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Original Article

Methylenetetrahydrofolate reductase TagSNPs contribute to the susceptibility to type 2 diabetes in a Chinese Han population

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Abstract: The potential function of single nucleotide polymorphisms (SNPs) in *methylenetetrahydrofolate reductase* (*MTHFR*) gene was predicted to be associated with the risk of type 2 diabetes mellitus (T2DM). The aim of our study was to evaluate the relationship between *MTHFR* tagging SNPs and the risk of T2DM. A hospital-based case-control study was conducted. Five hundred and two cases with T2DM and 782 controls were recruited. The SNPscan method was used to determine the genotypes. When we used the *MTHFR* rs4845882 GG homozygote genotype as the reference group, the AA genotype significantly increased the risk of T2DM (AA versus GG: OR = 1.73, 95% CI = 1.02-2.93, *P* = 0.041). When adjusted for age, sex, smoking, drinking status and BMI, the AA genotype still significantly increased risk of T2DM (AA versus GG: adjusted OR = 1.73, 95% CI = 1.02-2.95, *P* = 0.044). In the recessive model, when the *MTHFR* rs4845882 GG/GA genotype was used as the reference group, the AA homozygote genotype was also associated with a significantly increased risk of T2DM (OR = 1.85, 95% CI = 1.10-3.12, *P* = 0.020). Similarly when adjusted for age, sex, smoking, drinking status and BMI, the AA genotype still significantly increased risk of T2DM (adjusted OR = 1.84, 95% CI = 1.09-3.11, *P* = 0.024). Our study indicates that the AA genotype of the *MTHFR* rs4845882 G>A polymorphism significantly increased risk of T2DM in a Chinese Han population.

Keywords: *MTHFR*, polymorphism, type 2 diabetes mellitus, risk

Introduction

Diabetes is a chronic disease occurring when the body cannot efficaciously take advantage of the insulin, or alternatively, when the pancreases do not produce enough insulin [1]. The latest data of International Diabetes Federation (IDF) showed that 1 in 11 adults had diabetes globally. Meanwhile, 46.5% of adults with diabetes were undiagnosed. By 2040, 1 adult in 10 will be diagnosed. Three quarters of people with diabetes live in low and middle income countries and every 6 seconds a person dies from diabetes. By the end of 2015, diabetes has caused 5.0 million deaths [2]. Diabetes is the main factor contributing to non-traumatic amputations, blindness, and renal failure; the association between diabetes with weakened metabolic control and the high mortality due to coronary heart disease, neuropathies, retinopathy, and nephropathies has been well-established [3-5].

There are three main types of diabetes including type 1 diabetes mellitus, type 2 diabetes mellitus (T2DM), and gestational diabetes. T2DM is the most common type of diabetes. It usually occurs in adults, but is increasingly diagnosed in children and adolescents. In T2DM, the body can produce insulin but becomes insulin resistant so that the insulin is relatively ineffective. Over time, insulin level may subsequently become insufficient. Both insulin resistance (IR) and insulin deficiency result in high blood glucose level. Patients with

T2DM are well known to have a 2-4 fold higher risk of cardiovascular events contrasted with individuals without diabetes [6-8]. A recent meta-analysis concluded that the plasma homocysteine (HCY) level in subjects with T2DM was obviously higher than that in control subjects [9]. HCY is one of the sulfur-containing amino acids formed through the methionine demethylation. It exists in plasma in four different forms: 20-30% combines with itself or other thiols to form dimer HCY, about 1% circulates as free thiol, and 70-80% remains disulphide-combined to plasma proteins, especially albumin [10]. It is a neotype risk factor for cardiovascular disease and diabetic nephropathy that has gradually caused the interest of studies [11]. Several effects resulted from HCY on vascular endothelial cells have been determined, such as decrease of nitric oxide (NO) release from platelets [12] and endothelial cells [13], increase of arachidonic acid mobilization, more thromboxane A_2 and reactive oxygen species (ROS) production in human platelets [14], stimulation of smooth muscle cells proliferation [15] and potentiation of low-density lipoproteins oxidation [16]. The mechanism that HCY lowers NO content and promotes ROS generation leads to endothelial cell injury in vitro. Then it weakens NO-mediated inhibition of platelet aggregation [17] and modifies the vascular response to L-arginine [18] and the adhesion characteristic of endothelial cells [19]. The HCY level increased in diabetes patients [20]. Previous studies indicated that hyperhomocysteinemia (Hhcy) was associated with the increased oxidative stress in T2DM subjects [21]. ROS contribute to β-cell dysfunction, IR, and both the macrovascular and microvascular long-term complications of diabetes [22-25].

Familial Hhcy aggregation is consistent to the concept that this disorder may possess a genetic predisposition. An enzyme that has drawn particular attention in Hhcy is methylenetetrahydrofolate reductase (MTHFR), which catalyzes HCY methylation into methionine. The chemical reaction is the final segment in the sulfur-recycling pathway. The *MTHFR* gene has been suggested to be one of the genetic determinants of Hhcy [26]. The *MTHFR* gene is situated in 1p36.3 and includes 11 exons with a length of 1980 bp. In humans, the MTHFR enzyme exists in the form of dimers and each monomer has an N-terminal catalytic domain and a C-terminal regulatory domain [27]. Common mutations of this gene produce thermolabile variants of *MTHFR* which decreases the enzymatic activity and in turn results in higher total HCY concentrations in plasma [28].

The *MTHFR* gene contains more than 20 single nucleotide polymorphisms (SNPs) [29]. In the view of the *MTHFR* biological and pathologic importance, functional *MTHFR* genetic SNPs may contribute to risk of T2DM. In order to explore the association between risk of T2DM and *MTHFR* SNPs, we performed a hospital-based case-control study to evaluate the association between five *MTHFR* tagging SNPs (rs3753584 A>G, rs9651118 T>C, rs1801133 C>T, rs4846048 A>G and rs4845882 G>A) and susceptibility to T2DM in a Chinese Han population.

Materials and methods

Subjects

In our study, all participants, including 502 T2DM cases and 782 controls, were Chinese Han populations. All of them provided written informed consents. This study was approved by the Institutional Review Board of Fujian Medical University (Fuzhou, China) and Jiangsu University (Zhenjiang, China). All cases were recruited consecutively from Affiliated People's Hospital of Jiangsu University (Zhenjiang City, China) and Affiliated Union Medical College of Fujian Medical University (Fuzhou, China) from October 2014 through May 2016. A case of T2DM was diagnosed if the subject's reported glucose level conformed to the well-established T2DM diagnostic criteria recommended by international diabetes federation (IDF) [30]. All controls were free of diabetes and impaired glucose tolerance (IGT) determined by clinical examination or medical history and recruited from health check centers in two hospitals mentioned above. All of control individuals were matched to the cases for sex and age (±5 years).

Clinical data collection

Demographic variables, including age, gender, history of smoking, drinking, were collected through a standardized interview. Weight and height were measured and used to calculate the body mass index (BMI) [BMI = weight/height^2 (kg/m^2)]. Blood samples were extracted to detect the total cholesterol (TC), fasting blood

Table 1. Distribution of selected demographic variables and risk factors in type 2 diabetes cases and controls

Variable	Cases (n = 502)		Controls (n = 782)		*P*[a]
	n	%	n	%	
Age (years)	65.20±9.51		64.67±9.80		0.347
Age (years)					0.113
<65	227	45.22	389	49.74	
≥65	275	54.78	393	50.26	
Gender					0.819
Male	332	66.14	522	66.75	
Female	170	33.86	260	33.25	
Tobacco use					0.264
Never	333	66.33	542	69.31	
Ever	169	33.67	240	30.69	
Alcohol use					0.263
Never	453	90.24	690	88.24	
Ever	49	9.76	92	11.76	
Height (m)	1.68±0.08		1.66±0.07		0.015
Weight (kg)	67.63±11.42		64.62±9.96		**<0.001**
BMI (kg/m^2)	24.95±3.64		23.51±2.94		**<0.001**
BMI (kg/m^2)					**<0.001**
<24	210		436		
≥24	292		346		
Systolic pressure (mmHg)	135.08±17.83		134.02±17.71		0.297
Diastolic pressure (mmHg)	79.79±10.35		80.06±10.02		0.649
Fasting blood glucose (mmol/L)	8.08±2.76		5.13±0.49		**<0.001**
Total cholesterol (mmol/L)	4.61±1.24		4.88±1.02		**<0.001**
Triglyceride (mmol/L)	1.74±1.14		1.55±0.96		**0.001**
HDL-C (mmol/L)	1.13±0.37		1.30±0.37		**<0.001**
LDL-C (mmol/L)	3.00±1.07		3.14±0.82		**0.010**
LDL-C/HDL-C	2.90±1.66		2.59±1.01		**<0.001**

[a]Two-sided χ^2 test and student t test; Bold values are statistically significant (P<0.05). BMI, Body mass index; HDL-C, High-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol.

glucose (FBG), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C). We also collected the data of systolic pressure and diastolic pressure of two group subjects but not hypertension history. All biochemical parameters were measured in the laboratory of Affiliated People's Hospital of Jiangsu University and Affiliated Union Medical College of Fujian Medical University.

DNA extraction

Venous blood sample from each subject was collected and stored in EDTA-containing tubes at -20°C. Genomic DNA was extracted from white blood cells using DNA Blood Mini Kit (Promega, Madison, USA). DNA concentration was measured using the NanoDrop 2000 spectrophotometer. All sample concentration was standardized to the detectable level of 10 ng/μL.

SNP selection

Tagging SNPs were selected by retrieving Chinese Han population data from the HapMap project (http://www.hapmap.org/) and Haploview 4.2 software described previously [31, 32]. The following criteria were adopted to identify the candidate tagging SNPs: (1) SNPs located in the gene or within the 2-kb region flanking the gene, (2) a minor allele frequency (MAF) ≥0.05, and (3) other unselected SNPs

Table 2. Primary information for *MTHFR* rs1801133 C>T, rs3753584 A>G, rs4845882 G>A, rs4846048 A>G and rs9651118 T>C polymorphisms

	Chromosome	Function	Chr Pos (Genome Build 36.3)	MAF[a] for Chinese in database	MAF in our controls (n = 782)	*P* value for HWE[b] test in our controls	Genotyping method	% genotyping value
rs1801133 C>T	1	Missense	11778965	0.439	0.349	0.498	SNPscan	99.61%
rs3753584 A>G	1	NearGene-5	11787173	0.093	0.107	0.703	SNPscan	99.61%
rs4845882 G>A	1	Intron	11765754	0.198	0.259	0.112	SNPscan	99.61%
rs4846048 A>G	1	Intron	11768839	0.105	0.121	0.785	SNPscan	99.61%
rs9651118 T>C	1	Intron	11784801	0.382	0.470	0.021	SNPscan	99.61%

[a]MAF: Minor allele frequency; [b]HWE: Hardy-Weinberg equilibrium.

could be gained through one of the tagging SNPs with a linkage disequilibrium (LD) of $r^2 \geq 0.08$. As a result, a total of 5 tagging SNPs were identified.

Polymorphism genotyping

Genotyping was performed utilizing SNPscan method by a custom-by-design 2×48-Plex SNPscan™ Kit (Genesky Biotechnologies Inc., Shanghai, China) [33]. The SNPscan technique was exploited according to proprietary technology of SNP genotyping by Genesky Biotechnologies Inc., who provides a cost-saving and high-throughput SNP genotyping method based on multiplex fluorescence PCR and double ligation as previously described [34]. For quality control repeated tests were conducted by randomly selecting 4% total samples. This confirmed an accuracy rate of 100%.

Statistical analysis

The measurement data (age, height, weight, BMI, systolic pressure, diastolic pressure, fasting glucose, total cholesterol, triglyceride, HDL-C, LDL-C and LDL-C/HDL-C) were compared by the t-test. Differences in the demographic characteristics and genotype between T2DM cases and controls were calculated by the chi-squared (χ^2) test. We tested the Hardy-Weinberg equilibrium (HWE) in controls by an internet-based HWE calculator (website at http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) [29]. In order to evaluate the association between five *MTHFR* genotypes and susceptibility to T2DM, unconditional logistic regression analysis was performed to compute the odds ratios (ORs, crude or adjusted appropriately) and their 95% confidence intervals (CIs). All statistical analyses were performed with the SAS software (version 9.4, SAS Institute, Cary, NC). $P<0.05$ was defined statistically significant; with two-sided probabilities.

Results

Characteristics of the study population

The clinical and demographic characteristics of all subjects were summarized in **Table 1**. The cases and the controls were matched evenly for age ($P = 0.347$) and gender ($P = 0.819$). Tobacco and alcohol use were not significantly different between the cases and the controls ($P = 0.264$, $P = 0.263$, respectively). Meanwhile, systolic and diastolic pressure did not show significantly different between the cases and the controls ($P = 0.297$, $P = 0.649$, respectively). But diabetes-related indexes and traditional risk factors, such as BMI ($P<0.001$), FBG ($P<0.001$), TC ($P<0.001$), TG ($P = 0.001$), HDL-C ($P<0.001$), LDL-C ($P = 0.010$) and LDL-C/HDL-C ($P<0.001$) were significantly different between the cases and the controls.

Primary information of MTHFR SNPs

The primary information of *MTHFR* tagging SNPs was shown in **Table 2**. The molecular markers that we used consisted of rs37535-84 A>G, rs9651118 T>C, rs1801133 C>T, rs-4846048 A>G and rs4845882 G>A polymorphisms. These markers relatively randomly locate in introns, except rs1801133 C>T (missense). The minor allele frequencies (MAF) of five SNPs in our control range from 0.107 to 0.470 (according to the NCBI database). Genotyping was performed using SNPscan method. With exception rs9651118 T>C ($P = 0.021$), these SNPs genotype distribution in the controls accorded to the HWE ($P>0.05$). All of the genotyping values of five SNPs were 99.61% in all 1284 samples.

Table 3. SNPscan analyses of association between *MTHFR* polymorphisms and risk of type 2 diabetes

Genotype	Cases (n = 502)		Controls (n = 782)		Crude OR (95% CI)	*P*	Adjusted OR[a] (95% CI)	*P*
	n	%	n	%				
MTHFR rs1801133 G>A								
GG	228	45.88	327	41.82	1.00		1.00	
GA	215	43.26	364	46.55	0.83 (0.65-1.05)	0.122	0.82 (0.65-1.05)	0.118
AA	54	10.87	91	11.64	0.83 (0.57-1.21)	0.341	0.80 (0.55-1.17)	0.247
GA+AA	269	54.13	455	58.19	0.85 (0.68-1.06)	0.154	0.84 (0.66-1.05)	0.129
GG+GA	443	89.14	691	88.37	1.00		1.00	
AA	54	10.87	91	11.64	0.93 (0.65-1.32)	0.671	0.89 (0.62-1.28)	0.528
A allele	323	32.49	546	34.91				
MTHFR rs3753584 T>C								
TT	378	76.06	622	79.54	1.00		1.00	
CT	117	23.54	152	19.44	1.25 (0.95-1.64)	0.109	1.23 (0.93-1.62)	0.143
CC	2	0.40	8	1.02	0.41 (0.09-1.92)	0.256	0.43 (0.09-2.07)	0.295
CT+CC	119	23.94	160	20.46	1.22 (0.94-1.60)	0.142	1.21 (0.92-1.59)	0.177
TT+CT	495	99.60	774	98.98	1.00		1.00	
CC	2	0.40	8	1.02	0.39 (0.08-1.85)	0.236	0.42 (0.09-1.99)	0.273
C allele	121	12.17	168	10.74				
MTHFR rs4845882 G>A								
GG	310	62.37	477	61.00	1.00		1.00	
GA	155	31.19	277	35.42	0.85 (0.67-1.08)	0.181	0.87 (0.68-1.11)	0.247
AA	32	6.44	28	3.58	**1.73 (1.02-2.93)**	**0.041**	**1.73 (1.02-2.95)**	**0.044**
GA+AA	187	37.63	305	39.00	0.94 (0.75-1.18)	0.622	0.96 (0.76-1.21)	0.736
GG+GA	465	93.56	754	96.42	1.00		1.00	
AA	32	6.44	28	3.58	**1.85 (1.10-3.12)**	**0.020**	**1.84 (1.09-3.11)**	**0.024**
A allele	219	22.03	333	25.93				
MTHFR rs4846048 A>G								
AA	410	82.49	634	81.07	1.00		1.00	
AG	78	15.69	141	18.03	0.85 (0.62-1.14)	0.276	0.89 (0.65-1.21)	0.447
GG	9	1.81	7	0.90	1.96 (0.73-5.32)	0.184	1.93 (0.70-5.30)	0.202
AG+GG	87	17.50	148	18.93	0.91 (0.68-1.22)	0.523	0.95 (0.71-1.28)	0.735
AA+AG	488	98.18	775	99.10	1.00		1.00	
GG	9	1.81	7	0.90	2.04 (0.76-5.52)	0.159	1.99 (0.73-5.46)	0.182
G allele	96	9.66	155	12.07				
MTHFR rs9651118 T>C								
TT	184	37.02	280	35.81	1.00		1.00	
TC	241	48.49	401	51.28	0.89 (0.70-1.14)	0.351	0.91 (0.71-1.16)	0.445
CC	72	14.49	101	12.92	1.06 (0.74-1.51)	0.763	1.04 (0.72-1.48)	0.852
TC+CC	313	62.98	502	64.20	0.95 (0.75-1.20)	0.659	0.96 (0.76-1.22)	0.732
TT+TC	425	85.51	681	87.09	1.00		1.00	
CC	72	14.49	101	12.92	1.14 (0.83-1.58)	0.423	1.11 (0.80-1.54)	0.549
C allele	385	38.73	603	46.96				

[a]Adjusted for age, sex, smoking, drinking status and body mass index; Bold values are statistically significant ($P<0.05$).

Associations between MTHFR polymorphisms and the risk of T2DM

The genotype frequencies of the *MTHFR* rs4845882 G>A polymorphism were 62.37% (GG), 31.19% (GA) and 6.44% (AA) in T2DM patients, and 61.00% (GG), 35.42% (GA) and 3.58% (AA) in controls (**Table 3**). When we used the *MTHFR* rs4845882 GG homozygote genotype as the reference group, the AA genotype significantly increased risk of T2DM (AA versus GG: OR = 1.73, 95% CI = 1.02-2.93, $P = 0.041$). When adjusted for age, sex, smoking, drinking status and BMI, the AA genotype still significantly in-

creased risk of T2DM (AA versus GG: adjusted OR = 1.73, 95% CI = 1.02-2.95, *P* = 0.044). In the recessive model, when the *MTHFR* rs4845882 GG/GA genotype was used as the reference group, the AA homozygote genotype was also associated with a significantly increased risk of T2DM (OR = 1.85, 95% CI = 1.10-3.12, *P* = 0.020). Similarly when adjusted for age, sex, smoking, drinking status and BMI, the AA genotype still significantly increased risk of T2DM (adjusted OR = 1.84, 95% CI = 1.09-3.11, *P* = 0.024, **Table 3**).

None of the *MTHFR* rs3753584 A>G, rs9651118 T>C, rs1801133 C>T and rs4846048 A>G polymorphisms had a significantly different distribution between T2DM cases and controls. Logistic regression analyses also revealed that these polymorphisms were not associated with the susceptibility of T2DM (**Table 3**).

Discussion

In our study, we experimented on the association between *MTHFR* rs3753584 A>G, rs9651118 T>C, rs1801133 C>T, rs4846048 A>G and rs4845882 G>A polymorphisms and the risk of T2DM in a Chinese Han populations. We found that the homozygote genotype AA of *MTHFR* rs4845882 G>A polymorphism may increase the risk of T2DM in the homozygote and recessive models. However, the rs3753584 A>G, rs9651118 T>C, rs1801133 C>T and rs4846048 A>G polymorphisms were not associated with the risk of T2DM.

As the most prevalent metabolic disorder, diabetes mellitus is characterized by chronic hyperglycemia due to resistance against insulin action or defect of insulin secretion by beta cells of pancreas islets [35]. T2DM includes 90-95% of patients with diabetes. Patients with T2DM may be asymptomatic for a long time. Vascular complications such as cardiovascular disease, neuropathy, nephropathy and retinopathy may develop in these patients. The effect of genetic factors appears to be greater in T2DM compared to T1DM [36, 37]. The rising incidence of T2DM and the necessity of early detection and management have urged many investigators to investigate environmental and genetic risk factors for T2DM.

MTHFR gene codes a crucial enzyme (flavoprotein) in the metabolism of folate and HCY and is involved in methylation, DNA synthesis and nucleotide repair [38]. MTHFR is the enzyme that catalyzes the transformation of HCY to methionine via the re-methylation pathway [39]. The accumulation of HCY in the plasma is associated with metabolic syndrome, including T2DM [40]. Elevatory plasma levels of HCY, a condition defined as HCY, have been related to such T2DM characteristics as insulin resistance [41, 42], endothelial dysfunction and arterial stiffness [43], hypercoagulability and prothrombotic inflammation [44], nephropathy [45, 46] and macroangiopathy [41, 47]. Hhcy has also linked with coronary heart disease [48], atherosclerosis [49] and sudden death [50] among individuals with T2DM.

Genetic polymorphisms often vary between different ethnic groups. The MAF of the rs4845882 G>A polymorphism was 0.259 in 782 controls in our study, similar to MAF for Chinese in database (0.198). So far there were not any experimental research about the association between the rs4845882 G>A polymorphism and the risk of T2DM. And the studies about the rs4845882 G>A polymorphism were also rare. Tang W *et al.* did the first study about this polymorphism in the role of esophageal squamous cell carcinoma (ESCC) risk and concluded that it was associated with the decreased risk of ESCC [31]. Hereafter, we conducted the study about this polymorphism in the role of gastric cardia adenocarcinoma (GCA) but we found no association about them [29]. These results implied that the rs4845882 G>A polymorphism might have different functions in different diseases.

In 1977, the discovery of intronsbrought the molecular biology world a big surprise [51]. In eukaryotes, intronsare also called as spliceosomalintrons which are discovered in the nuclear genomes [52]. They have quasi-random sequences and generally lack open reading frames (ORFs), but they have been shown or proposed to carry out many functions. In nonsense-mediated decay (NMD), the transcript is targeted for degradation if a transcriptional error causes to a stop codon upstream of an adjacent intron-exon boundary [53]. Some studies have indicated that many introns involved in exon shuffling [54-56]. And the introns in genes increase the recombination rate between parts of the codingregion, leading to the creation of new products and enhancing the efficiency of selection [57-60]. The rs4845882 G>A polymorphism is situated in the intron of the *MTHFR*

gene and has almost complete linkage disequilibrium (LD) with rs1801131 A>C [31]. The polymorphism may affect the *MTHFR* enzyme activity through the mechanism mentioned above. These evidence could indicate that the rs4845882 G>A polymorphism influences the risk of T2DM.

Several limitations of our present study must be noted. Firstly, this study was a hospital-based case-control study. The controls were select from Health Check Centers in two hospitals. Since the control subjects did not completely represent the overall Chinese Han population, selection bias was unavoidable. Secondly, although we selected tagging SNPs to study the association between *MTHFR* polymorphisms and risk of T2DM, the included polymorphisms investigation might not offer a comprehensive view on the *MTHFR* genetic function. In the future, a fine-mapping study is needed. Thirdly, for the number of cases is limited, a single case-control and repeated study is insufficient to comprehensively interpret the association between the *MTHFR* polymorphisms and the susceptibility to T2DM. Studies with larger participants are required to verify our findings. Fourthly, HCY is an important factor involved in the development of T2DM. We will bring it into our future studies. Finally, the risk of T2DM is probably influenced by gene-environment and gene-gene interactions, such studies are needed to explain the pathogenesis of T2DM.

In conclusion, our study indicates that the AA genotype of the *MTHFR* rs4845882 G>A polymorphism significantly increases the risk of T2DM in a Chinese Han population. However, further studies are warranted to confirm our results.

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Disclosure of conflict of interest

None.

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