Material and methods

Participants

This study was carried out under approvement of Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology Ethics Committee (No. TJ-IRB20170506), and performed under the Declaration of Obstetrics Department. And informed consent of all participants included in this study were obtained. All participants included were recruited from obstetrics inpatient department in Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei, China from October 2017 to December 2019. This study consisted of 856 pregnant women, including 320 pregnant women with abnormal 75 g oral glucose tolerance test (OGTT) (gestational diabetes mellitus (GDM) group) and 536 normal pregnancy women (normal pregnancy (NP) group). Potential participants were evaluated independently by two researchers, and then participant lists were compared and inconsistency records were discussed and revalidated. Clinical and laboratory parameters of all pregnant women were obtained from electronic medical records and presented in table 1.

OGTT was used to diagnose GDM between 24 and 28 weeks of gestation, and the diagnostic criteria was based on the International Association of Diabetes and Pregnancy Study Groups (IADPSG). The recommended GDM diagnosis are as follows: fasting plasma glucose (FPG)≥5.1 mmol/L, 1-hour plasma glucose≥10.0 mmol/L, 2-hour plasma glucose≥8.5 mmol/L. One or more values of plasma glucose that reach or exceed the critical value can be diagnosed as GDM. The inclusion criteria for GDM group was Chinese nationality and singleton pregnancy with a positive result of OGTT. And the inclusion criteria for NP group was Chinese nationality and singleton pregnancy with a negative result of OGTT. The exclusion criteria for GDM and NP groups were pregnant women with other pregnancy complications or comorbidities, such as pre-diabetes, hypertension, thyroid disease, and other metabolic diseases.

Blood and placental samples collection

Peripheral blood (5 mL) of each pregnant woman was collected in tubes containing ethylenediamine tetraacetic acid (EDTA). All blood samples were centrifuged at 800 r/min for 5 min, then the supernatant and pellet were collected in 1.5 mL centrifuge tubes separately and stored at -80°C for future genotype discrimination analysis. The placental tissues of each pregnant woman (17 cases for each group, collected from September to December 2019) were collected immediately

after placental expulsion. The tissue was cut into ~0.5 cm^3 pieces from four placental quadrants and washed by phosphate buffered saline. A tissue section from each quadrant was fixed with polyoxymethylene (4%), and the rest tissues were snap frozen using liquid nitrogen and kept at -80°C for future Melatonin receptor 1B (MT2) expression detection.

Genomic DNA extraction and genotype discrimination

Extraction protocol of genomic DNA samples has been mentioned in our previous study (Wei et al., 2019). Genomic DNA samples were genotyped using TaqMan-MGB probe quantitative polymerase chain reaction (quantitative PCR, qPCR) assays (Tsingke Biotechnology, China), conducted by Bio-rad CFX CONNECT Real-Time System (Bio-rad, USA). Each allelic discrimination qPCR reaction was carried out in a volume of 15 μL consisting 7.5 μL of 2 × T5 Fast qPCR Mix (Tsingke Biotechnology, China), 50 ng of genomic DNA and 0.75 μL of Probe & Primer Mix (Tsingke Biotechnology, China). The qPCR was carried out in a standard protocol: preincubation at 95°C for 1 min followed by 40 cycles of denaturation at 95°C for 10 sec and annealing at 60°C for 30 sec. Ten percent of the samples were randomly selected and re-genotyped for the accuracy of genotyping results. And the results were 100% concordant. The sequences for primer and TaqMan MGB probes used were shown in Table S1.

Table S1 Sequences for primer and TaqMan MGB probes used

Locus rs10830963	5′ to 3′ sequences	SNP change
Primer	Forward- CCCAGTGATGCTAAGAA	C/G
	Reverse- CTCAGTCAACAAGTTGGA	
TaqMan probe	FAM-ACACCATCTGCTATC-MGB	
	VIC-CACCATCTCCTATCC-MGB	

Cell culture and treatment

HTR-8/SVneo cell was gotten from Wuhan Servicebio Technology (Wuhan, China) and cultured in RPMI-1640 medium (Invitrogen, USA) containing 10% (v/v) fetal bovine serum (CellMax, China) at 37 °C in an incubator with an atmosphere of 5% CO_2. Increasing concentrations of melatonin (0.25, 0.5, and 1.0 mM) (HY-B0075, MedChem Express, USA) and corresponding control (Dimethyl sulfoxide, DMSO) were added to the medium respectively. The small interfering

RNAs (siRNA) against MTNR1B (si-MT2) and corresponding control (si-NC) were obtained from Guangzhou RiboBio (China). For the experiments, HTR-8/SVneo cells were adherently cultured in 12-well plates and reached 50～60% density before use. si-MT2 (target sequence CGACTTGCGGAGCTTTCTA, Guangzhou RiboBio, China). Opti-MEM™ Reduced Serum Medium (Thermo Fisher Scientific, USA) was used to dilute Lipofectamine 3000 reagent (Thermo Fisher Scientific, USA) and siRNA separately, and the mixture was incubated for 15 min before adding to the cell medium.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from placental tissues by TRIzol reagent (Takara, Japan) according to the manufacturer's protocol. In brief, 100 mg placental tissue was homogenized with 1.0 mL of TRIzol solution. Total RNA was selectively isolated away from proteins and DNA using traditional method based on liquid-phase separation. The quantity and quality of RNA were measured using the Nanodrop 2000 (Thermo, USA). 1.0 μg of total RNA was used for cDNA synthesis using HiScript II Q RT SuperMix for qPCR Kit (Vazyme, China). Gene expression was detected by RT-PCR using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China). Each RT-PCR reaction was carried out in a volume of 20 μL consisting 10 μL of ChamQ Universal SYBR qPCR Master Mix (Vazyme, China), 20 ng of cDNA and 0.2 μM of primers. The qPCR was carried out in a standard protocol: preincubation at 95°C for 30 sec followed by 40 cycles of denaturation at 95°C for 10 sec and annealing at 60°C for 30 sec. The reactions were performed in a Bio-rad CFX CONNECT Real-Time System (Bio-rad, USA). Relative expression of MTNR1B mRNA was analyzed using $2^{-\Delta\Delta Ct}$ method with ACTB as internal control. The sequences of primers for RT-PCR were shown in Table S2.

Table S2　Sequences of primers for RT-PCR

Gene	5′ to 3′ sequences
MTNR1B	Forward- TGCGACCCACGCATCTATTCC
	Reverse- AGGTAGCAGAAGGACACGACA
ACTB	Forward- CCTTCCTGGGCATGGAGTC
	Reverse- CCTTCCTGGGCATGGAGTC

Western blot analysis

Placental tissues and HTR-8/SVneo cells were lysed with RIPA buffer (P0013B, Beyotime, China) supplemented with protease inhibitors (G2008-1ML, Servicebio, China) and phosphatase inhibitors (G2007-1ML, Servicebio, China), and protein concentration were detected by a BCA Protein Assay Kit (P0012, Beyotime, China). 20 μg of each protein sample was electrophoresed on an 11% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (PVDF). The membranes were blocked in 2% nonfat milk at room temperature for 30 min, then incubated at 4°C for at least 12 h with corresponding primary antibodies: anti-MTNR1B (Melatonin Receptor 1B) rabbit polyclonal antibody (NLS932SS; 1:1000; Novus Biologicals, USA), anti-GLUT1 (Glucose transporter 1) rabbit monoclonal antibody (A11727; 1:1000; ABclonal, China), anti-GLUT3 rabbit monoclonal antibody (A4137; 1:1000; ABclonal, China), anti-GLUT4 rabbit polyclonal antibody (A7637; 1:1000; ABclonal, China), anti-PPARγ (Peroxisome proliferator-activated receptor gamma) rabbit polyclonal antibody (A16958; 1:1000; ABclonal, China), anti-β-actin mouse monoclonal antibody (M20011S; 1:1000; Abmart, China), anti-β-tubulin mouse monoclonal antibody (M30109S; 1:5000; Abmart, China). The next day, the membranes were incubated with secondary antibodies at room temperature for 1 h: HRP (horseradish peroxidase)-conjugated affinipure goat anti-rabbit IgG (SA00001-2; 1:10000; Proteintech, China) or HRP-conjugated affinipure goat anti-mouse IgG (SA00001-1; 1:10000; Proteintech, China), and detected using enhanced chemiluminescence (K-12045-D50, Advansta, USA) by G:BOX Chemi XRQ (Syngene, UK). The intensity of protein bands was analyzed and normalized to β-actin or β-tubulin by ImageJ.

Immunofluorescence

The placenta tissue fixed in polyoxymethylene (4%) was dehydrated for paraffin embedding. The section was first incubated with anti-MTNR1B antibody (Abcam, ab203346) in combination with anti-CK7 (Cytokeratin 7) antibody (YM3054, Immunoway, Beijing, China) at 4°C overnight. Then, the section was incubated with secondary antibody at 37°C for 1 h: donkey anti-mouse IgG (FITC) (GB22401; 1:100; Servicebio, China) in combination with donkey anti-rabbit IgG (Cy3) (GB21403; 1:100; Servicebio, China). Placental morphology was imaged using immunofluorescence microscope (Olympus BX53, Tokyo, Japan).

Glucose consumption

Glucose levels in HTR-8/SVneo cell culture medium were determined by glucose assay kit (Sigma-Aldrich, USA), based on the manufacturer's instructions. HTR8/SVneo cells culture supernatants were collected and centrifuged to remove cell debris. 50 uL of each supernatant sample and 100 uL of catalase assay buffer containing o-diphenylamine and catalase were added to each well of 96-well plate, and incubated at 37°C for 30 min. Reaction was stopped with 100 uL of 6 mol/L sulfuric acid and then the absorbance at 540 nm was measured by microplate reader (Biotek, USA). The glucose concentration of each supernatant sample was calculated according to the standard curve. And the glucose consumption was the difference of glucose content in the corresponding supernatant samples from the beginning to the end of cell treatment period normalized to corresponding protein amounts.

Statistical analysis

The continuous variables were presented as mean ± standard deviation (SD). SPSS 25.0 was utilized for statistical analyses, and GraphPad Prism 8.0 was utilized for drawing graphics. Comparisons between two independent groups were carried out by student's t-test or non-parametric test. Comparisons between multiple groups and treatments were performed using two-way ANOVA. Comparisons between multiple groups were performed through one-way ANOVA. Hardy-Weinberg equilibrium (HWE) of individual polymorphisms were performed using Chi-Square test. Comparisons the odd ratios, 95% confidence intervals and the correlative *P* values of risk alleles in three genotype models (dominant model, recessive model and additive model) were performed using logistic regression analysis (Khan et al., 2019). Comparisons of relatively metabolic parameters in three genotype models were performed using linear regressions analysis, adjusted for gestational age and body mass index (BMI). A *P* value with a two-side less than 0.05 was considered to be statistically significant.

References

Khan IA, Jahan P, Hasan Q, et al., 2019. Genetic confirmation of T2DM meta-analysis variants studied in gestational diabetes mellitus in an Indian population. *Diabetes Metab Syndr*, **13**(1):688-694. DOI: 10.1016/j.dsx.2018.11.035

Wei LJ, Zhou X, Zhu SL, et al., 2020. Genetic Variants of Potassium Voltage-gated Channel Subfamily J Member 11 in Gestational Diabetes Mellitus: A Case-control Study. Maternal-Fetal Medicine, 2(1):12-16.