

## Association between the rs4753426 polymorphism in *MTNR1B* with fasting plasma glucose level and pancreatic $\beta$ -cell function in gestational diabetes mellitus

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**ABSTRACT.** We investigated the association between rs4753426 single nucleotide polymorphisms in the melatonin receptor 1B (*MTNR1B*) gene and the risk of developing gestational diabetes mellitus (GDM). A total of 516 gravidas (186 with GDM and 330 non-diabetic controls) were enrolled in the study. Genotype and allele frequencies of rs4753426 in the *MTNR1B* gene were detected by DNA sequencing. Fasting plasma glucose and fasting insulin levels were measured to calculate the homeostasis model assessment for insulin resistance (HOMA-IR) and for  $\beta$ -cell function. Three genotypes (CC, CT, and TT) were found in both groups. The frequencies of CC, CT, and TT genotypes for the GDM group were 70.97, 22.58, and 6.45% vs 53.03, 39.70, and 7.27% in the control group, respectively. Significant differences were observed in genotype frequencies between groups ( $P < 0.05$ ). T and C allele frequencies in the GDM group were 17.74 and 82.26%, respectively, and in the control group were 27.12 and 72.88%, respectively. Significant differences in T and C allele frequencies

were found between groups ( $P < 0.05$ ). In the GDM group, the C allele was associated with increased fasting plasma glucose level and reduced pancreatic  $\beta$ -cell function ( $P < 0.05$ ). There were no significant differences in total cholesterol, triglyceride, low-density lipoprotein, high-density lipoprotein concentration, or HOMA-IR between groups ( $P > 0.05$ ). The single nucleotide polymorphism rs4753426 in *MTNR1B* may be a susceptibility gene locus for GDM, and the C allele may contribute to the increased fasting plasma glucose level and reduced pancreatic  $\beta$ -cell function.

**Key words:** Diabetes mellitus; Genetic susceptibility; Pregnancy; Melatonin receptor; MT2; Single nucleotide polymorphisms

## INTRODUCTION

Gestational diabetes mellitus (GDM) is commonly defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Although the etiology and exact mechanisms of GDM are not fully understood, GDM is generally recognized as a clinical syndrome resulting from a combination of genetic and environmental risk factors (Mao et al., 2012). Melatonin receptor 1B (*MTNR1B*) is a novel candidate gene contributing to type 2 diabetes mellitus (T2DM) (Chambers et al., 2009). Currently, several large-scale genome-wide association analyses demonstrate that the common variants in or near the *MTNR1B* gene are robustly associated with T2DM (Kwak et al., 2012). Specifically, there are significant associations between increased risks of developing T2DM and GDM and single nucleotide polymorphism (SNP) rs10830963, a widely researched locus (Kim et al., 2011; Vlassi et al., 2012). In this study, we focused on SNP rs4753426 in the *MTNR1B* gene, which is located in the 5' promoter regions as well in the regulatory region. Previous studies showed that the DNA sequence around this locus may provide a binding site for a specific transcription factor, which is likely correlated with the expression level of melatonin (Gałecka et al., 2011). A previous study, which investigated the *MTNR1B* SNP rs4753426 (C/T) locus in the global human population, found that the C allele of SNP rs4753426 in *MTNR1B* was strongly associated with elevated fasting plasma glucose (FPG) levels (Ji et al., 2010). In the present study, we investigated the differences in genotype and allele frequencies of SNP rs4753426 between the GDM group and control group by DNA sequencing following polymerase chain reaction (PCR). We also evaluated the levels of FPG and fasting plasma insulin (FIN), and then calculated and compared homeostasis model assessment for insulin resistance (HOMA-IR) as well as homeostasis model assessment for  $\beta$ -cell function (HOMA- $\beta$ ) between different phenotypes in a GDM group to explore the association between GDM and SNP rs4753426.

## MATERIAL AND METHODS

### Subjects

All subjects included in this study were outpatients or inpatients from the Department of Obstetrics in Affiliated Hospitals of Qingdao University from June 2012 to July 2013, including 186 subjects with GDM as the treatment group [mean maternal age  $30.85 \pm 9.46$

years; mean gestational age  $25.65 \pm 3.84$  weeks; and mean body mass index (BMI)  $22.99 \pm 4.97$  kg/m<sup>2</sup>] and 330 subjects with normal pregnancy as the control group (mean maternal age  $29.96 \pm 8.52$  years; mean gestational age  $24.84 \pm 4.96$  weeks; and mean BMI  $23.47 \pm 5.48$  kg/m<sup>2</sup>). There were no significant differences between the 2 groups in terms of the maternal age, gestational age, parity, and BMI ( $P > 0.05$ ). In this study, GDM was diagnosed according to the criteria established by the American Diabetes Association (Galecka et al., 2011); GDM was diagnosed when at least one value was above normal ( $>5.1$ ,  $>10.0$ , and  $>8.5$  mM at 0, 60, and 120 min, respectively). All subjects were Han Chinese women residing in northern region of China. Women with multiple pregnancies and pathological conditions such as chronic hypertension, type 1 or 2 diabetes mellitus, infection, renal dysfunction, rheumatoid arthritis, and systemic lupus erythematosus were excluded from the study. This study was conducted in accordance with the Declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Affiliated Hospitals of Qingdao University. Written informed consent was obtained from all participants.

### Blood sampling and laboratory evaluations

All pregnant women were offered a 75-g oral glucose tolerance test after overnight fasting (6-8 h) and a 5-mL fasting venous blood sample was taken simultaneously to measure levels of FIN and FPG. FIN levels were assayed using a radioimmunoassay kit (Linco Research, Inc., St. Charles, MO, USA). FPG, total cholesterol, triglyceride, low-density lipoprotein, and high-density lipoprotein concentrations were measured using a 7600-2020 biochemical automatic analyzer (Hitachi, Ltd., Tokyo, Japan). Another 3-mL venous blood sample was drawn from all women to extract DNA, and then ethylenediaminetetraacetic acid-treated and stored at  $-20^{\circ}\text{C}$ . Height and weight were recorded for all women at the first prenatal office visit (gestational age 6-12 weeks) to calculate the early-pregnancy BMI ( $\text{BMI} = \text{weight}/\text{height}^2$ , kg/m<sup>2</sup>). The HOMA index was used to calculate insulin resistance ( $\text{HOMA-IR} = \text{FPG} \times \text{FIN} / 22.5$ ) and pancreatic  $\beta$ -cell function [ $\text{HOMA-}\beta = 20 \times \text{FIN} / (\text{FPG} - 3.5)$ ].

### SNP genotyping

For genotype analysis, genomic DNA was extracted from whole blood using the method described by Miller with some modifications, and stored at  $-20^{\circ}\text{C}$  after confirming that the concentration of genomic DNA was 10-20 mg/L. PCR was performed to amplify the target genes. The primers for promoter SNPs were designed using the PRIMER 5 software. Primer sequences were as follows: upstream primer, 5'-CTCAATACCCACCCTCAA-3'; downstream primer, 5'-CCAACAGAAGAATGGATAAG-3'. PCR was carried out using 0.2  $\mu\text{L}$  genomic DNA in a total volume of 20  $\mu\text{L}$ , with 35 cycles of initial denaturation at  $95^{\circ}\text{C}$  for 5 min, denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $57^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 30 s, with a final extension step at  $72^{\circ}\text{C}$  for 7 min. PCR products were evaluated by running 2  $\mu\text{L}$  on 1.5% agarose-TBE gels, using 250 base pairs as the standard molecular weight. PCR products and upstream primers were sequenced by the Shanghai Shensu Biological Technology Company (Shanghai, China). DNA sequence analysis was performed using the dideoxy chain-termination method with an ABI PRISM 3730XL sequencing machine (Applied Biosciences, Foster City, CA, USA).

## Statistical analysis

SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The normality of variables was analyzed using the Kolmogorov-Smirnov test. Quantitative data are reported as means  $\pm$  standard deviation, and a comparison of the 2 groups was obtained using the Student *t*-test. Genotype and allele frequencies are presented as percentages (%), which were compared using the chi-square test. Furthermore, distributions of genotype group representation in the GDM group and control group were tested to determine whether they were in Hardy-Weinberg equilibrium.

## RESULTS

### Clinical and laboratory characteristics

As shown in Table 1, the levels of FPG, FIN, and HOMA-IR were significantly higher in GDM patients than in controls ( $P < 0.05$ ), while the level of HOMA- $\beta$  was significantly lower ( $P < 0.05$ ).

**Table 1.** Clinical and laboratory characteristics of the studied pregnant women in two groups.

	Controls (N = 330)	GDM (N = 186)	<i>t</i>	P
Age (years)	29.96 $\pm$ 8.52	30.85 $\pm$ 9.46	1.094	0.27
GA (weeks)	24.84 $\pm$ 4.96	25.65 $\pm$ 3.84	1.760	0.06
BMI (kg/m <sup>2</sup> )	23.47 $\pm$ 5.48	22.99 $\pm$ 4.97	0.987	0.32
FPG (mM)	4.62 $\pm$ 1.87	6.43 $\pm$ 2.75	5.653	0.00
FIN (mU/L)	15.37 $\pm$ 11.26	19.96 $\pm$ 15.41	3.878	0.00
CH (mM)	4.86 $\pm$ 1.56	5.12 $\pm$ 2.46	1.467	0.14
LDL (mM)	2.21 $\pm$ 1.09	2.27 $\pm$ 1.36	0.573	0.58
HDL (mM)	1.55 $\pm$ 0.69	1.43 $\pm$ 0.81	1.779	0.08
TG (mM)	2.33 $\pm$ 1.31	2.37 $\pm$ 1.72	0.275	0.77
HOMA-IR	2.96 $\pm$ 2.79	5.95 $\pm$ 4.26	9.074	0.00
HOMA- $\beta$	274.46 $\pm$ 50.21	130.59 $\pm$ 65.35	18.391	0.00

GA = gestational age; BMI = body mass index; FPG = fasting plasma glucose; FIN = fasting plasma insulin; CH = total cholesterol; LDL = low-density lipoprotein; HDL = high-density lipoprotein; TG = triglyceride; HOMA-IR = homeostasis model assessment for insulin resistance; HOMA- $\beta$  = homeostasis model assessment for  $\beta$ -cell function.

### Distributions of genotype and allele frequencies of SNP rs4753426

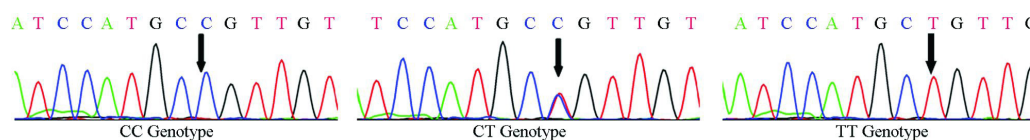
The distributions of genotype and allele frequencies regarding SNP rs4753426 as well as statistical comparisons are reported in Table 2. The results of DNA sequencing using PCR amplification products revealed 3 genotypes at the rs4753426 locus, including the CC, CT, and TT genotypes (Figure 1). This study applied the case-control method. The control group ( $P = 0.939$ ) was in Hardy-Weinberg equilibrium. Cases in the GDM group were from selection ( $P = 0.018$ ) and were not in Hardy-Weinberg equilibrium.

The frequencies of the CC, CT, and TT genotypes were 70.97% (132/186), 22.58% (42/186), and 6.45% (12/186), respectively, in women with GDM, and were 53.03% (175/330), 39.70% (131/330), and 7.27% (24 of 330), respectively, in healthy controls. The frequencies of the 3 genotypes differed significantly between the GDM group and the control group ( $P < 0.05$ ). The frequency of allele C of rs4753426 was higher in the GDM group than in the normal group [82.26% (306/372) vs 72.88% (481/660)], whereas the frequency of allele T was

lower [17.74% (66/372) vs 27.12% (179/660)]. Significant differences were observed between the C and T allele frequencies in the GDM group compared to the control group (Table 2).

**Table 2.** Comparison of the genotype and allele frequencies of SNP rs4753426 in *MTNR1B* gene between the GDM and control groups.

	N	Genotype frequencies [N (%)]			Allele frequencies [N (%)]	
		CC	CT	TT	C	T
GDM	186	132 (70.97%)	42 (22.58%)	12 (6.45%)	306 (82.26%)	66 (17.74%)
Control	330	175 (53.03%)	131 (39.70%)	24 (7.27%)	481 (72.88%)	179 (27.12%)
$\chi^2$		10.327			11.560	
P		0.00			0.00	



**Figure 1.** DNA direct sequencing results of the *MTNR1B* rs4753426 SNP.

### Levels of FPG, FIN, HOMA-IR, HOMA- $\beta$ , and BMI in different genotypes in the GDM group

In the GDM group, women with the CC and CT genotype showed significantly higher FPG levels ( $P < 0.05$ ), but lower HOMA- $\beta$  levels ( $P < 0.05$ ) compared with women with the TT genotype. There were no significant differences in regards to blood lipid, BMI, FIN, and HOMA-IR levels between the CC, CT genotypes and the TT genotype ( $P > 0.05$ ) (Table 3).

**Table 3.** Comparison of the HOMA-IR and HOMA- $\beta$  in women with GDM according to the different genotypes of the SNP rs4753426 in the *MTNR1B* gene.

	CC+CT (N = 174)	TT (N = 12)	<i>t</i>	P
FPG (mM)	6.768 $\pm$ 2.195	5.396 $\pm$ 2.779	2.057	0.041
FIN (mU/L)	18.593 $\pm$ 10.171	17.872 $\pm$ 9.519	0.456	0.511
BMI (kg/m <sup>2</sup> )	23.519 $\pm$ 5.933	22.738 $\pm$ 4.561	0.396	0.656
TC (mM)	5.098 $\pm$ 2.562	4.983 $\pm$ 2.424	0.151	0.880
LDL (mM)	2.245 $\pm$ 1.319	2.268 $\pm$ 1.298	0.253	0.756
HDL (mM)	1.443 $\pm$ 0.839	1.502 $\pm$ 0.827	0.449	0.647
TG (mM)	2.457 $\pm$ 1.665	2.319 $\pm$ 1.703	0.719	0.325
HOMA-IR	6.476 $\pm$ 4.406	5.641 $\pm$ 3.255	0.643	0.521
HOMA- $\beta$	124.640 $\pm$ 53.212	169.319 $\pm$ 47.490	2.830	0.005

## DISCUSSION

### Association between the *MTNR1B* gene and GDM risk

Recent epidemiological studies have demonstrated that women with GDM are susceptible to T2DM, possibly because GDM and T2DM share similar pathophysiology, includ-

ing insulin resistance caused by impaired pancreatic  $\beta$ -cell function (Yang, 2012). In addition, SNPs of many genes have been implicated in the pathogenesis of GDM and T2DM (Robitaille and Grant, 2008). *MTNR1B* is closely related to FPG levels and pancreatic  $\beta$ -cell function (Tam et al., 2010), and the genetic pathogenesis contributing to T2DM and GDM have been widely examined. *MTNR1B* is located on human chromosome 11 q21-q22, composed of 2 exons and 1 intron, and encodes the MT2 protein, which is a 362-amino acid melatonin receptor. Numerous studies reported that *MTNR1B* is mainly expressed in the retina and brain as well as in pancreatic  $\beta$ -cells and regulates circadian rhythm and blood glucose homeostasis by inducing regulation of insulin release by altering the melatonin signaling pathway. Thus, in the melatonin signaling pathway, abnormal variants of *MTNR1B* may contribute to the pathogenesis of GDM (Müssig et al., 2010).

To date, the human genome project has revealed that variants in the *MTNR1B* gene, such as SNPs rs10830963, rs1387153, and rs4753426, increase the future risk of developing T2DM. Moreover, a number of studies have focused on SNP rs10830963 and found significant associations between the CG and GG genotypes of this locus as well as FPG levels and the risk of developing T2DM (Vlassi et al., 2012; Stuebe et al., 2014). The risks of T2DM and GDM conferred by the G allele of SNP rs10830963 were also reported previously in China (Mao et al. 2012; Li et al., 2013). However, the association between the SNP rs4753426 and GDM has not been examined.

SNP rs4753426 is located in the upstream regulatory region of the *MTNR1B* gene. Qiu et al. (2007) detected a potential transcription factor of the SNP rs4753426 DNA sequence using the TFSEARCH software and found a nearby transcription factor-binding site for v-Myb. Furthermore, SNP rs4753426 alleles were found in the same study to result in variation of *MTNR1B* expression level (Qiu et al., 2007). The C allele was found to act as a mutant gene, and a close link between CC and CT genotype and risks of T2DM were observed in a dominant model (Dietrich et al., 2011).

In this study, we examined the association between 186 pregnant women and GDM and 330 healthy controls. Our results showed that the CC, CT, and TT genotype frequencies in the GDM group were 70.97, 22.58, and 6.45%, respectively, whereas in the control group these values were 53.03, 39.70, and 7.27%, respectively. Comparison of genotype frequencies in the 2 groups revealed a significant difference. The frequency of allele C in the GDM group was 82.26%, which was significantly higher than that in the control group with 72.88%; the C allele of SNP rs4753426 was increased markedly in women with GDM, indicating that the C allele may contribute to GDM risk.

### Effects of SNPs in *MTNR1B* rs4753426 on GDM risk

The underlying pathophysiology of GDM involves insufficient pancreatic  $\beta$ -cell function and insulin resistance during pregnancy. A large-scale study including 1578 non-diabetic subjects, reported by Staiger et al. (2008), indicated that the C allele of SNP rs4753426 in *MTNR1B* was associated with FPG levels and lower insulin levels at 30 min during the oral glucose tolerance test. The purpose of this study was to detect 5 *MTNR1B* gene loci, including rs4753426, after oral glucose tolerance test or intravenous glucose tolerance test in the subjects. The same study demonstrated a significant association between the C allele and increased FPG levels as well as decreased HOMA- $\beta$ , but the impact on HOMA-IR remained unknown (Staiger et al., 2008). In our study, we investigated the difference between the CC or CT genotype and the TT genotype in the GDM group and found that FPG levels increased



significantly while HOMA- $\beta$  levels decreased markedly in women with the C allele; this supports that the C allele is related to increased FPG levels and decreased HOMA- $\beta$ .

Consistent with the results of previous studies, our results indicate that women with GDM show insulin resistance and hyperinsulinemia, which arose from mildly impaired pancreatic  $\beta$ -cell function due to their relatively short-term GDM course. Hormone changes and cytokine release during pregnancy are thought to induce increased insulin secretion, which serves a compensatory function to achieve blood glucose homeostasis. Unfortunately, these changes worsen insulin resistance in GDM pregnant women. With the development of insulin resistance, this may eventually lead to  $\beta$ -cell failure. Genome-wide association studies and Chinese research have highlighted the SNP rs10830963 locus and revealed variants in *MTNR1B* that play an essential role in the effect on fasting blood glucose as well as the increased risk of T2DM development (Rönn et al., 2009). Several studies have reported that the G allele of SNP rs10830963 is an important functional *MTNR1B* polymorphism, which is associated with hepatic insulin resistance (Sparsø et al., 2009). Nevertheless, the link between *MTNR1B* gene polymorphisms and insulin resistance remained unclear. Therefore, in the present study, we performed preliminary observations of GDM women carrying the C allele of SNP rs4753426. However, no significant difference was observed between pregnant women with the CC or CT genotype and the TT genotype in FIN levels and BMI. HOMA-IR levels in the CC or CT genotype pregnant women were slightly higher than that in TT genotype women, but the difference was not statistically significant.

In conclusion, the SNP rs4753426 in *MTNR1B* gene may be a susceptibility gene locus for GDM, and the C allele may contribute to increased FPG level and reduced pancreatic  $\beta$ -cell function. The frequency of the C allele in rs4753426 loci was high in pregnant women with GDM. Pregnant women carrying the C allele may have a higher risk of GDM development. In addition, the C allele may lead to elevated FPG level and islet  $\beta$ -cell dysfunction in pregnant women with GDM.

Although the *MTNR1B* gene is known to be strongly associated with T2DM development, many of its SNP sites appear to be associated with genetic susceptibility to T2DM. Thus, additional studies are necessary to determine the functions of SNPs in GDM pathogenesis.

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