Is the C677T polymorphism in methylenetetrahydrofolate reductase gene or plasma homocysteine a risk factor for diabetic peripheral neuropathy in Chinese individuals?☆

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Abstract

The present study enrolled 251 diabetic patients, including 101 with neuropathy and 150 without neuropathy. Of the 150 patients, 100 had no complications, such as retinopathy, nephropathy, or neuropathy. Polymerase chain reaction-restriction fragment length polymorphism analysis was used to identify methylenetetrahydrofolate reductase gene variants. Plasma homocysteine levels were also measured. Homocysteine levels and the frequency of hyperhomocysteinemia were significantly higher in patients with diabetic peripheral neuropathy compared with diabetic patients without neuropathy ($P < 0.05$). In logistic regression analysis with neuropathy as the dependent variable, the frequency of C677T in methylenetetrahydrofolate reductase was significantly higher in patients with diabetic peripheral neuropathy compared with patients without diabetic complications. Homocysteine levels were significantly higher in patients with diabetic peripheral neuropathy carrying the 677T allele and low folic acid levels. In conclusion, hyperhomocysteinemia is an independent risk factor for diabetic neuropathy in Chinese patients with diabetes. The C677T polymorphism in methylenetetrahydrofolate reductase and low folic acid levels may be risk factors for diabetic peripheral neuropathy in Chinese patients with diabetes.

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Key Words

homocysteine; methylenetetrahydrofolate reductase; type 2 diabetes mellitus; diabetic peripheral neuropathy; neural regeneration

Research Highlights

(1) Hyperhomocysteinemia is an independent risk factor for diabetic peripheral neuropathy in Chinese individuals.
(2) The C677T polymorphism in the methylenetetrahydrofolate reductase gene and low folic acid levels may be risk factors for diabetic peripheral neuropathy.
(3) This large-scale study was the first to investigate associations of homocysteine levels and the C677T mutation in the methylenetetrahydrofolate reductase gene in patients with diabetic peripheral neuropathy.

Abbreviations

DPN, diabetic peripheral neuropathy; MTHFR, methylenetetrahydrofolate reductase

INTRODUCTION

Diabetic peripheral neuropathy (DPN), which affects up to 50%[1] of diabetic patients, is a serious chronic microangiopathic complication of diabetes mellitus. Although the pathophysiological mechanisms underlying DPN are still debated, recent studies have shown that vascular factors and metabolic interactions are involved in all stages of DPN[1-2]. Homocysteine is a sulfydryl amino acid that is believed to play an important role in the regulation of the methylenetetrahydrofolate reductase (*MTHFR*) gene, which leads to amino acid substitution from alanine to valine, resulting in a thermolabile phenotype with impaired activity[3].

Numerous longitudinal and cross-sectional studies have shown that hyperhomocysteinemia and the C677T mutation in *MTHFR* is associated with an increased risk of diabetic microangiopathies, particularly retinopathy and nephropathy[4-6]. However, the association between hyperhomocysteinemia and neuropathy has received little attention, nor has the potential role of homocysteine[6-7]. Ambroscht *et al* [8] and Li *et al* [9] recently reported hyperhomocysteinemia could be an independent risk factor for DPN in patients with type 1 or type 2 diabetes mellitus, after adjusting for related variables. This is partly because of the limited number of sample, the lack of objective criteria for assessing DPN, and poorly understood pathogenic mechanisms underlying hyperhomocysteinemia. Therefore, detecting and analyzing the clinical relevance of the C677T polymorphism in *MTHFR* is an important issue.

The present study examined the hypothesis that *MTHFR* polymorphisms and abnormal homocysteine concentrations could be risk factors for DPN, defined using the newest diagnostic criteria[10], in Chinese individuals. Diabetic patients with or without DPN, and those without diabetic complications were compared to investigate the relationship between hyperhomocysteinemia and DPN, as well as the possible pathogenic mechanisms. We also examined whether the plasma total homocysteine level is a risk factor for DPN or diabetic microangiopathy in Chinese individuals, and whether it is associated with the C677T polymorphism in *MTHFR*. Finally, we evaluated the effects on other factors.

RESULTS

Quantitative analysis of patients

A total of 251 consecutive patients with type 2 diabetes mellitus were enrolled in this study. Of these, 101 were diagnosed with DPN according to the Diabetic Neuropathy Expert Panel Meeting held in Toronto, in 2011. Of the 150 patients without DPN, 100 patients had no evidence of diabetic retinopathy, nephropathy, or neuropathy, and were considered to have no diabetic complications.

Baseline characteristics and standard laboratory data

According to the criteria for diagnosis of DPN, 101 of 251 patients were classified as having DPN. All of the patients suffered from peripheral sensorimotor neuropathy, and 14 also had autonomic neuropathy. The clinical characteristics of the patients are shown in Table 1. All of the groups of patients were well matched for age, sex, body mass index, smokers, family history, insulin therapy, and blood pressure ($P > 0.05$). The duration of diabetes tended to be longer ($P < 0.01$) and the levels of glycated hemoglobin (HbA$_{1c}$; $P < 0.05$), creatinine ($P < 0.05$), and triglyceride ($P < 0.01$) were significantly higher in patients with DPN compared with patients without diabetic complications.

Relationship between homocysteine level and DPN

Plasma homocysteine levels and the prevalence of hyperhomocysteinemia were significantly higher in patients with DPN compared with patients without diabetic complications ($P < 0.01$ and $P = 0.001$, respectively) and patients without neuropathy ($P = 0.01$ and $P = 0.001$, respectively). Moreover, 61(60%) patients with neuropathy had hyperhomocysteinemia, which was significantly greater than the rate in patients without neuropathy (70, 47%; chi-square test; $P = 0.022$)

Pearson correlation analysis showed that DPN was significantly correlated with homocysteine levels ($r = 0.429$, $P = 0.002$), duration of diabetes ($r = 0.623$, $P = 0.01$), HbA$_{1c}$ ($r = 0.417$, $P = 0.02$), and serum folic acid levels ($r = -0.270$, $P = 0.002$). In logistic regression analysis, the models were adjusted.

For factors that may influence homocysteine and neuropathy, including age, sex, family history, frequency of smoking, duration of diabetes, body mass index, blood pressure, HbA$_{1c}$, homocysteine, serum folic acid, vitamin B$_{12}$, total cholesterol, triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol. In this analysis, hyperhomocysteinemia, duration of diabetes, HbA$_{1c}$, homocysteine, and serum folic acid were significantly associated with DPN. Among patients with hyperhomocysteinemia, the odds ratio (*OR*) for DPN was 5.232 [95% confidence interval (*CI*) 1.101–25.323].

Table 1 Baseline characteristics and standard laboratory variables in different groups

Item	Patients without diabetic complications (n = 100)	Patients without DPN (n = 150)	Patients with DPN (n = 101)
Sex (n, M/F)	53/47	77/73	51/50
Age (mean ± SD, year)	62.4±11.3	60.8±12.5	61.8±14.0
Family history [n(%)]	45 (45)	69 (46)	49 (49)
Frequency of smokers (%)	11.0 (11.0)	16.8 (11.2)	12.5 (12.4)
BMI (mean ± SD, kg/m^2)	21.5±4.0	23.5±4.0	24.2±3.9
Insulin therapy [n(%)]	76 (76)	114 (76)	83 (82)
Systolic blood pressure (mean ± SD, mmHg)	137.0±21.7	135.0±19.7	127.0±16.5
Diastolic blood pressure (mean ± SD, mmHg)	75.2±11.0	75.5±11.0	76.0±8.3
Duration of diabetes (mean ± SD, year)	11.2±3.9	7.2±3.96[b]	8.84±4.5
BUN (mean ± SD, mM)	6.5±2.1	6.7±2.6	6.8±3.1
Cr (mean ± SD, mM)	73±15	73±15	95±23[a]
HbA$_{1c}$ (%)	8.1±2.4	10.1±2.0	11.2±1.8[a]
Triglyceride (mean ± SD, mM)	8.9±3.1	6.9±2.7	5.0±1.2[a]
Vitamin B$_{12}$ (mean ± SD, pM)	328.9±124.4	307.6±59.2	277.8±27.6[ab]
Serum folate (mean ± SD, nM)	15.9±6.9	14.3±11.0	11.0±6.6[a]
Hcy (mean ± SD, μM)	17.5±8.94	18.1±10.5	20.9±13.8[ab]
Hyperhomocysteinemia [n(%)]	30 (30)	71 (47)	61 (60)[ab]
LDL-C (mean ± SD, mM)	3.8±7.2	3.6±2.2	3.0±1.9

[a]P < 0.05 *vs.* patients without diabetic complications; [b]P < 0.05 *vs.* patients without DPN. All values are expressed as mean ± SD. Hyperhomocysteinemia was defined as homocysteine concentration ≥ 15 μM. P-values were determined by independent samples *t*-tests or one-way analysis of variance for continuous variables, or chi-square tests for categorical variables. M: Male; F: female; DPN: diabetic peripheral neuropathy; BMI: body mass index; SD: standard deviation; BUN: blood urea nitrogen; Cr: creatine; Hcy: homocysteine; LDL-C: low-density lipoprotein cholesterol. 1 mmHg = 0.133 kPa.

Homocysteine was significantly associated with the prevalence of neuropathy, as the *OR* for neuropathy for each 1 M increase in homocysteine was 1.518 (95% *CI* 0.417–4.601; P = 0.026).

Relationship between *MTHFR* genotypes and DPN
The frequencies of the genotypes observed in the 251 patients were in accordance with the Hardy-Weinberg equilibrium (P = 0.831). Overall, 19% of patients had the CC genotype, 60% had the CT genotype, and 21% had the TT genotype. The allele frequencies of the T and C mutations were 51.0% and 49.0%, respectively. The distribution of *MTHFR* genotypes in the DPN group was compared between patients without diabetic complication and patients without diabetic neuropathy. The genotype distribution differed significantly between these two groups, as the frequencies of the CC, CT, and TT genotypes were 19%, 64%, and 17%, respectively, among patients without diabetic complications versus 20%, 49%, and 31%, respectively, among patients with DPN (χ^2 = 1.569, P = 0.021). The *OR* for DPN in patients with the TT genotype was 1.732 (95% *CI*: 0.731–4.105). The frequency of the T allele was similar in both groups (49% and 55%, respectively; Table 2). In 150 patients without neuropathy, the allele frequency of the T mutation was 48.0%, which insignificantly differed from that in patients with neuropathy (55.0%; χ^2 = 2.932, P = 0.087). The frequencies of the CC, CT, and TT genotypes were

19%, 67%, and 14%, respectively, in patients without neuropathy. The corresponding rates in patients with neuropathy were 20%, 49%, and 31%. Therefore, the genotype distribution was not significantly different between patients with and without neuropathy (χ^2 = 3.218, P = 0.073).

Table 2 Distribution of *MTHFR* genotypes and alleles [n (%)] in patients without diabetic complications and in patients with DPN

SNPs	Patients without diabetic complications (n = 100)	Patients with DPN (n = 101)	χ^2	P	OR (95% *CI*)
Genotype					
C677C	19 (19)	20 (20)			
C677T	64 (64)	50 (49)			
T677T	17 (17)	31 (31)	1.569	0.021[a]	1.732 (0.731–4.105)
Allele					
677C	102 (51)	90 (45)			
677T	98 (49)	112 (55)	1.673	0.196[b]	1.295 (0.875–1.917)

a: Chi-square test with 2 *df*; b: chi-square test with 1 *df*. The overall power was calculated as the mean power for the genotyped single nucleotide polymorphisms (SNPs) in *MTHFR*.

DPN: Diabetic peripheral neuropathy; *OR*: odds ratio; *CI*: confidence interval; *MTHFR*: methylenetetrahydrofolate reductase.

Correlations between *MTHFR* genotype and plasma homocysteine levels
Plasma homocysteine levels were higher in patients with

the TT genotype than those with the CC or CT genotypes in all subgroups. However, the difference in homocysteine levels between patients with the TT genotype and either the CT or CC genotype was only statistically significant in patients with DPN when tested with one-way analysis of variance ($P = 0.023$ and $P = 0.016$, respectively; Table 3). Plasma homocysteine levels were higher in patients with the T allele than in patients with the C allele in all groups. However, the difference in homocysteine levels between the T allele and the C allele were only statistically significant in patients with DPN when tested with one-way analysis of variance ($P = 0.033$; Table 3).

Table 3 Correlations between *MTHFR* genotypes and plasma homocysteine levels

SNPs	Patients without diabetic complications ($n = 100$)	Patients without DPN ($n = 150$)	Patients with DPN ($n = 101$)
Genotype			
C677C	13.92±4.85	15.81±5.44	17.78±7.56
C677T	15.25±6.67	18.98±4.85	20.92±7.63[a]
T677T	18.62±3.10	19.13±7.37	23.61±16.20[ab]
Allele			
677C	15.09±3.97	17.09±5.11	18.58±7.51
677T	16.74±4.87	19.09±5.66	22.10±14.28[c]

[a]$P < 0.05$, *vs.* C677C; [b]$P < 0.05$, *vs.* C677T; [c]$P < 0.05$, *vs.* C allele. All values are expressed as mean ± SD. *P*-values were determined by independent samples *t*-tests or one-way analysis of variance. SNP: Single nucleotide polymorphism; DPN: peripheral diabetic neuropathy; *MTHFR*: methylenetetrahydrofolate reductase.

Comparison of plasma homocysteine levels among *MTHFR* genotypes and serum folic acid concentrations (Table 4)

We hypothesized that allelic variations in *MTHFR*, a folic acid-related enzyme, as well as baseline homocysteine levels, may contribute to the observed heterogeneity. In the present study, the median serum folic acid concentration in the diabetic patients was 13.65 nM. Subjects homozygous for the 677T allele were more likely to have elevated levels of total homocysteine in the presence of low folic acid levels, as reported earlier. Accordingly, these patients may require higher folic acid levels to regulate total homocysteine levels.

DISCUSSION

Few studies have examined the clinical relevance of hyperhomocysteinemia and the C677T polymorphism in *MTHFR* in DPN. The present study showed that plasma homocysteine levels were significantly higher in patients with DPN compared with those without neuropathy. However, the prevalence of homozygosity for the C677T mutation in *MTHFR* was significantly different from that in patients without diabetic complications, but compared with patients without neuropathy.

The current results also indicate that homocysteine levels and the prevalence of hyperhomocysteinemia were strongly associated with DPN. There were significant differences in the prevalence of hyperhomocysteinemia and homocysteine levels between patients with neuropathy and those without neuropathy. Hyperhomocysteinemia was significantly associated with DPN in multiple logistic regression models that adjusted for factors that may influence homocysteine levels and neuropathy. Recent data published by Ambroscht *et al* [8] and Li *et al* [9] suggest that high homocysteine levels could be an independent risk factor for DPN in patients with either type 1 or type 2 diabetes after adjustment for related variables. Data from animal models and *in vitro* experiments have linked homocysteine to neuropathy, as suggested by Sheng *et al* [11], Hofmann *et al* [12], and El Boghdady *et al* [13]. DPN was defined using the criteria proposed by the Diabetic Neuropathy Expert Panel Meeting[10].

Many previous cross-sectional studies of patients with diabetes have reported positive associations between *MTHFR* polymorphisms and diabetic microangiopathies, including nephropathy and retinopathy[4-6, 14-15]. However, little is known about the relationship between *MTHFR* polymorphisms and DPN. The results of the present study indicate that a thermolabile variant of *MTHFR* was more frequent in DPN patients, particularly compared with that in patients without diabetic complications, but not compared with patients without neuropathy.

Table 4 Correlation between *MTHFR* genotype and plasma homocysteine levels according to serum folic acid concentrations

Genotype	Folic acid (median, 13.65 ± 3.01 nM)	C677C	C677T	T677T
Patients without diabetic complications ($n = 100$)	≥ median (nM)	10.65±3.42	12.27±4.51	17.57±1.50[a]
	< median (nM)	15.19±4.96[b]	19.03±3.53[b]	21.68±6.19[ab]
Patients with PDN ($n = 101$)	≥ median (nM)	15.10±2.87	19.30±5.42	22.12±5.95[a]
	< median (nM)	18.32±7.03	21.04±8.02	26.15±5.10[ab]

[a]$P < 0.05$, *vs.* C677C; [b]$P < 0.05$, *vs.* ≥ median folic acid concentration. All values were expressed as mean ± SD. *P*-values were determined by independent samples *t*-tests or one-way analysis of variance. DPN: Diabetic peripheral neuropathy.

It is possible that the patients without neuropathy had retinopathy or nephropathy; in other words, the C677T polymorphism in *MTHFR* may be a risk factor for diabetic microangiopathy, but not neuropathy. Nevertheless, other factors should be considered. First, the association between *MTHFR* polymorphisms and diabetic microangiopathy may be confounded by other factors, such as ethnicity.

Meta-analyses showed that ethnicity was a possible confounder, particularly among western Asians and Africans, as were sex and the duration of diabetes mellitus, in studies of diabetic nephropathy[7]. Second, there are multiple causes of DPN, and it seems likely that a specific population may have genetic factors that protect against the development of DPN, despite the elevations in homocysteine levels that are associated with the C677T mutation.

Analysis of the distribution of homocysteine levels among patients with different *MTHFR* genotypes (CC, CT, and TT) showed that patients with the T mutant genotype generally exhibited higher homocysteine levels compared with patients without this mutation[5]. This trend was observed in all of the diabetic groups, but the difference between the T and C alleles only reached statistical significance in patients with DPN. Therefore, this polymorphism in *MTHFR* gene is an important genetic risk factor for hyperhomocysteinemia, consistent with the results of previous studies[16-18]. The C677T polymorphism leads to the formation of a thermolabile variant of *MTHFR*. Therefore, patients with the CC genotype may show accelerated *MTHFR* activity. The DNA mutation responsible for the heat-labile variant was identified as a C-to-T mutation at nucleotide 677, which substitutes a valine for alanine at position 114 in *MTHFR*[19].

In the present study, serum folic acid concentrations may interact in the genotype-phenotype relationships. Folic acid supplementation usually lowers homocysteine levels, but polymorphisms in *MTHFR* may contribute to some variability in the reduction of homocysteine levels[20-24]. Researchers reported that about one third of the heterogeneity in the responsiveness was attributable to baseline homocysteine and folic acid levels, as well as multivitamin use[26]. The results of two screening studies, in which plasma folic acid levels were correlated with total plasma homocysteine levels[20-21], and prospective studies[22-24], in which folic acid supplementation was tested, indicate that high serum folic acid levels restore total plasma homocysteine levels to normal, and overcome the reduction of *MTHFR* activity associated with the C677T mutation generating a thermolabile enzyme. The present study suggested a possible genetic sensitivity to the detrimental effects of low folic acid intake.

Moreover, subjects homozygous for the 677T *MTHFR* allele had greater decreases in plasma homocysteine levels after folic acid supplementation compared with that in homozygotes for the 677C allele, particularly in subjects with higher baseline folic acid levels[16]. Therefore, the response to folic acid supplementation is affected by the number of 677T alleles in *MTHFR*, and subjects with the TT genotype showed the most robust response to the homocysteine-lowering effects of folic acid.

However, there were some limitations to this study. First, a methionine loading test, a stimulation test that detects up to 27% of patients with otherwise-undiagnosed hyperhomocysteinemia, was not performed. Moreover, the sample size was relatively limited.

In conclusion, the C677T mutation in *MTHFR* is relatively common among Chinese individuals. The present study showed that this polymorphism and hyperhomocysteinemia were independently associated with the prevalence of DPN in a limited number of patients with type 2 diabetes mellitus. However, the prevalence of homozygosity for the C677T mutation in *MTHFR* in patients with DPN was significantly different to that in patients without diabetic complications, but compared with patients without neuropathy. Subjects with the TT genotype showed the most robust response to the homocysteine-lowering effects of folic acid.

SUBJECTS AND METHODS

Design
A case-control study.

Time and setting
The study was performed at the Laboratory of Department of Neurology, Peking University Third Hospital, China between October 2011 and December 2011.

Subjects
A total of 251 consecutive patients with type 2 diabetes mellitus (117 females/134 males) who attended the diabetes clinics at Peking University Third Hospital were enrolled. The patients underwent screening for diabetes-related complications (retinopathy and/or microalbuminuria).

Diagnostic criteria
Type 2 diabetes mellitus was diagnosed according to the American Diabetes Association criteria (2000)[25], as follows: fasting plasma glucose ≥ 7.0 mM, or symptoms of hyperglycemia and a random plasma glucose ≥   11.1

mM, or 2-hour plasma glucose ≥ 11.1 mM during an oral glucose tolerance test. Fasting was defined as no food intake for at least 8 hours and random glucose was defined as a value measured at any time of day without considering the time since the patient's last meal. Symptoms of hyperglycemia included polyuria, polydipsia, and unexplained weight loss. Oral glucose tolerance tests were performed as described by the World Health Organization with a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water. Only patients with HbA1c < 15% were included.

The onset and progression of DPN were assessed based on the modified criteria established by Dyck, in accordance with the recommendations of the Diabetic Neuropathy Expert Panel Meeting[10]. Of the 251 enrolled patients, 101 were diagnosed with DPN.

The presence and staging of peripheral neuropathy was based on Neurological Symptom Score, Neurological Disability Score, Autonomic Function Testing, and Quantitative Sensory Examination. All of the tests were performed in each patient by the same experienced physician.

The Neurological Symptom Score[26] was based on patient-reported symptoms including subcategories of sensory, motor and autonomic symptoms. A score of > 1 was defined as abnormal.

The Neurological Disability Score[26] was calculated from an abbreviated, scored neurological examination. It included subcategories of sensory, motor, and reflex function. A score of ≥ 6 was defined as abnormal.

The Autonomic Function Testing was calculated based on an acetylcholine sweatspot test[27] and autonomic cardiovascular reflex tests[28].

The Quantitative Sensory Examination[29] consisted of thermal and vibration sensitivity testing of the great toe using the NTE-2 Thermal Sensitivity tester and the Vibratron® Vibration Sensitivity Tester (Medoc, Ltd., Jerusalem, Israel). The examination was considered abnormal if both the thermal and vibration threshold scores were abnormal.

Patients without complications

Among 251 patients with diabetes, 100 showed no evidence of diabetic complications (*e.g.*, retinopathy, nephropathy, or neuropathy) and were included as a control group.

Inclusion criteria were as follows: time since first clinical signs of diabetes of > 10 years; the eyes were normal on ophthalmoscopy and/or fluorescein angiography (diabetic retinopathy was evaluated in dilated pupils by an experienced ophthalmologist); albumin excretion rate < 20 mg/min; urinary albumin excretion rate < 20

g/min at least three times; and creatinine < 37 mg/g. Patients meeting any of the following criteria were excluded: treatment for medical conditions known to increase homocysteine (*e.g.*, dysthyroidism), type 1 diabetes, severe renal disease (creatinine > 150 M), liver disease, heart failure, or any other conditions known to cause neuropathy or peripheral vascular disease. Patients who had used vitamin B supplements within the last 6 months were also excluded.

In accordance with the *Administrative Regulations on Medical Institutions*, issued by the State Council of China[30], the investigators provided each patient with information about the project and its possible risks, and obtained informed consent before enrolment.

Methods

PCR-restriction fragment length polymorphism analysis of MTHFR polymorphisms

Genomic DNA was extracted from ulnar vein peripheral blood leukocytes by the phenol-chloroform method. PCR was performed using the primers described by Frosst *et al*[31]. The forward primer was 5'-CAG GTT ACC CCA AAG GCC AC-3' and the reverse primer was 5'-AGG ACG GTG CGG TGA GAG TG -3'. The PCR mixture contained 1 µL of DNA template (1 µg/µL), 0.4 µL of each primer (10 pmol/µL), 0.3 µL dNTP (10 mM), 0.9 µL MgCl$_2$ (25 mM), 10.38 µL pure water, and 2.5 units of Taq polymerase (5 U/µL, Sangon, Shanghai, China) in a final volume of 15 µL. The DNA fragment was amplified for 35 cycles with predenaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute, followed by final extension at 72°C for 30 seconds in a thermal cycler (DNA Engine, MJ Research, Watertown, MA, USA), followed by reextension at 72°C for 7 minutes after the last cycle. Then, 10 µL of the 255-bp PCR product were digested with 0.4 µL of *Hinf*1 (10 U/µL, Sangon), and the resulting digests were separated by electrophoresis on 3% agarose gels (FMC, Rockland, ME, USA) containing ethidium bromide. The presence of the 677 C to T mutation in *MTHFR* gene caused a digestion of the 255 bp PCR product into two DNA fragments of 176 bp and 79 bp. Therefore, subjects homozygous for this mutation had two DNA fragments at 176 bp and 79 bp, whereas homozygous subjects had a single DNA fragment of 255 bp. Heterozygous subjects had three DNA fragments of 255 bp, 176 bp, and 79 bp. Three genotypes were identified, namely 255/255 CC, 176/79 TT, and 255/176/79 TC. The mutation was identified by agarose electrophoresis and ethidium bromide fluorescence. The frequencies of each genotype or allele were quantified based on the results of

PCR.

Laboratory parameters

HbA_{1c} was measured by high-performance liquid chromatography (Series 1100, Hewlett Packard, Cleveland, Ohio, USA). Plasma homocysteine concentrations were determined using an IMx System (Abbott Diagnostics, Wiesbaden, Germany) with a fluorescence polarization immunoassay that quantifies total L-homocysteine in human serum or plasma samples. Plasma folic acid and vitamin B_{12} concentrations were measured by microparticle enzyme immunoassays (Abbott Laboratories). Renal function was assessed by measuring blood urea nitrogen, creatinine, creatinine clearance rate, and urinary albumin excretion rate. Blood urea nitrogen and creatinine were measured by enzymatic spectrophotometric tests on an autoanalyzer (Hitachi 75, Boehringer, Mannheim, Germany). Creatinine clearance was calculated at follows: creatinine clearance = (urine creatinine concentration × 24 hour urine capacity) / (blood creatinine concentration × 1 440).

Statistical analysis

All the tests used were two-tailed and values of $P < 0.05$ were considered statistically significant. Continuous variables are presented as mean ± SD or as medians for non-normally distributed variables. All analyses were conducted using SPSS software version 11.5 (SPSS, Chicago, IL, USA). Independent samples *t*-tests and one-way analysis of variance were performed to determine the significance of differences in mean values between each group. The chi-square test for goodness of fit with 1 *df* was used to assess whether the observed genotypes were in accordance with the Hardy-Weinberg equilibrium. Chi-square tests were also used to compare genotype distribution between each group. Pearson's correlation analysis was used to determine correlations of homocysteine levels with glucose, total cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, uric acid, creatinine, and HbA_{1c}. Multiple logistic regression was used to adjust for factors likely to affect homocysteine levels or known risk factors for DPN.

Author contributions: Hongli Wang provided, integrated, and analyzed the experimental data, and wrote the manuscript. Dongsheng Fan and Tianpei Hong conducted the experiments.
Conflicts of interest: None declared.
Ethical approval: The project was approved by the Ethical Committee of Beijing Medical University, China.

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