIC-5′-CTGCGGGAGCC GA-3′-MGB. The primers and probes were designed by Applied Biosystems. The amplification conditions consisted of denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, and then by annealing and elongation at 65 °C for 60 s. To verify the genotypes, PCR products from 50 patients were randomly selected for DNA sequencing. The forward primer used for PCR was 5′-GTCTCTTCATCCCTCGCCTT-3′ and the reverse primer was 5′-GAACTCAGCGAACTCAGCAC-3′. The products were analyzed by ABI 3500 (Applied Biosystems).

Statistical analysis

The baseline characteristics age, body mass index (BMI), blood pressure, duration of diabetes, hemoglobin A1C, total cholesterol (TC), high-density lipoprotein–cholesterol (HDL-C), low-density lipoprotein–cholesterol (LDL-C), triglyceride (TG), and Hcy showed a non-Gaussian distribution, and the data were presented as median (interquartile range). The differences in clinical characteristics between the DN and control groups were analyzed by Wilcoxon signed rank test. Genotype distribution and allelic frequency were analyzed using the χ^2 test. A goodness of fit χ^2 test was used to evaluate whether the genotypic distribution of the *MTHFR* C677T polymorphism was in accordance with Hardy–Weinberg equilibrium in the DN and control groups.

We also used multivariate logistic regression to analyze the association between *MTHFR* C677T polymorphism and DN adjusted for age, BMI, duration of diabetes, history of hypertension, smoking, and TC and TG levels using three genetic models (additive, recessive, and dominant models). TT and CT were coded as 1 while CC was coded as 0 in the dominant model; TT was coded as 1 while CC and CT were coded as 0 in the recessive model; and TT, CT, and CC were coded as 2, 1, and 0, respectively, in the additive model. Data were analyzed with SPSS version 17.0 software. $P < 0.05$ was considered a significant difference, and $P < 0.01$ was considered a highly significant difference.

Results

General characteristics of the study population

We included 321 DN patients (DN group) and 334 T2DM patients (control group), and all 655 participants were male. BMI, history of hypertension, smoking status, blood pressure, Hcy, LDL-C, and TG in the DN group were significantly higher than those in the control group ($P < 0.05$) (Table 1). However, HDL-C in the DN group was significantly lower compared with that in the control group ($P < 0.05$) (Table 1). There were no significant differences in

Table 1 The characteristics of the study participants

Variables	DN ($n = 321$)[a]	Control ($n = 334$)[a]	P
Age, y	60.0 (53.0, 69.0)	61.0 (54.0, 68.0)	0.77
BMI, Kg/m^2	26.26 (24.39, 28.70)	25.31(23.0, 27.64)	< 0.01
Duration of diabetes, y	15.0 (9.0, 21.0)	14.0 (10.0, 19.0)	0.43
History of hypertension (%)	77.9 (250/321)	51.5 (172/334)	< 0.01
Smoking (%)	53.0 (170/321)	39.8 (133/334)	< 0.01
SBP (mm Hg)	138.00 (125.00,150.00)	126.00 (120.00,140.00)	< 0.01
DBP (mm Hg)	80.0 (75.0, 86.0)	80.0 (70.0, 81.0)	< .01
A1C (%)	7.7 (6.7, 9.4)	7.9 (6.8, 9.2)	0.45
Hcy (μmol/L)	13.52 (11.16, 16.41)	11.57 (9.65, 13.42)	< 0.01
TC (mmol/L)	3.99 (3.30, 5.02)	4.12 (3.50, 4.75)	0.77
HDL-C (mmol/L)	0.92 (0.77, 1.13)	0.97 (0.81, 1.19)	0.03
LDL-C (mmol/L)	2.39 (1.82, 3.08)	2.40 (1.93, 2.95)	0.75
TG (mmol/L)	1.62 (1.12, 2.55)	1.49 (1.02, 2.20)	0.03

A1C hemoglobin A1C, *BMI* body mass index, *DBP* diastolic blood pressure, *Hcy* homocysteine, *HDL-C* high-density lipoprotein–cholesterol, *LDL-C* low-density lipoprotein–cholesterol, *SBP* systolic blood pressure, *TC* total cholesterol, *TG* triglyceride

[a]Data are shown as median (interquartile range) or %.

age, duration of diabetes, hemoglobin A1C, TC, and LDL-C between the two groups ($P > 0.05$).

Genotypic and allele frequency of *MTHFR* C677T polymorphism

Genotypic and allelic frequencies for the *MTHFR* C677T polymorphism are presented in Table 2. The distribution of allele frequencies of the polymorphism among the study population was in accordance with the Hardy–Weinberg equilibrium in both the DN and control groups. The T allele frequency of *MTHFR* C677T in the DN group (59.2%) was significantly higher than that in the control group (51.05%) ($P < 0.01$). Genetic distribution of *MTHFR* C677T (CC, CT, and TT) in the two groups differed significantly ($P < 0.01$) (Table 2).

Association between *MTHFR* C677T polymorphism and DN

To confirm the association between the risk of DN and *MTHFR* C677T polymorphism, further multivariate logistic regression analysis was performed. The additive, dominant, and recessive models were significantly different in participants with DN than in the control group ($P = 0.003$, 0.005, and 0.03, respectively) (Table 3). TT genotype significantly increased the risk of DN compared with the CC genotype (OR = 2.05, 95% CI = 1.30–3.24, and $P = 0.002$), and CT genotype was also found to increase the risk of DN compared with the CC genotype (OR = 1.62, 95%

Table 2 Genotypic and allele frequency of *MTHFR* C677T in DN and control groups

	Genotypic frequencies				Allele frequencies		
MTHFR	CC	CT	TT	P	C	T	P
DN, n (%)	48 (15.0)	166 (51.7)	107 (33.3)	< 0.01	262 (40.8)	380 (59.2)	< 0.01
Control, n (%)	79 (23.7)	169 (50.6)	86 (25.7)		327 (49.0)	341 (51.0)	

Table 3 Odds ratios for DN under three genetic models

Genetic models	Unadjusted		Adjusted[a]		Adjusted[b]	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Additive	1.41 (1.13–1.77)	0.003	1.40 (1.11–1.76)	0.004	1.46 (1.14–1.85)	0.002
Recessive	1.44 (1.03–2.02)	0.03	1.44 (1.02–2.03)	0.04	1.55 (1.07–2.23)	0.02
Dominant	1.76 (1.18–2.62)	0.005	1.73 (1.16–2.59)	0.007	1.76 (1.15–2.68)	0.009
CT vs. CC	1.62 (1.06–2.45)	0.02	1.59 (1.04–2.43)	0.03	1.57 (1.01–2.45)	0.047
TT vs. CC	2.05 (1.30–3.24)	0.002	2.01 (1.27–3.21)	0.003	2.15 (1.32–3.51)	0.002

CI confidence interval, *DN* diabetic nephropathy, *OR* odds ratio

[a]Adjusted for age, BMI

[b]Adjusted for age, BMI, duration of diabetes, history of hypertension, smoking,TC and TG levels

Table 4 Association of *MTHFR* C677T with risk of DN in different smoking status

Smoking status	Unadjusted			Adjusted[a]	
	Genotype	OR (95% CI)	*P*	OR (95% CI)	*P*
Smoking	Additive	1.60 (1.14–2.26)	0.006	1.75 (1.21–2.52)	0.003
	Recessive	1.83 (1.11–3.02)	0.02	2.12 (1.23–3.67)	0.007
	Dominant	1.88 (1.02–3.45)	0.04	1.98 (1.04–3.76)	0.04
Non-smoking	Additive	1.22 (0.90–1.66)	0.19	1.24 (0.89–1.72)	0.20
	Recessive	1.11 (0.69–1.78)	0.66	1.16 (0.70–1.92)	0.56
	Dominant	1.59 (0.94–2.70)	0.09	1.56 (0.89–2.73)	0.12

DN diabetic nephropathy

[a]Adjusted for age, BMI, duration of diabetes, history of hypertension, TC and TG levels

Table 5 Odds ratios for the combined effect of *MTHFR* C677T and smoking on DN

Genotype		DN	Control	Unadjusted		Adjusted[a]	
Smoking status	*MTHFR*	321	334	OR (95% CI)	*P*	OR (95% CI)	*P*
Non-smoking	CC	26	50	1 (Ref)		1 (Ref)	
Non-smoking	CT	82	98	1.61 (0.92–2.81)	0.09	1.61 (0.92–2.81)	0.09
Non-smoking	TT	43	53	1.56 (0.84–2.90)	0.16	1.56 (0.84–2.90)	0.16
Smoking	CC	22	29	1.46 (0.70–3.02)	0.31	1.46 (0.70–3.02)	0.31
Smoking	CT	84	71	2.28 (1.29–4.02)	0.004	2.28 (1.29–4.02)	0.004
Smoking	TT	64	33	3.73 (1.98–7.02)	< 0.001	3.73 (1.98–7.02)	< 0.001

[a]Adjusted for age, BMI, duration of diabetes, history of hypertension, TC and TG levels

CI = 1.06–2.45, $P = 0.02$). The data indicated that the T allele of *MTHFR* C677T polymorphism was a potential risk factor for DN progression. After adjusting for age, BMI, duration of diabetes, history of hypertension, smoking, and TC and TG levels, results were similar.

Association of *MTHFR* C677T polymorphism with DN risk in smoking and nonsmoking groups

Participants were divided into two groups based on smoking status. The additive, recessive, and dominant genetic models showed significant associations with DN in the smoking group in the unadjusted models ($P = 0.006$, 0.02 and 0.04, respectively), indicating that the T allele carriers in the smoking group were at higher risk of DN. However, no significant association was observed in the nonsmoking group ($P = 0.19$, 0.66, and $P = 0.09$, respectively) in the unadjusted models. After multivariate adjustment, we achieved similar results in the two groups (Table 4).

Association of the combined effect of *MTHFR* C677T polymorphism and smoking status on DN risk

Combination of *MTHFR* TT or CT genotype and smoking carried a higher risk of DN compared with the control group (non-smoking with CC genotype; OR = 3.73 and 2.28; $P <$ 0.001 and $P = 0.004$), whereas the combined effects of *MTHFR* CC genotype and smoking compared with those of

MTHFR CT or TT genotype and non-smoking were not observed. After adjusting for age, BMI, duration of diabetes, history of hypertension, TC and TG levels, results were similar (Table 5).

Discussion

In this study, we systematically explored the effects of the *MTHFR* C677T polymorphism, smoking, and their interactions on the development of DN among Chinese men with T2DM. This is one of the few studies to describe the association of the combined effect of *MTHFR* C677T polymorphism and smoking on DN. The effect of the T allele of *MTHFR* C677T polymorphism resulted in a higher risk of DN in all three genetic models (additive, dominant, and recessive). When participants were divided into two groups based on smoking status, significant association of T allele of *MTHFR* C677T polymorphism with the risk of DN was observed only in the smoking group in all three genetic models, whereas in the non-smoking group no association was observed. Furthermore, the combined effect of the *MTHFR* TT and CT genotype with smoking resulted in a higher risk of DN compared with the control group (non-smoking with CC genotype). We conclude that the T allele of *MTHFR* C677T appears to impart susceptibility to DN in Chinese men with T2DM in interaction with smoking.

It has been shown that high plasma Hcy is an independent risk factor for DN progression in T2DM patients [16], which is associated with *MTHFR* C677T polymorphism [17]. This was also shown in our previous study [15]. Elevated Hcy level, predominantly caused by polymorphisms in key enzyme genes involved in Hcy metabolism, is a risk factor for DN. Furthermore, the frequency of the CT and TT genotypes was 43.9% and 23.2%, respectively, in the Chinese population, which is higher compared with many other populations worldwide [18, 19]. Higher Hcy levels are found in the Chinese population due to the *MTHFR* C677T polymorphism [20]. In the present study, 48 (15%), 166 (51.7%), and 107 (33.3%) patients in the DN group had the CC, CT, and TT genotypes of *MTHFR*, respectively, while 79 (23.7%), 169 (50.6%), and 86 (25.7%) in the control group had these genotypes, respectively. The genotype distribution differed significantly in two groups. The T allele frequency in the DN group was significantly higher than in the control group, which indicated that the increased T allele frequency might be related to the DN incidence.

In order to detect the association between *MTHFR* C677T polymorphism and DN, we performed logistic regression analysis using additive, recessive, and dominant genetic models. Our results indicated that the T allele of *MTHFR* C677T polymorphism was an potential risk factor in DN progression, and TT genotype was more likely to promote DN development compared with CC genotype in unadjusted models, which was in accordance with another study [9]. Previous meta-analyses have suggested a potential association between the *MTHFR* C677T polymorphism and DN susceptibility; however, the results of these studies were inconsistent [7–9, 21]. Our data further revealed that the *MTHFR* C677T polymorphism was closely related to DN development in Chinese men with T2DM.

Recently, growing emphasis has been attached to the combined influence of genetic and environmental factors on DN development [22]. One study showed that the *MTHFR* C677T polymorphism might be related to DN progression in the Chinese population, and gene–environment interactions should be further studied [23]. Smoking, as a novel risk factor, has been considered to play a key role in DN development [13, 24, 25], and smoking might influence DN progression induced by *MTHFR* polymorphism. In our study, the number of smokers was higher in the DN group than the controls, and all the participants were male. In order to explore the relationship between smoking and *MTHFR* polymorphism-induced DN progression, we analyzed smoking status stratification. *MTHFR* polymorphism was significantly associated with DN in the smoking group using the additive, recessive, and dominant genetic models. However, no significant association was observed in the nonsmoking group. The results indicated that smoking has

an important effect on the association between *MTHFR* C677T polymorphism and DN progression.

Although the precise mechanisms by which smoking and *MTHFR* C677T polymorphism interact to influence DN development remain unclear, it has been shown that smoking might have a major impact on renal structure and function. First, nephrotoxic effects of smoking have been reported, including production of pro-inflammatory factors, enhanced oxidative stress, endothelial dysfunction, glomerulosclerosis, and tubular atrophy [26, 27]. Second, glycotoxins in cigarette smoke can cause production of advanced glycation end products [28], which can induce vascular nephropathy [29]. Third, smoking is related to insulin resistance in patients with diabetes [30] and accelerates renal atherosclerosis [31]. Furthermore, in order to study the joint effect of *MTHFR* polymorphism and smoking risk factors on DN progression, we compared the ORs for the combined effect of *MTHFR* C677T and smoking on DN. Our data showed that the smokers with TT and CT genotypes had a higher risk of DN compared with the control group, whereas no combined effect was observed among the remaining three groups (smoking with CC genotype, or non-smoking with CT or TT genotype). Therefore, the combined interaction of smoking and *MTHFR* genotype might increase the risk of DN in Chinese men with T2DM.

There were some limitations to this study. First, the study population was small when stratified by smoking status, which might have influenced the statistical power to detect associations. Second, although we identified the rs1801133 polymorphism in the *MTHFR* gene as a susceptibility variant in Chinese men with T2DM, screening more loci within the *MTHFR* gene to clarify the interaction between polymorphisms and DN is clearly warranted. Third, the result is warranted to be replicated in another independent cohort in the future. Finally, more environmental risk factors should be investigated in future studies, and the precise biological mechanism of the combined effects of smoking and *MTHFR* C677T polymorphism on susceptibility to DN needs further elucidation.

In conclusion, this study suggested that the T allele of *MTHFR* C677T is associated with DN development. Additionally, these results suggest that a synergistic effect of *MTHFR* C677T and smoking appears to be significantly associated with development of DN in the Chinese men with T2DM. Our findings provide a further insight into the gene–environment relationships involved in DN pathogenesis. Therefore, future large, well-designed studies in Chinese, and other populations are needed to validate our findings. Furthermore, based on our observations, smoking cessation should be considered in carriers with high genetic risk, which can selectively decrease the risk of DN.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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