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Association of Two Methylenetetrahydrofolate Reductase Polymorphisms (rs1801133, rs1801131) with the Risk of Type 2 Diabetes in South-East of Iran

Maryam Poodineh[1], Ramin Saravani*[2,3], Mahboubeh Mirhosseini[1], Saman Sargazi[2]

Abstract

Background: DNA methylation has been linked to the development and progression of multiple disorders including T2D. One significant enzyme involved in DNA methylation is methylene tetrahydrofolate reductase (*MTHFR*). This study was designed to evaluate the association between rs1801133 and rs1801131 polymorphisms, located in the *MTHFR*, and T2D in an Iranian population.

Methods: Blood samples from 151 patients with T2D and 136 healthy individuals were collected and DNA was extracted using the salting out method. Variants were genotyped using amplification tetra-refractory mutation system-polymerase chain reaction analysis. The data were analyzed via independent sample t-test and x^2 tests.

Results: The rs1801131 A/C polymorphism significantly increased the risk of T2D in codominant heterozygous AC (P=0.008), homozygous CC (P=0.01), and recessive CC (P=0.001) genotypes. Significant correlations were found regarding rs1801133 T/C gene polymorphism and the risk of T2D in codominant heterozygous TC (P=0.001), homozygote CC (P=0.001), and recessive CC (P=0.0001) models. The presence of the C allele is a potential risk factor for T2D for rs1801133 T/C (P=0.001) and rs1801131 A/C (P=0.04) polymorphisms.

Conclusions: Both the rs1801133 T/C and rs1801131 A/C *MTHFR* polymorphisms significantly increased the risk of T2D in our population. Further studies in other ethnicities are necessary to verify our findings.

Keywords: Gene Polymorphism, *MTHFR*, Type 2 diabetes.

Introduction

Diabetes mellitus (DM), the most common metabolic endocrine disorder, is characterized by increased glucose levels and disturbed carbohydrate, fat, and protein metabolism (1). Generally, DM is categorized into several subtypes, the most common of which are types 1 and 2 (T1D and T2D, respectively) (2). Type 2 diabetes is most common, gradually progresses with age, and unlike type 1, does not result in ketoacidosis (3). The prevalence of DM in western Asia, including Iran, was 11.37 % in 2011, and is expected to increase (4). Type 2 diabetes is more prevalent in developed countries, where it is a major health problem, than in undeveloped countries (5). Female gender, high blood pressure, increased basic metabolic index (BMI), and positive family history are independent risk factors for DM (6). Type 2 diabetes is a polygenic and multifactorial disease as various genetic loci play crucial roles in its development (7). Single-nucleotide polymorphisms (SNPs) have been identified in several genes associated with the increased risk of T2D (8).

The methylene tetrahydrofolate reductase gene (*MTHFR*) is located at the end of the short arm of

1: Department of Biology, Payame Noor University of Taft, Yazd, Iran.
2: Cellular and Molecular Research Center, Zahedan University of Medical Sciences, Zahedan, Iran.
3: Department of Clinical Biochemistry, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran.
***Corresponding author: Ramin Saravani; Tel: + +98-543329892, Fax: +98-543329892; E-mail: saravaniramin@yahoo.com.**
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chromosome 1 and has 11 exons. Based on reads per kilo base per million mapped reads (RPKM) information, *MTHFR* expression differs between organs, being higher in the lung (RPKM 7.5) and thyroid (RPKM 7.2) than in other tissues (9). The active MTHFR enzyme plays a central role in folate metabolism and converts 5, 10-methylene tetrahydrofolate to 5-ethyltetrahydrofolate, the dominant form of folate in the blood (10). Homocysteine is an amino acid that contains sulfur formed during methionine metabolism. Homocysteine converts to methionine by receiving one methyl group from 5-methyltetrahydrofolate, which also plays a role in the biosynthesis of S-adenosyl methionine (11). Earlier studies have established that insulin plasma levels affect homocysteine metabolism through the effective activation of enzymes involved in homocysteine metabolism, such as MTHFR (12, 13). rs1801133 (677C>T, MAF=0.31) and rs1801131 (1298A/C, MAF=0.29) are two common polymorphisms located within *MTHFR* (14). Studies in China showed a significant association between these two missense genetic variants and T2D (15). Basically, *MTHFR* polymorphisms reduce the frequency of mutations in *MTHFR*, causing decreased enzymatic activity in some races (16); in some cases, enzymatic activity is reduced by up to 50% (17, 18). Also, hypermethylation in the *MTHFR* promoter region is linked with complications of DM (19) and the susceptibility to essential hypertension, cardiovascular diseases, and homocysteinemia (20). Kheradmand et al. showed that genetic mutations in *MTHFR* coding regions are significantly higher in patients with insulin resistance than in those without (21).

To our knowledge, a possible link between rs1801133 T/C and rs1801131 A/C polymorphisms and the risk of developing T2D has not been investigated in the southeast Iranian population, therefore we aimed to investigate this association in various inheritance models.

Materials and methods

Subjects

The study protocol was approved by the Ethics Committee of the Payam Noor University of Taft, Yazd (Grant No: 3713). One hundred thirty-six T2D patients referring to the diabetes center of Boo-Ali Hospital in Zahedan and 151 healthy individuals with normal blood glucose levels and no history of systemic diseases or family relationships with T2D patients were enrolled. The inclusion criteria included blood glucose concentration greater than 126 mg/dL, hemoglobin A1C greater than 6.5%, and agreement of at least two endocrinologists. All participants comprehended the informed consent forms and the demographic information including age, sex, body mass index (BMI), fasting blood glucose (FBS), triglyceride (TG) and total cholesterol (TC), and these were collected from both groups (Table 1).

Table 1. Association between clinicopathological characteristics of T2D patients and healthy individuals.

Parameters evaluated	T2D (n=136) (n±SD)	Controls (n=151) (n±SD)	P Value
Sex female/male	94/42	101/50	**0.39**
Age (year)	52.16±9.3	50.53±9.02	**0.08**
FBS (mg/dl)	182.72±68	95.16±10.37	**<0.001**
TG (mg/dl)	148.34±81	138±64	**0.41**
TC (mg/dl)	171/36±53	165.01±45	**0.24**

DNA extraction, primer design, and genotyping

Four mL of venous whole blood were collected for DNA extraction (2 mL in 0.5 M EDTA-containing tubes) and biomedical parameters (2 mL in blood-drawing tubes containing an integral serum-separating barrier gel). DNA was extracted using the salting out method (22). The quality and the quantity of extracted DNA was assessed using 2% agarose gel electrophoresis and UV spectrophotometry. The tetra-primer amplification refractory mutation system (Tetra-ARMS) method was used to genotype *MTHFR* polymorphisms rs1801131 A/C and rs1801133 T/C. The specific primers were designed using BatchPrimer3 (23) (Table 2).

Table 2. The primers used for detection of rs1801131 A/C and rs1801133 T/C polymorphisms.

Gene polymorphism	Primers	Sequence (5′ to 3′)
rs1801131 A/C	FO	GCAGAAGAAGTTTGCATGCTTGTGGTTG
	RO	ACTTACCCTTCTCCCTTTGCCATGTCCA
	FI	GTGGGGGGAGGAGCTGACCAGTGAGGA
	RI	GGTAAAGAACGAAGACTTCAAAGACACCTG
rs1801133 C/T	FO	CATATCAGTCATGAGCCCAGCCACTCAC
	RO	AGGAGATCTGGGAAGAACCAGCGAACTC
	FI	TTGAAGGAGAAGGTGTCTGCGGGCGT
	RI	CAAAGAAAAGCTGCGTGATGATGAAATAGG

FO: Forward outer; RO: Reverse outer; FI: Forward inner; RI: Reverse inner.

For each PCR, 0.5 µL (10 ng/mL) of each primer (Pishgaman Co.Iran), 12 µL of Master Mix (Ampliqon, Denmark), 2 µL (~1ng/mL) of genomic DNA, and 10 µL of DNase-free distilled water (Parstous Biotech Co., Iran) were mixed to a final volume of 25 µL. The tetra ARMS-PCR temperature/time schedule is presented in *Table 3*. At least 40% of the PCR products were evaluated on 2% agarose gels using a UV transilluminator set at 254 nm (Ultra-Lum Electronic UV Transilluminator, USA).

Table 3. PCR conditions for tetra-ARMS PCR genotyping of rs1801131 A/C and rs1801133 T/C polymorphisms.

Polymorphism	Cycle	Temperature (ºC)	Time (minutes)	Number of cycles
rs1801133 T/C	Initial Denaturation	95	5:00	**1**
	Denaturation	95	0:30	
	Annealing	66	0:30	**35**
	Extension	72	0:45	
	Final extension	72	05:00	**1**
rs1801131 A/C	Initial Denaturation	95	05:00	**1**
	Denaturation	95	0:30	
	Annealing	54	0:30	**35**
	Extension	72	0:45	
	Final extension	72	05:00	**1**

Statistical analyses

The results were analyzed using SPSS V.16 for Windows (SPSS Inc., Chicago, IL, USA). An independent t-test was used to compare the data between the patient and control groups. Chi-square and logistic regression were used to determine the relationship between T2D and the SNPs. P values less than 0.05 were considered significant.

Results

Patients and controls were matched in terms of age and sex. No significant differences were found between the two groups for either parameter while the mean glucose level significantly differed between the two groups ($P<0.05$, Table 1). Because the patients in this study were monitored by endocrinologists and receiving prescriptions, no significant differences were found between the two groups for either triglyceride or total cholesterol ($P > 0.05$ for both, *Table 1*). Comparison of the frequency of *MTHFR* rs1801131 A/C polymorphism between T2D patients and healthy controls revealed that in the codominant model genotype CC (OR = 3.03, 95% CI, (1.25-7.36), P = 0.01) and in the recessive model genotype CC (OR = 5.8, 95% CI, (2.80-12.31), P = 0.001) and allele C (OR = 1.37, 95% CI (0.99-1.91), P = 0.04) of this polymorphism were risk factors for T2D (Table 4).

Table 4. Genotypic and allelic frequencies of *MTHFR* polymorphism (rs180131 A/C) in T2D patients and control subjects.

Inheritance Models	Genotypes/Alleles	T2D, n (%)	Control, n (%)	OR (95%CI)	P-Value
Codominant					
	AA	29 (21)	22 (14)	1.00	
	AC	67 (50)	119 (79)	0.42 (0.22-0.8)	**0.08**
	CC	40 (29)	10 (7)	3.03 (1.25-7.36)	**0.01**
Recessive					
	AA+ AC	40 (000)	10 (000)	1.00	
	CC	96 (000)	141 (000)	5.8 (2.80-12.31)	**0.001**
	A	125	163	1.00	
	C	147	139	1.37 (0.99-1.91)	**0.04**

Regarding the rs1801133 (T>C) polymorphism, we found that inheritance models of codominant heterozygote TC (OR = 7.39, 95%= CI (4.21-12.97), P = 0.001), homozygous CC (OR = 7.78, 95% = CI (3.40-17.79), P = 0.001), and recessive CC (OR: 3.17, 95% = CI (1.46-6.88, P = 0.001) genotypes were significantly associated with T2D susceptibility. Also, the presence of allele C (OR = 2.77, 95% = CI (1.87-4.09) P = 0.001) could be a risk factor for T2D (Table 5).

Table 5. Genotypic and allelic frequencies of *MTHFR* polymorphism (rs180133 T/C) in T2D patients and control subjects.

Inheritance Models	Genotypes/Alleles	T2D, n (%)	Control, n (%)	OR (95%CI)	P-Value
Codominant					
	TT	35 (26)	109 (72)	1.00	
	TC	76 (56)	32 (21)	7.39 (4.21-12.97)	**0.001**
	CC	25 (18)	10 (7)	7.78 (3.40-17.79)	**0.001**
Recessive					
	TT+TC	25 (18)	10 (7)	1.00	
	CC	111 (82)	141 (93)	3.17 (1.46-6.88)	**0.001**
	T	174 (64)	251(83)	1.00	
	C	98 (36)	51(17)	2.77 (1.87-4.09)	**0.001**

Discussion

Studies have shown that both hyperglycemia and insulin resistance trigger a cascade of responses, which ultimately leads to enhanced DNA methylation in various tissues (24). DNA methylation has attracted much attention in recent years and occurs under various physiochemical conditions. To date, DNA methylation has been well demonstrated in DM (25). Hyperinsulinemia affects MTHFR activity, while the high level of homocysteine, due to mutations in MTHFR, is associated with insulin resistance (26). The relationship between *MTHFR* C677T and A1298C polymorphisms and complications of T2D has been reported in an Emirati population (16), but no study has investigated a possible link between these two variants and T2D susceptibility in the Iranian population.

In our study, we found that the presence of C allele increased the risk of T2D progression and development in both *MTHFR* polymorphisms. Also, the evaluation of codominant, recessive models indicated that the rs1801131 and rs1801133 polymorphisms were significantly linked with T2D susceptibility in our population. In contrast to our findings, Zhong et al. (2013) reported no significant association between the rs1801133 polymorphism and risk of T2D (18). Raza et al. (2017) reported that the rs1801133 polymorphism might also be correlated with dyslipidemia development in T2D patients (27).

Furthermore, Raza et al. (2012) found a significant correlation between the rs1801131 polymorphism and T2D in an Indian population (28). Mtiraoui et al. (2007) reported that CT and TT genotypes in the rs1801133 locus indicated increased risk of T2D, while no significant association was found between the rs1801131 polymorphism and DM in a Tunisian population (29). Zhong et al. (2013) reported that the allele C of rs1801133 locus has a protective effect against T2D (18), which agreed with our findings in this work. Additionally, our results are consistent with those reported by Yan et al. (2013) on a Chinese population sample, indicating that rs1801131 was associated with an increased risk of DM (30). Zhou et al. (2014), inferred that the rs1801131 CC genotype significantly increased the risk of ischemic stroke, indicating that *MTHFR* polymorphisms might influence susceptibility to several disorders by significantly altering total homocysteine levels and MTHFR enzymatic activity (31). The differences in the results obtained in the current work and those of other investigations could be attributed to differences in race, genetics, geographic area, and lifestyle, as well as the limited sample size. Further studies with a larger cohort in different populations could provide more details on the relationship between these two non-synonymous *MTHFR* SNPs and T2D.

We conclude that the *MTHFR* rs1801131 A/C and rs1801133 T/C polymorphisms might be genetic biomarkers for T2D progression and development in this Iranian population sample. However, the authors suggest further investigation on a larger sample and other ethnicities to verify these results.

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