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Association Between a Melatonin Receptor 1B Genetic Polymorphism and Its Protein Expression in Gestational Diabetes Mellitus

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Abstract

Aims: This study was conducted to investigate the relationship between a genetic polymorphism and the expression of melatonin receptor 1B (MTNR1B) in the placenta of Han Chinese women with gestational diabetes mellitus (GDM). **Methods:** In this study, 215 patients with GDM and 243 healthy controls were genotyped using direct sequencing for the MTNR1B single-nucleotide polymorphism rs10830963. The expression of MTNR1B in placenta was detected by immunohistochemistry and Western blotting. The association of rs10830963 with the expression of MTNR1B, plasma glucose, and insulin levels as well as blood lipid levels was investigated. **Results:** The genotype and allele frequencies of rs10830963 were significantly different between women with GDM and controls ($P < .05$). Fasting blood glucose, fasting insulin, and homeostasis model assessment for insulin resistance in women with GDM with the GG and GC genotypes were significantly higher than those with the CC genotype ($P < .05$). The expression level of MTNR1B in placenta was significantly higher in the GDM group than in the control group ($P < .05$). The expression of MTNR1B was significantly higher in all participants with the GG and GC genotypes (1.31 [0.74]) than in pregnant women with the CC genotype (0.92 [0.52], $P < .05$). **Conclusions:** The genetic polymorphism rs10830963 in MTNR1B and its protein expression levels in placenta are associated with an increased risk of developing GDM. Furthermore, rs10830963 may tag a molecular mechanism leading to insulin resistance in Han Chinese women with GDM.

Keywords

polymorphisms, single nucleotide, diabetes, gestational, insulin resistance, MTNR1B

Introduction

Gestational diabetes mellitus (GDM) is a common obstetric complication and is defined as glucose intolerance first diagnosed during pregnancy.[1] Approximately 5% to 10% of Asian pregnant women have GDM, and the prevalence of GDM is continually rising in China.[2] Gestational diabetes mellitus is associated with short- and long-term complications for both mothers and offspring.[3] The pathogenesis of GDM is not clear but is generally recognized as a combination of genetic and environmental risk factors.[4] As GDM and type 2 diabetes mellitus (T2DM) share similarities in their pathogenesis with respect to impaired insulin secretion and increased insulin resistance,[5] it is possible that GDM is a multigenic disease related to T2DM.

Melatonin receptor 1B (MTNR1B), which belongs to a class of G-protein-coupled receptors, has recently attracted considerable attention as a novel candidate gene contributing to T2DM.[6] *MTNR1B* has been reported to be associated with T2DM in various ethnic groups.[7-9] Several large-scale genome-wide association studies (GWAS) have demonstrated that variation in the *MTNR1B* gene is associated with increased fasting plasma glucose (FPG).[10] Further associations between common *MTNR1B* variants with impaired insulin release and insulin resistance have been reported in recent studies.[9,11] To date, human genetic studies have revealed that many common variants (such as rs10830963, rs4753426, rs1387153, and rs1374645) in *MTNR1B* have independent effects on increased FPG and T2DM risk. Specifically, rs10830963, located in an unconserved sequence across species on human chromosome 11q21-22, might represent a key *MTNR1B* variant associated with T2DM risk. The effect of the G allele of this single-nucleotide polymorphism (SNP) on FPG has been observed in T2DM.[7] Further studies have confirmed significant

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associations of rs10830963 with both fasting plasma insulin and insulin resistance.[12,13] These findings suggest that rs10830963 may be a key *MTNR1B* variant associated with metabolic levels of blood glucose.

Melatonin easily crosses the placental barrier, which is involved in placental function and fetal development.[14] The human placenta is one of the main target organs of melatonin, and it expresses both melatonin receptors and produces melatonin itself.[15,16] Furthermore, the placenta is exposed to the intrauterine environment in common with the fetus and it is affected by maternal metabolic derangements.[17] Studies have demonstrated that the abnormal expression of placental MTNR1B is related to pregnancy complications.[18] Accordingly, placental MTNR1B levels may be altered in pregnancy complicated by GDM. However, until now, there has been no study on the relationship between *MTNR1B* SNPs and gene expression in the placenta with the development of GDM.

In the current study, we studied the relationship between a polymorphism in *MTNR1B*, the expression of this gene in the placenta, and the pathogenesis of GDM. Our results provide additional insights into the mechanisms underlying the association between *MTNR1B* genetic variants and the risk of developing GDM.

Participants and Methods

Participants

The study was approved by The Affiliated Hospital of Qingdao University Ethics Committee, and informed consent was obtained from the patients. Only women with a singleton pregnancy who delivered by planned cesarean delivery at term were enrolled into the study. None of the pregnant woman had a history of chronic medical conditions or impaired glucose tolerance before pregnancy. The sample consisted of 458 pregnant women. Among them, 215 women were diagnosed with GDM and 243 were in the normal pregnancy group. All pregnant women underwent a 75-g oral glucose tolerance test at weeks 24 to 28 of pregnancy, according to the International Association of Diabetes and Pregnancy Study Groups (capillary whole blood; fasting glucose >5.1 mmol/L, 1 hour >10.0 mmol/L, 2 hours >8.5 mmol/L; GDM was defined as one or more blood glucose values above these limits). The blood samples were taken from the peripheral blood before cesarean delivery. Two tissue samples of 2 cm × 2 cm × 2 cm were taken from the same central part of the placenta after delivery, and 1 full-thickness placental tissue was fixed in 4% buffered formalin and embedded in paraffin. Villous tissue isolated from the second placental tissue was immediately frozen in liquid nitrogen and stored at −80°C for protein extraction after being flushed with saline.

All patients in the GDM group were treated with a program of diet and exercise first. Twenty percent of patient's blood glucose was poorly controlled according to their blood glucose, Glycosylated Hemoglobin values.

Table 1. Clinical and Biochemical Parameters of 2 Groups.[a]

Characteristic	GDM Group	Controls	t/χ^2 Value	*P* Value
Number	215	243		
Age, years	31.41 (3.07)	30.85 (3.28)	1.959	.051[b]
Gestational age, weeks	38.91 (1.41)	39.12 (1.29)	1.664	.096[b]
Pre-BMI	25.32 (4.47)	24.85 (4.65)	1.099	.272[b]
Gravidity	2.3 (1.4)	2.0 (1.3)	1.584	.113[b]
Parity	1.5 (0.8)	1.6 (0.9)	1.249	.212[b]
Gestational weight gain, kg	14.8 (4.1)	14.2 (3.4)	1.767	.077[b]
FPG, mmol/L	5.52 (1.13)	4.71 (0.62)	9.652	<.0001[b,c]
FIN, mIU/L	15.23 (4.56)	11.71 (4.30)	8.498	<.0001[b,c]
HOMA-IR	3.75 (1.64)	2.88 (1.51)	5.909	<.0001[b,c]
CH, mmol/L	5.01 (2.16)	4.88 (1.67)	0.724	.468[b]
LDL, mmol/L	2.17 (1.36)	2.11 (1.08)	0.525	.599[b]
HDL, mmol/L	1.45 (0.58)	1.55 (0.64)	1.743	.081[b]
TG, mmol/L	2.27 (1.23)	2.31 (1.32)	0.334	.738[b]
Birth weight (g)	3461.25 (532)	3382.91 (525)	1.523	.128[b]
Apgar score	9.79 (0.22)	9.85 (0.17)	0.547	.584[b]
Percentage of fetal gender (male)	59.06	51.02	2.978	.084[d]

Abbreviations: BMI, body mass index; CH, total cholesterol; FIN, fasting insulin; FPG, fasting plasma glucose; GDM, gestational diabetes mellitus; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; LDL, low-density lipoprotein; SD, standard deviation; TG, triglycerides.
[a]Values are mean (SD) or percentage.
[b]Using *t* test.
[c]$P < .05$.
[d]Using χ^2 test.

The patients were treated with insulin at the Department of Incretion of The Affiliated Hospital of Qingdao University. Clinical and laboratory characteristics of the study population are summarized in Table 1.

Biochemical Measurements

Glucose, total cholesterol (CH), triglycerides (TGs), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) concentrations were measured enzymatically on an automated biochemical analyzer (Beckman Coulter Inc, Brea, California). Fasting insulin (FIN) was determined by radioimmunoassay. Fasting plasma glucose was determined by glucose oxidase method. The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated according to the following equation[19]: FPG (mmol/L) × FIN (mU/L)/22.5.

Genotyping of rs10830963

rs10830963 was genotyped by polymerase chain reaction (PCR) restriction fragment length polymorphism analyses. The PCR primer sequence was CTG GCT CCA TAG GCA CAG CCA. The reverse primer sequence was GCA AGG AAC AGG GGC CAC AG. The PCR was performed as follows: 5 minutes preincubation at 94°C to activate the Taq DNA polymerase,

Table 2. Genotype and Allele Frequencies of SNP rs10830963 in MTNR1B in Patients With GDM and Healthy Woman.[a]

	Genotype, n (%)			Allele, n (%)	
SNP	GG	GC	CC	G	C
GDM	59 (27.4)	102 (47.4)	54 (25.1)	220 (51.2)	210 (48.8)
Control	35 (14.40)	121 (51.03)	87 (35.80)	191 (39.3)	295 (60.7)
χ^2 value	13.810		12.978		
P value	.001[b]		<.001[b]		
OR value	1.663 (95% CI, 1.109-2.492)[c]		1.618 (95% CI, 1.245-2.104)		

Abbreviations: C, control group; CI, confidence interval; GDM, gestational diabetes mellitus; MTNR1B, melatonin receptor 1B; OR, odds ratio; SNP, single-nucleotide polymorphism.
[a]Using χ^2 test.
[b]$P < .05$.
[c]GG + GC versus CC.

35 cycles of PCR consisting of denaturation at 95°C for 30 seconds, and then annealing at 56°C for 45 seconds and extension at 72°C for 30 seconds, followed by a final extension step at 72°C for 5 minutes. The products of PCR were visualized by electrophoresis on 2% agarose gel and stored at 4°C. The PCR products were sequenced by the Shanghai Shensu Biological Technology company.

Localization of MTNR1B Expression

Placental samples (n = 20) were fixed in 4% paraformaldehyde and embedded in paraffin. The sections (5 μm) were dewaxed in xylol (20 minutes) and rehydrated prior to antigen retrieval in a pressure cooker (5 minutes) using sodium citrate buffer. The slides were cooled to room temperature and blocked with hydrogen peroxide (0.3%) for 1 hour at room temperature. Followed by, they were incubated with the primary antibody (1:100, anti-MTNR1B antibody; Abcam, Cambridge, UK) for overnight at room temperature and then with anti-rabbit conjugated secondary antibody (1:200; Sigma Aldrich, Saint Louis, USA) for 1 hour at room temperature. The slides were counterstained with hematoxylin and dehydrated in ethanol before mounting. For the negative control, the primary antibody was omitted. Images were observed at ×10 and ×40 with an Olympus microscope (Tokyo, Japan). Two examiners reviewed these images until consensus was reached.

Detection of MTNR1B Expression Level

Proteins were isolated from villous tissue homogenates using radioimmunoprecipitation assay buffer and the bicinchoninic acid protein assay kit was used to quantify them. Protein samples (30 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, which was followed by transfer onto polyvinyl difluoride membranes. The membranes were probed with primary antibody (Amyjet Scientific Inc, Cambridge, UK), at a 1:1000 dilution (5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 [TBST]), overnight at 4°C with gentle shaking, followed by washing with TBST 4 times (10 minutes each time). Washed membranes were then incubated with secondary goat anti-rabbit antibody (Novus Biologicals) at a 1:5000 dilution in blocking buffer for 1 hour at room temperature with gentle shaking. The washed membranes were then incubated in Pierce-enhanced chemiluminescence Western blotting substrate (Thermo Scientific) and were visualized using Geldoc (Bio-Rad Laboratories, California, USA). Densitometry analysis of relative protein levels was performed using the ImageJ version 13.0.

Statistical Analysis

In this study, data are presented as mean (standard deviation). SPSS version 21.0 was used for statistical analysis. We determined the differences in allele and genotype frequencies between the control and GDM groups using χ^2 tests. The differences in sample data were assessed using a *t* test. We determined that this case–control study was sufficiently powered ($1 - \beta > .8$) to detect effect sizes as small as 20%. Data were considered statistically significant at *P* value <.05. Graphs were made using Prism 5 (GraphPad, San Diego, California, USA).

Results

The SNP of rs10830963 and Key Phenotypic Traits of Pregnant Women

Gestational diabetes mellitus was strongly associated with higher FPG and FIN and HOMA-IR (all $P < .001$). We observed no significant differences in pre–body mass index (BMI), age, gravidity, parity, gestational age at delivery, birth weight, Apgar score, fetal gender, CH, LDL, HDL, or TG between pregnant women with GDM and the healthy control group (*P*s > .05).

Table 2 presents the distribution of rs10830963 between the 2 groups. As shown, no significant deviation of genotype frequencies from Hardy-Weinberg equilibrium was detected in the GDM and control groups for the SNP ($P > .05$). The frequencies of the 3 genotypes differed significantly between the GDM group and the healthy group ($P = .001$). Furthermore, the frequency of the G allele of rs10830963 was higher among

Table 3. Relationships Between SNP rs10830963 in MTNR1B and Key Phenotypic Traits in Patients With GDM.

Genotype	GG + GC (n = 161)	CC (n = 54)	*t*	*P*
Age, years	32.16 (4.35)	31.56 (4.57)	0.866	.387
Gestational age, weeks	38.74 (1.68)	39.06 (1.57)	1.230	.219
Pre-BMI	25.65 (4.72)	24.71 (4.11)	1.306	.192
FPG, mmol/L	6.01 (1.27)	5.12 (1.13)	4.576	<.001[a]
FIN, mIU/L	15.28 (4.62)	14.75 (5.19)	0.706	.480
HOMA-IR	3.93 (1.80)	3.01 (1.49)	3.385	<.001[a]
CH, mmol/L	5.10 (2.41)	4.99 (2.32)	0.292	.769
LDL, mmol/L	2.28 (1.24)	2.16 (1.38)	0.597	.550
HDL, mmol/L	1.42 (0.57)	1.56 (0.70)	1.471	.142
TG, mmol/L	2.34 (1.25)	2.16 (1.17)	0.930	.353

Abbreviations: BMI, body mass index; CH, total cholesterol; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; LDL, low-density lipoprotein; FIN, fasting insulin; FPG, fasting plasma glucose; MTNR1B, melatonin receptor 1B; SNP, single-nucleotide polymorphism; TG, triglycerides.
[a]$P < .05$.

pregnant women with GDM than the control group ($P = .002$, odds ratio = 1.522 [95% confidence interval, 1.169-1.981]).

Table 3 presents the levels of FPG, FIN, HOMA-IR, and BMI in the different genotypes in the GDM group. In the GDM group, the pregnant women with the GG and GC genotypes had higher FPG and HOMA-IR compared with those with the CC genotype ($P < .05$). Moreover, there were no statistically significant differences in the clinical and biochemical characteristics of pregnant women of the control group with different genotypes ($P > .05$).

Localization and Expression of MTNR1B in Placental Tissues

The hematoxylin and eosin immunostaining for MTNR1B is shown in Figure 1. Immunohistochemical analysis showed that MTNR1B was specifically detected in villous cytotrophoblast, syncytiotrophoblast, and fetal capillary.

Figure 2 shows a representative immunoblot of the MTNR1B protein in the GDM and control villous tissue. Semiquantitative Western blot analysis demonstrated that the protein had increased expression in the GDM group when compared with the control group (1.04 [0.31] vs 0.82 [0.37], $P < .05$; Figure 2).

The SNP of rs10830963 and Expression Level of MTNR1B

The expression level of MTNR1B in villous tissue in all participants was compared among the participants with different MTNR1B genotypes. The expression level of MTNR1B in villous tissue with GG genotype was the highest (0.97 [0.28], n = 94), followed by intermediate expression level of MTNR1B with GC genotype (0.92 [0.33], n = 223), and the expression level of MTNR1B in villous tissue with CC genotype was lowest (0.86 [0.34], n = 141). The expression level of MTNR1B in villous tissue differed significantly between the 3 genotypes ($F = 3.40$, $P < .05$). Furthermore, all participants with GG and GC genotypes had higher expression level of MTNR1B (0.94 [0.32], n = 317) compared with the participants with the CC genotype (0.86 [0.34], $P < .05$; Figure 3).

Discussion

Association Between the rs10830963 Variant in MTNR1B and GDM Risk

Melatonin is a circulating hormone secreted primarily from the endocrine cells of the pineal gland.[20] The function of melatonin is mainly mediated by MTNR1A and MTNR1B. The *MTNR1B* gene is located on human chromosome 11q21-22 and is composed of 2 exons and 1 intron. It has become clear that *MTNR1B*, while associated with T2DM, is involved in insulin secretion.[21] The present study shows an association between rs10830963 and GDM. The GG and GC genotypes were more common among pregnant women with GDM than in the healthy group ($P < .05$). Furthermore, the G allele was found to be more frequent in the GDM group than in the control group, which supports a potential association of this polymorphism with an increased risk of developing GDM. A study in the Greek population revealed that the GG genotype and the G allele of rs10830963 were significantly associated with an increased risk for GDM.[22] Meanwhile, studies in Mexican Americans concluded that rs10830963 is strongly associated with insulin secretion, contributing to risk of GDM.[23] All of the above studies support our conclusions. In addition, our study indicated that rs10830963 affects FPG and insulin resistance in women with GDM. Women with GDM who carried the genotypes GG and GC of rs10830963 displayed significantly increased FPG and HOMA-IR compared with women carrying the CC genotype. Consistent with our findings, numerous studies in ethnically diverse populations have shown that the G allele of rs10830963 was associated with FPG.[24-26] However, no marked differences were observed between the glucose and insulin levels of healthy women with different SNP genotypes. A variant in the MTNR1B receptor was associated with an increase in fasting glucose and predicted future GDM, most likely through impairment of insulin secretion from pancreatic β-cell function. The *MTNR1B* gene can directly inhibit cyclic guanosine monophosphate formation and affect insulin secretion, contributing to elevated glucose level.[19] Therefore, it may affect the activity of MTNR1B to influence melatonin signaling, ultimately playing a role in glucose homeostasis and insulin release in the GDM group. This indicates that rs10830963 may be associated with insulin resistance and contribute to the pathogenesis of GDM.

Effects of Abnormal Expression of MTNR1B in the Placenta on GDM Risk

The placenta is an important organ responsible for pregnancy well-being, which synthesizes and releases numerous

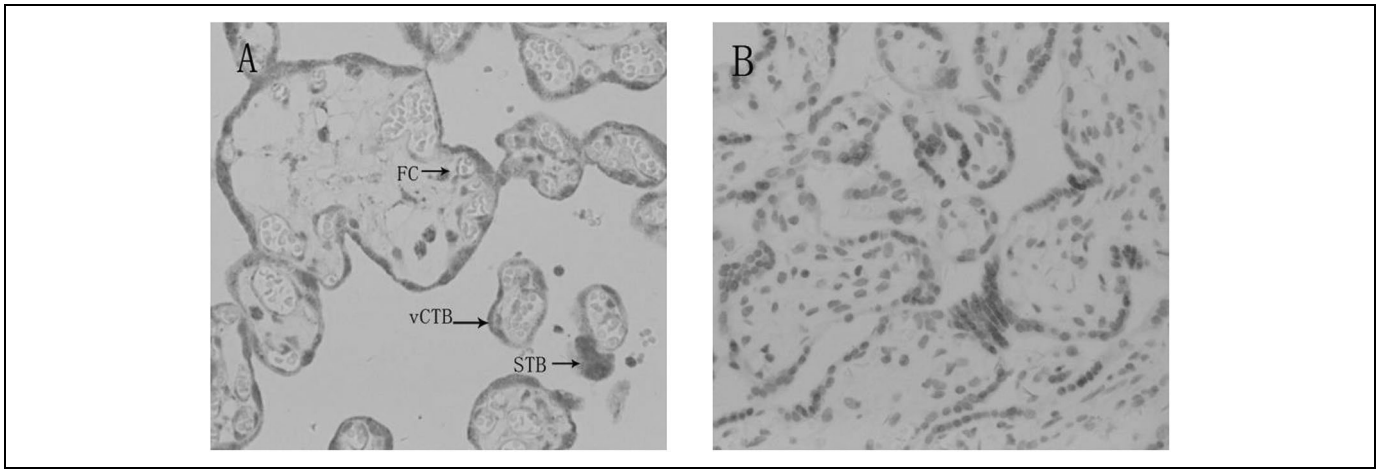

Figure 1. Immunohistochemical analysis of MTNR1B expression in human term placental tissue. A, A negative control was incubated without primary antibodies. B, Pictures were taken at a magnification of ×200. Staining was performed in 10 GDM and 10 control placenta. Pictures are representative of at least 3 independent experiments from 3 different placentas. FC, fetal capillaries; GDM, gestational diabetes mellitus; MTNR1B, melatonin receptor 1B; STB, syncytiotrophoblast; vCTB, villous cytotrophoblast.

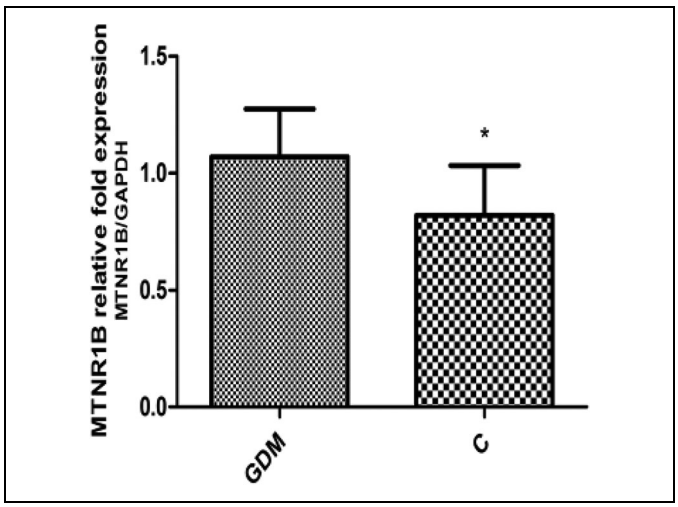

Figure 2. Expression of MTNR1B in villous tissue from healthy woman and GDM pregnancies. *$P < .05$. GAPDH was used to normalize protein expression. C indicates control group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDM, gestational diabetes mellitus; MTNR1B, melatonin receptor 1B.

hormones and cytokines. Hormonal changes and cytokine release during pregnancy are thought to induce increases in insulin secretion, which is used as a compensatory mechanism to achieve blood glucose homeostasis. However, these changes worsen insulin resistance in pregnant women with GDM.[27] Any alteration in placental development or function may contribute to insulin resistance; therefore, the placenta is a good model to study changes in metabolic programming in women with GDM.

Our study found that women with GDM showed higher expression levels of MTNR1B in the placenta compared with those of healthy women, indicating that placental melatonin might be implicated in the pathogenesis of GDM. Supporting our results, Elmar et al reported that patients with T2DM showed upregulated expression of MTNR1B compared with the control group.[28] Peschke et al also reported that patients with T2DM had higher mRNA transcript levels of *MTNR1B* compared to the control group.[29] The potential mechanism by which patients with T2DM show increased expression of MTNR1B cannot presently be explained. Higher plasma glucose levels lead to transcriptional upregulation of other genes, which may be one of the reasons in this case as well.[30] Stumpf et al reported that MTNR1B plays a role in mediating the insulin-inhibiting effect of melatonin.[31] The insulin-inhibiting effect could be partly reversed by an MTNR1B-specific antagonist. This indicates that MTNR1B is an important component of the melatonin-signaling pathway, and abnormal expression of MTNR1B in the placenta might trigger aberrant changes in melatonin that leads to impaired insulin function, ultimately contributing to the development of GDM.

Association Between rs10860963 and Expression Level of MTNR1B

Results of previous studies that provided evidence for this SNP showed that it may have an influence on transcription and expression, which results in variation of MTNR1B expression level.[32] Our study revealed that G allele of rs10830963 occurs more frequently in the GDM group than in the control group, supporting a potential association between this polymorphism and the development of GDM. Additionally, women with GDM showed higher expression of MTNR1B in the placenta compared with healthy women. Further exploration revealed that individuals carrying the G allele showed higher expression of MTNR1B in the placenta as compared with carriers of the C allele. These findings suggest that rs10830963 may be associated with the

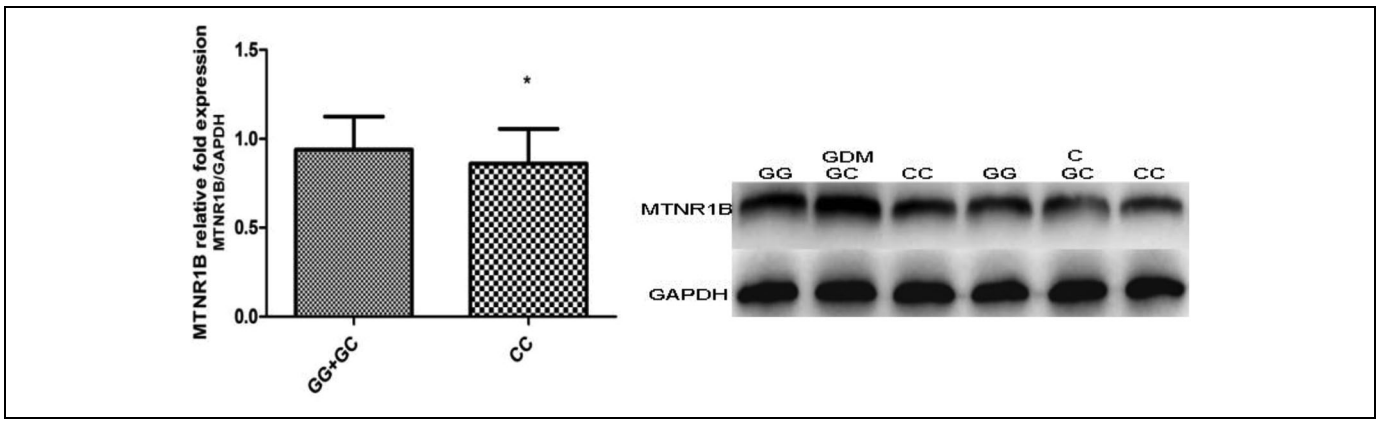

Figure 3. Expressions of MTNR1B in villous tissue with difference genotypes. Representative immunoblots of the 37-kDa housekeeping protein GAPDH was used to illustrate the protein load of all samples. The 40-kDa immunoreactive MTNR1B protein was observed in the GDM group and control samples. *$P < .05$. GAPDH was used to normalize protein expression. C indicates control group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDM, gestational diabetes mellitus; MTNR1B, melatonin receptor 1B.

expression of MTNR1B, which may be elevated by the effect of the G allele of rs10830963. This SNP is localized in the 5′ promoter region of the *MTNR1B* locus and may thus have an influence on transcription and expression.[33] Activation of MTNR1B by melatonin would block activation of intracellular second messengers (cyclic adenosine monophosphate [cAMP] or cyclic diguanylate).[34] Thus, cellular cAMP levels may be lower when MTNR1B is upregulated in the placenta. The SNP of MTNR1B may via both protein kinase A-dependent and protein kinase A-independent mechanisms plays a role in insulin secretion. Hence, the potentiating effects from the MTNR1B would be diminished, with subsequent detrimental effects on insulin secretion. Lyssenko et al reported that the T2DM risk allele (G) of rs10830963, identified in GWAS, was associated with increased expression of MTNR1B in human islets.[34] However, Bonnefond and colleagues suggested that MTNR1B variants may result in impaired MTNR1B function, rather than increased MTNR1B expression, leading to impaired insulin secretion and contributing to T2DM.[35] The association between rs10830963 and MTNR1B expression levels requires further examination.

In conclusion, our study suggests that the rs10830963 variant in *MTNR1B* and abnormal expression level of MTNR1B in the villous tissue are associated with an increased risk of developing GDM among Han Chinese women. We showed that the common genetic variant of rs10830963 is linked to fasting glucose levels and insulin resistance. Here, we also replicated a significant association of an *MTNR1B* variant with increased expression levels of MTNR1B. Further studies are required in order to investigate the function of this *MTNR1B* variant and abnormal expression in GDM pathogenesis.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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