ORIGINAL RESEARCH

C-reactive protein, vitamin B12 and C677T polymorphism of N-5,10-methylenetetrahydrofolate reductase gene are related to insulin resistance and risk factors for metabolic syndrome in Chinese population

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Abstract

Purpose: Metabolic syndrome (MS) and type 2 diabetes mellitus (T2DM) are complex diseases affected by both dietary intake and genetic background. Whether N-5, 10-methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism, high-sensitivity C-reactive protein (hs-CRP) and dietary components folate and vitamin B12 are associated with MS in Asian has not been determined.

Methods: We hypothesized that MTHFR gene C677T, folate, vitamin B12 and hs-CRP are associated with MS and factors related to MS in northern Han Chinese. To test this hypothesis, MTHFR C677T gene polymorphism was determined by PCR-RFLP, serum insulin, folate and vitamin B12 levels by radioimmunoassay, and hs-CRP by immunoturbidimetry in newly diagnosed T2DM patients with MS (118) and without MS (40), and in 55 healthy subjects.

Results: Results indicated that MS-associated T2DM accounts for 75% of newly diagnosed T2DM in Han Chinese.

Serum hs-CRP was higher and serum vitamin B12 was lower in subjects with TT genotype in comparison with those with CC or CT genotypes. Total T frequency was significantly higher in MS-associated T2DM patients (45.3%) compared to 26.3% in non-MS-associated T2DM patients. MTHFR C677T gene polymorphism and vitamin B12 levels were associated with MS-associated T2DM.

Conclusion: MTHFR C677T gene polymorphism may contribute to insulin resistance in Han Chinese with MS by increasing hs-CRP and decreasing vitamin B12, and consequently play an important role in development of MS-associated T2DM.

List of Abbreviations

MS	metabolic syndrome
hs-CRP	high-sensitivity C-reactive protein
Hhcy	hyperhomocysteinemia
MTHFR	methylenetetrahydrofolate reductase

T2DM	type 2 diabetes mellitus
UA	serum uric acid
TCHO	total cholesterol
TG	triglyceride
HDL	high density lipoprotein
LDL	low density lipoprotein
FINS	fasting insulin
FPG	fasting plasma glucose

Methylenetetrahydrofolate reductase (MTHFR) catalyses the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate and methyl donor of homocysteine remethylation. In addition, the activity of MTHFR is important for metabolism homeostasis. Mutation of MTHFR gene at nucleotide position 677 from C to T (MTHFR677C>T) decreases its enzymatic activity, leading to accumulation of homocysteine, an emerged novel independent risk factor for cardiovascular diseases¹ and type 2 diabetes mellitus (T2DM).²⁻⁴

It is known that dietary inadequacy can result in hyperhomocysteinemia (Hhcy), presumably by affecting plasma folate and vitamin B12 concentrations. Low levels of dietary folate and B12 are related to metabolic syndrome (MS)⁵ and cardiovascular disease.^{6,7}

MTHFR 677C/T polymorphism was found to be significantly associated with Hcy levels among Turks but not independently with CHD, despite the fact that CHD was reported to be independently associated with folate and Hcy levels.⁸ Whether C667T polymorphism of the MTHFR gene is related to MS needs to be fully investigated.

The prevalence of T2DM and MS are rapidly increasing in Asia. The MS prevalence is even higher in DM patients and insulin resistance is believed to be the underlying cause for both T2DM and the MS.⁹ MS and T2DM are complex diseases affected by both dietary intake and genetic background.¹⁰ Long-term dietary inadequacy can lead to diabetes and MS.⁴ We¹¹ and others¹² have reported that the presence of the

MTHFR gene is correlated with T2DM complications such as diabetic nephropathy and diabetic retinopathy in northern Chinese. Whether MTHFR gene C677T polymorphism, hs-CRP, folate and vitamin B12 are associated with MS in different ethnic groups has not been well studied, especially in the Asian population. We hypothesized that MTHFR gene C677T, folate, vitamin B12 and hs-CRP are associated with MS and factors related to MS in northern Han Chinese. To test this hypothesis, MS prevalence was investigated in patients newly diagnosed with T2DM in northern Han Chinese. The correlation between MS and MTHFR C677T polymorphism was investigated, and the correlation between the presence of this polymorphism and the levels of serum folate, vitamin B12 and hs-CRP was evaluated. This study provides support for treatment of T2DM and MS at an early stage using combined dietary and pharmacological agents.

Materials and Methods

Subjects

A total of 158 patients and 55 healthy subjects participated in this study. The patients were enrolled in the Second Hospital at Lanzhou University and newly diagnosed with T2DM according to WHO's criteria. Healthy participants were recruited as control subjects during health screening; they had normal glycemia (3.3–6.1mmol/L) and were not diagnosed with metabolic diseases such as hypertension, hyperlipidemia or obesity. The inclusion criteria were that all subjects were Han Chinese and were unrelated to each other. Patients did not have a history of folate, vitamin or other drugs use, which could interfere with the folate and vitamin B12 measurements as well as homocysteine metabolism. Exclusion criteria included anemia and any related disease, potential vitamin or folate deficiency, inflammatory diseases, mental or emotional problems, and those that had untreatable chronic diseases such as cancer, liver disease, kidney disease, coronary heart disease and depression. Subjects gave

their informed consents for collection of DNA samples. The study was approved by the local ethics committee.

Assays

Blood was collected from fasting subjects and used for all laboratory measurements. Serum uric acid (UA), total cholesterol (TCHO), triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) were measured using commercially available kits with an autoanalyzer. Plasma glucose concentration was determined with a glucose oxidase-based analyzer (Beijing Strong Biotechnologies, Inc.). Serum insulin (Siemens Shanghai Medical Equipment Ltd.) and folate and vitamin B12 (Simul TRAC-SNB, folate^[125I]/B12^[57Co], American ICN Pharmaceuticals, Inc.) were analyzed by radioimmunoassay. High sensitive C-reactive protein (hs-CRP) was assayed with immunoturbidimetry. Homeostasis model assessment (HOMA-IR) was calculated according to the following formula:

$HOMA-IR = \ln (FINS \times FPG / 22.5)$, where FINS stands for fasting insulin ($\mu\text{U/ml}$) and FPG stands for fasting plasma glucose (mmol/l).

Definition of metabolic syndrome

MS was diagnosed according to the definition proposed by Chinese Diabetes Society.¹³ Patients with MS must meet at least two of the following criteria besides those for T2DM: 1) fasting serum triglyceride concentration $>1.7 \text{ mmol/l}$ (150 mg/dl); 2) cholesterol concentration of serum high density lipoprotein (HDL) $<0.9 \text{ mmol/l}$ (36 mg/dl) for men or $<1.0 \text{ mmol/l}$ (40 mg/dl) for women; 3) systolic blood pressure $\geq 140 \text{ mmHg}$ or diastolic blood pressure $\geq 90 \text{ mmHg}$; and 4) body mass index $\geq 25 \text{ kg/m}^2$.

Genotyping/genetic analysis

The C677T polymorphism of MTHFR gene was detected using the polymerase chain reaction (PCR)-re-

striction fragment length polymorphism method. Genomic DNA was extracted from blood leukocytes from all subjects using the PuregeneTM DNA isolation kit (Shanghai Sangon Co. Ltd, China) according to the manufacturer's protocol and amplified by PCR using primers 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3'.¹⁴ The amplicons were digested with restriction enzyme Hinf I (Takara Bio Inc. Otsu, Japan) followed by agarose electrophoresis and ethidium bromide staining. Three genotypes, wildtype CC, heterozygote CT and variant homozygote TT, were revealed by their sizes: 195bp for wildtype, 175 bp for variant TT and mixture of 175 bp and 195 bp for heterozygote CT.

Statistical analysis

All data were analyzed by SPSSv16.0 statistical software and presented as mean $\pm$ standard deviation (SD). Student's t test, one-way analysis of variance (ANOVA) and Chi square test were used for comparison of group differences. A p value less than or equal to 0.05 was considered as statistically significant.

Results

The general indices of all subjects were examined. Among the 158 patients with T2DM, 118 (75%) were diagnosed with MS. Table 1 lists the characteristics of all subjects: patients with MS-associated T2DM had higher BMI, TCHO, TG, LDL, UA, FPG, systolic blood pressure (SBP), diastolic blood pressure (DBP), HOMA-IR and CRP than their healthy counterparts ($p < 0.05$). In comparison with those patients with non-MS-associated T2DM, patients with MS-associated T2DM had significantly higher levels of TCHO, TG, UA, blood pressure and HOMA-IR ($p < 0.05$), and significantly lower levels of serum folate and vitamin B12 ($p < 0.05$). Furthermore, patients with MS-associated T2DM had slightly, but not significantly higher levels of BMI and CRP compared with those patients with non-MS-associated T2DM. All other in-

TABLE 1. Subjects' characteristics in healthy control, T2DM without MS and T2DM with MS groups

Subjects	Control	T2DM without MS	T2DM with MS
Number (male/female)	55(26/29)	40 (28/12)	118 (70/48)
Age (year)	56.07±11.48	57.15±12.09	58.85±11.52
Body mass index (kg/m ²)	21.17±1.86 ***	22.98±2.13*	23.95±3.47
Total cholesterol (mmol/L)	4.00±0.73***	4.47±0.96 **	5.11±1.40
Triglycerides (mmol/L)	1.31±0.38***	1.37±0.50**	2.34±1.68
HDL (mmol/L)	1.24±0.38	1.27±0.30	1.28±0.35
LDL (mmol/L)	2.09±0.60***	2.49±0.91	2.79±1.13
UA (mmol/L)	269.95±48.80***	252.49±62.18**	311.41±97.92
FPG (mmol/L)	5.11±0.52***	11.19 ±5.06	11.39±5.24
SBP(mmHg)	116.27±9.23***	124.10±12.57**	139.92±24.33
DBP(mmHg)	74.13±6.57***	74.62±9.14**	82.70±12.23
HOMA-IR	0.87±0.77***	2.03 ±1.02**	2.68±1.16
C-reactive protein (mg/L)	1.92±1.19***	3.29±2.14*	4.28±3.15
Serum folate (ng/mL)	44.79 ±24.32***	42.12 ±23.34**	32.46 ±22.74
Serum vitamin B12 (pg/mL)	633.30±298.44***	621.16±265.91**	506.08±249.57

* $P < 0.05$ for control group vs T2DM without MS; ** $P < 0.05$ for T2DM without MS group vs T2DM with MS group;

*** $P < 0.05$ for control group vs T2DM with MS.

TABLE 2. MTHFR genotype and various clinical characteristics in T2DM with MS

Variables	MTHFR genotypes			F	P value
	CC	CT	TT		
Age (year)	57.97±10.77	58.43±12.26	61.26±10.70	0.640	0.529
Body mass index (kg/m ²)	23.92±3.92	24.31±3.16	23.01±3.55	1.175	0.31
Triglycerides (mmol/L)	1.74±1.07	2.63±1.75	2.46±2.04	3.312	0.04*
HDL (mmol/L)	1.32±0.32	1.25±0.37	1.32±0.34	0.595	0.553
UA (mmol/L)	244.54±81.29	328.97±89.98	363.70±91.48	15.134	0.000***
FPG (mmol/L)	10.79±4.77	10.99 ±4.11	13.32±7.79	1.986	0.142
SBP(mmHg)	136.91 ±24.26	140.49±24.64	142.83±24.20	0.437	0.647
DBP (mmHg)	81.47±10.21	82.39±12.97	85.34±13.09	0.726	0.486
HOMA-IR	1.95±1.24	2.69 ±0.88	3.73±0.84	22.025	0.000***
C-reactive protein (mg/L)	3.17 ±1.82	4.43±3.09	5.54±4.25	4.257	0.016*
Serum folate (ng/mL)	32.98±21.69	33.72±23.84	28.36 ±21.74	0.472	0.625
Serum vitamin B12 (pg/mL)	599.73±265.99	498.25±235.66	388.42±213.20	5.350	0.006**

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for homogeneities among genotypes by ANOVA followed with Scheffe' multiple comparison.

dices tested were within normal range and there were no significant differences among all subjects.

Next, the data was analysed to determine whether the MTHFR genotype was associated with specific clinical characteristics in patients with MS-associated T2DM. As listed in Table 2, the serum levels of UA, TG, HOMA-IR and CRP were significantly higher in T2DM patients with TT genotype in comparison with

those with CC or CT genotype ($p < 0.05$ for TG and CRP, and $p < 0.001$ for UA and HOMA-IR, ANOVA). By contrast, serum vitamin B12 levels in patients with TT genotype were lower than those of patients with CC or CT genotypes ($p < 0.01$). There were no significant differences in other clinical characteristics such as BMI, SBP, DBP, HDL and serum level of folate

TABLE 3. Allelic frequencies were in the range described for population. Genotype distributions were compatible with the Hardy-Weinberg Equilibrium.

Group	Number	Genotype frequency						Allele frequency			
		CC		CT		TT		T		C	
		n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Control	55	34	61.8	17	30.9	4	7.3	25	22.7*	85	77.3
Type 2 diabetic without MS	40	23	57.5	13	32.5	4	10	21	26.3*	59	73.7
Type 2 diabetic with MS	118	34	28.8	61	51.7	23	19.5	107	45.3*	129	54.7

T allele frequency were compared between three groups by Chi-square test.

* $\chi^2=20.665$, $df=2$, $p<0.001$.

between patients with TT genotype and patients with CC or CT genotypes.

To further explore whether the distribution of MTHFR genotype was associated with MS in T2DM patients, the allele frequency of MTHFR was analysed. As shown in Table 3, the frequencies of MTHFR homozygote TT, and heterozygote CT in the MS-associated T2DM group were 19.5% and 51.7%, respectively, which were not only higher than those in the healthy subjects (7.3% and 30.9%, respectively), but also higher than those in non-MS-associated T2DM (10%, and 32.5%, respectively). Total T frequency was significantly higher in MS-associated T2DM patients (45.3%) compared to 22.7% in healthy subjects ($\chi^2=8.26$, $p<0.01$) and 26.3% in non-MS-associated T2DM patients ($\chi^2=9.03$, $p<0.01$), suggesting that the T allele is linked to susceptibility to MS-associated T2DM.

Discussion

Type 2 diabetes results from inadequate insulin secretion by pancreatic cells or insufficient response to insulin in peripheral tissues; in other words, insulin resistance. The most commonly described insulin resistance is metabolic syndrome, also known as Syndrome X, Reaven's syndrome, and insulin resistance syndrome. MS is characterized by the combination of insulin resistance, hyperglycaemia, hypertension, dyslipidaemia (low HDL, high small dense LDL and triglycerides), hyperuricaemia and obesity.¹⁵ The correlation of genetic and dietary factors with insulin resistance in Asian has not been well investigated. In

this study, it was found that in patients of Han Chinese origin with newly diagnosed T2DM, 75% (118/158) were also diagnosed with MS; and among these MS-associated T2DM patients, 71.4% (70/98) were male and 80% (48/60) female. These data indicate that the prevalence of MS in patients with T2DM is much higher in Han Chinese than has been reported in other countries in Asia including Korea, where levels were 46.9% for males and 65.1% for females.^{16,17}

T2DM results from alterations in a wide variety of genes; thus, its inheritance patterns are complex and environmental factors also play important roles in its development. Nutrigenetics has emerged as a tool for development of "personalized medicine" and provided great impetus for nutraceutical products. One of the best-known examples of applicability of nutrigenetics is for the treatment of MTHFR single nucleotide polymorphisms (SNPs).¹⁸ MTHFR irreversibly reduces N-5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate and methyl donor for remethylation of homocysteine to methionine. Mutation of the MTHFR gene at nucleotide position 677 from C to T (MTHFR677C>T) results in reduced MTHFR enzymatic activity and accumulation of homocysteine, a risk factor for venous thromboembolic disease and ischemic arterial disease.^{19,20} The relationship between MTHFR gene polymorphism and insulin resistance has not been reported previously. The present study analyzed MTHFR genotype in 158 patients with T2DM and 55 healthy subjects by PCR-RFLP analysis. This is the first epidemiological study on the cor-

relation of MTHFR gene with levels of folate, vitamin B12 and hs-CRP, and MS in Asia population.

These results indicate that levels of serum uric acid and triglycerides are significantly higher in people with MTHFR TT genotype than those with CC and CT genotype. The association of MTHFR C677T gene polymorphism with hyperuricemia observed in Han Chinese is consistent with those reported in Arabian,²¹ Italian,²² and Japanese²³ populations.

Pathologic and clinical data suggest a prominent role for inflammation at every stage of atherogenesis.²⁴ Increased levels of inflammatory markers, such as the high-sensitivity C-reactive protein (hs-CRP), are predictive for increased risk of cardiovascular diseases and associated with atherosclerotic disease and insulin resistance.^{25,26} Our study indicates that patients with MS-associated T2DM, the serum hs-CRP level and HOMA-IR are higher in those patients with MTHFR TT genotype than those with CC and CT genotypes. In contrast, the serum level of vitamin B12 in those with TT genotype is lower than those with CC and CT genotypes. These results are in agreement with the observation that vitamin B12 concentration is associated with MS.^{5,27}

Although erythrocyte folate concentration is a better index of tissue folate stores and may be a more reliable indicator of long-term supply of the vitamin and homocysteine status, whether serum folate or erythrocyte folate better reflect fasting plasma homocysteine status is less clear. According to a study by Chenga in Taiwanese adults [28], serum folate has been shown to correlate well with fasting plasma homocysteine; only serum folate concentration was associated with lower rate of fasting Hhcy. Therefore, we selected serum folate as a marker of folate status. Despite this, we did not find significant differences in serum levels of folate between patients with TT genotype and patients with CC or CT genotypes of MS-associated T2DM.

This study also shows that the C677T mutation of MTHFR gene is related to MS-associated T2DM, and the allele T may be a susceptible gene for MS. The TT genotype of MTHFR gene may contribute to insulin

resistance in Han Chinese with T2DM by increasing the serum levels of UA and CRP and decreasing the serum level of B12: an important player in the development of MS-associated T2DM. MTHFR gene polymorphism is related to hs-CRP level, suggesting that MTHFR may participate in the inflammation-related processes of insulin resistance, T2DM and atherosclerosis; however, the mechanisms involved are not clear. Our results suggest that improved serum levels of B12 may help to decrease the risk of MS. Further research on individual differences in genetic profiles and nutrient requirements will help to establish nutrigenetics as an essential discipline for practice of nutrition and dietetics. Our study is only one example of specific genetic polymorphisms in the context of nutrigenetics and metabolic disease. Generally, supplementation of folic acid and vitamin B12 helps to overcome the negative health effect of SNPs in MTHFR gene.²⁹⁻³¹ In meta-analysis of Hcy-lowering studies, oral supplement of 0.5–5 mg folic acid daily resulted in a reduction of Hcy by 25% ($P < 0.001$). Oral supplement of 0.5 mg vitamin B12 daily further reduced Hcy level by another 3% to 10%.³²⁻³⁴ It will be very interesting to explore whether supplementation of folic acid and vitamin B12 to patients with MS-associated T2DM can reduce the levels of uric acid and hs-CRP in individuals with the TT genotype and whether changing in capital lifestyle in conjunction with drug treatment can alter the characteristics of MS. Further epidemiological studies on the correlations of MTHFR gene polymorphism with MS in other ethnic populations are required.

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