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Formyl peptide receptor-2 is upregulated in the blood and placenta of patients with gestational diabetes mellitus

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Abstract

Aim: To investigate the expression of formyl peptide receptor 2 (FPR2) in maternal blood, umbilical blood, and placenta of patients with gestational diabetes mellitus (GDM), and to analyze the changes of other pro-inflammatory cytokines in blood, including interleukin 33 (IL-33), IL-1β, tumor necrosis factor alpha (TNF-α), and C-reactive protein (CRP), so as to reveal the pathogenesis of GDM.

Methods: FPR2, IL-33, IL-1β, T TNF-α, and CRP in maternal blood and umbilical cord blood of 50 pregnant women with GDM and 30 normal pregnant women were analyzed by ELISA method to explore the correlation between inflammatory factors and blood glucose. The expression of FPR2 in placental tissues was analyzed by PCR and immunohistochemistry.

Results: The expression of FPR2 in maternal blood of gestational diabetes patients was significantly higher than that of normal pregnant women, and other inflammatory factors IL-33 and IL-1β in maternal blood were also significantly increased. The expression of FPR2 in umbilical cord blood of gestational diabetes was higher than that of normal pregnant women, but the difference was not significant. Other inflammatory factors IL-33, IL-1β, and CRP in umbilical cord blood were also significantly increased. The expression of FPR2mRNA and protein in placental tissues of gestational diabetes was significantly higher than that of normal pregnant women.

Conclusions: The level of FPR2, IL-33, and IL-1β in maternal blood was related to the pathogenesis of GDM and these inflammatory factors could be used as special candidate direction of marks for the prevention, clinical treatment and drug design of GDM, laying a new theoretical foundation for the treatment of GDM.

Key words: FPR2, GDM, inflammatory cytokines, pathogenesis.

Introduction

As a type of diabetes diagnosed during pregnancy, gestational diabetes mellitus (GDM) belongs to endocrine and metabolic diseases with abnormal glucose metabolism to various extents. The incidence of GDM greatly varies in terms of region, race, and living environment. Recently, the incidence of GDM has been significantly increased. According to the diagnostic standards issued by the International Association of Diabetes and Pregnancy Study Groups (IADPSG), the approximate incidence of GDM is 17.8% worldwide.[1] The highest prevalence is observed in the Southeast Asia (24.2%). The most cases (87.6%) of hyperglycemia

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during pregnancy are found in low- and middle-income regions.[2] In China, the prevalence of GDM has been reported to be 9.3% in a survey consisting of 18 589 women in Tianjin between 2010 and 2012.[3] Women with untreated GDM may experience a higher risk of adverse pregnancy outcomes, and there is an increased risk of developing type 2 diabetes later in life for both mother and offspring.[4]

With the improvement of life quality and the changes of the lifestyle, the incidence of diabetes in China has been significantly increased, with a huge elevation in youth. The corresponding incidence of GDM has been also increased rapidly. The major causes can be attributed to the changes of dietary habits due to the concern about intrauterine fetal malnutrition. On the other hand, exercise management is unreasonable.[5] Some people immediately decrease the activities after they are pregnant, especially for the patients with a history of abortion. Other risk factors for GDM in Chinese population include advanced age, pre-pregnancy body mass index (BMI), Han-nationality, hypertension, family history of diabetes, weight gain during pregnancy, and habitual smoking.[3] GDM can impair the health of both mothers and their offspring and even endanger the perinatal life.[6,7] Women with GDM can suffer from several complications, including miscarriage, gestational hypertension, hydramnios, fetal macrosomia, postpartum hemorrhage, and puerperal infection. The rate of cesarean delivery and the risk of postnatal type 2 diabetes have been increased.[8–10] The fetus under the condition of elevated blood sugar may suffer from more complications, such as fetal distress, intrauterine growth restriction, premature birth, and increased malformation rate. High blood sugar from GDM women can stimulate fetal insulin secretion, leading to fetal hypoglycemia and respiratory disease.

In recent years, with the development of molecular genetics, molecular biology, and molecular immunology research for GDM, the pathogenesis of GDM may be related to insulin resistance (IR) during pregnancy, reduced sensitivity to insulin in maternal tissue, insulin receptor and receptor signal transduction disorder, free fatty acid (FFA), inflammation, genetic susceptibility, and autoimmune disease.[11,12] IR and islet beta-cell dysfunction are considered to play a critical and central role in the pathogenesis of GDM. As a metabolic factor, IR has been correlated to an inflammatory condition, which is a central feature in the progression of type 2 diabetes mellitus (DM) since different factors synergistically inhibit the insulin production partly through the secretion of pro-inflammatory cytokines and the regulation of pro-inflammatory signaling pathways. In addition, activation of pro-inflammatory signaling pathways contributes to the progression of GDM through fatty acids, amino acids, reactive oxygen species (ROS), and markers of oxidative stress.[13]

As a seven-transmembrane receptor with 351 amino acids, formyl peptide receptor 2 (FPR2) is located on human chromosome 19q13.3-q13.4.[14] FPR2 has been reported to be expressed in inflammatory, microglial, astrocytoma, and neuroblastoma cells as well as multiple organs, including spleen, lung, testes, ovaries, placenta, brain, and bone marrow.[15] It is involved in the defensive responses, inflammation, and disorders of glucose and lipid metabolism. Specific ligands can activate FPR2, leading to ameliorated regulation of inflammatory reactions,[16] which plays a critical role in the development and function of human placenta. FPR2 modulates inflammatory events in the human endometrium and decidua of early pregnancy, and it participates in the secretion of the pregnancy hormone human chorionic gonadotropin and activation of inflammatory cascade related to both physiological and pathological labor.[17] Liu et al.[18] have found that the FPR2 expression is significantly increased in the blood of patients with type 2 diabetes. FPR2 is also significantly increased in the blood of GDM patients in the Illumina microarray platform. [19]

Given the role of FPR2 in inflammatory regulation, fat metabolism, IR, and diabetes, we aimed to explore the expression of FPR2 in the blood and placenta of GDM patients in the south of China in the present study. In addition, we also investigated the changes of other pro-inflammatory cytokines, such as interleukin 33 (IL-33), IL-1β, tumor necrosis factor alpha (TNF-α), and C-reactive protein (CRP) in order to reveal more information underlying the pathogenesis of GDM. Collectively, our findings provided useful information for diagnosis, prevention, clinical treatment, and drug design of GDM.

Materials and Methods

Clinical specimens

All the clinical specimens, including 50 GDM patients and 30 healthy gravid subjects (control group), were obtained from the Department of Obstetrics in the Third Affiliated Hospital of Soochow University (Changzhou, China) between March 2018 and December 2018. The cases of GDM were diagnosed

according the GDM criteria set using the thresholds of several indices in a 75-g oral glucose tolerance test (OGTT), fasting glucose ≥5.1 mmol/L, 1-h result ≥10.0 mmol/L, or 2-h result ≥8.5 mmol/L.[20] The OGTT should be carried out in the morning following an overnight starvation of at least 8 h during the gestational week of 24–28. All studied objects were ethnic Chinese and GDM cases in our experiment were treated just by dietary control. Detailed clinicopathologic variables of the participants in terms of maternal age and weight, BMI before pregnancy and after admission, history of DM, fetal birth weight, amniotic fluid volume, and blood volume during delivery were listed in Table 1. Our current study protocol was ratified by the ethics committee of Soochow University. Written informed consent was provided by each participant.

Sample collection

The experimental specimens were collected as follows. (1) The fasting peripheral venous blood was drawn from pregnant women after admission during gestational week 37–41 before delivery, and umbilical cord blood was obtained after delivery. Blood specimens were conveyed to the laboratory where serum was separated by centrifugation. Briefly, maternal blood samples were centrifuged at 3000*g* for 10 min, while the umbilical cord blood was centrifuged at 1500*g* for 10 min, and then the serum was removed and stored at −80°C prior to further ELISA analysis. (2) The maternal side of the placenta was immediately dissected after parturition. Placenta specimens were rinsed with saline to eliminate blood, snap-frozen in liquid nitrogen and stored at −80°C until RNA isolation. (3) The placental tissue was immediately fixed in formalin solution and paraffin-embedded for immunohistochemistry.

Antibodies and reagents

ELISA kit for human FPR2 was purchased from Abcam (cat number: MBS723956). ELISA kit for human IL-33 was obtained from Invitrogen (cat number: BMS2048 or MS2048TEN). ELISA kit for human CRP was provided by Abcam (cat number: 99995). ELISA kit for human IL-1β was supplied by Abcam (cat number: 100562). ELISA kit for human TNF-α was purchased from Invitrogen (cat number: KHC3011).

Real time-polymerase chain reaction

Placenta samples stored in liquid nitrogen were taken out and rinsed twice with phosphate buffer saline (PBS) to remove the remaining blood. Tissues were cut into small pieces. Next, about 100 mg tissue sample was added with 1000 μL Trizol (Invitrogen) and ground thoroughly for RNA extracting. RNA was then reversely transcribed into

Table 1 Clinicopathological features between GDM and healthy pregnant women

Variation	Control ($n = 30$)	GDM ($n = 50$)	p-Value
Age (year)	31.9 ± 1.6	29.7 ± 0.9	0.2224
Gravidity	2.2 ± 0.3	2.3 ± 0.2	0.8431
Parity	1.5 ± 0.1	1.6 ± 0.1	0.5296
PCOS (%)	0%	4%	1
History of DM (%)	6.66%	32%	0.012
MBW (g)	3017 ± 75.22	3060 ± 75.28	0.7047
Weight before pregnancy (kg)	55 ± 1.5	59.86 ± 1.87	0.0787
Height (cm)	160.1 ± 0.86	160.1 ± 0.96	0.9699
BMI before pregnancy (kg/m^2)	21.46 ± 0.60	23.34 ± 0.72	0.0792
Admission weight (kg)	70.7 ± 2.13	74.58 ± 2.21	0.2472
Admission BMI (kg/m^2)	27.58 ± 0.83	29.34 ± 0.91	0.1984
FBG (OGTT)	4.57 ± 0.06	5.54 ± 0.17	0.0002
1 h sugar (OGTT)	7.00 ± 0.38	9.85 ± 0.32	<0.0001
2 h sugar (OGTT)	6 ± 0.31	7.67 ± 0.34	0.0016
HbA1c	4.07 ± 0.11	5.17 ± 0.17	<0.0001
FBW (g)	3307 ± 80.37	3497 ± 112.5	0.2461
Blood volume during delivery (mL)	388 ± 39.91	473.2 ± 42.35	0.1837
Rate of vaginal delivery	60%	40%	0.117
amniotic fluid volume (mL)	530 ± 56.44	566.5 ± 73.91	0.7337

Abbreviations: FBG, fasting blood glucose; FBW, fetal birth weight; MBW, maternal birth weight.

cDNA using PrimeScript RT-PCR Kit II (TakaRa, A160847A). Real-time polymerase chain reaction (PCR) was conducted on an ABI 7500 system (Applied Biosystems, USA) based on the manufacturer's instructions, and SYBR Green was used as a DNA-specific fluorescent dye. *GAPDH* was selected as a housekeeping gene. Primer sequences of both *GAPDH* and target gene *FPR2* were as follows: *GAPDH* forward, 5′-TGACTTCAACAGCGACACCCA-3′, *GAPDH* reverse, 5′-CACCCTGTTGCTGTAGCCAAA-3′; *FPR2* forward, 5′-AGTCTGCTGGCTACACTGTTC-3′ and *FPR2* reverse, 5′-TGGTAATGTGGCCG TGAAAGA-3′.

The concentration and purity of RNA were determined according to the spectrophotometric analysis at wavelengths of 260 and 280 nm. Gene expression was determined using one-step RT-PCR protocol in a 20-μL reaction system consisting of 10 μL 2 × One-Step TB Green RT-PCR Buffer 4, 0.8 μL PrimeScript 1 Step Enzyme Mix 2, 0.8 μL forward primer (10 μM), 0.8 μL reverse primer (10 μM), 0.4 μL Rox Reference Dye II (50×), and 7.2 μL placental RNA template (1:10 diluted). The reverse transcription was performed at 42°C for 5 min. Subsequently, after an initial denaturation step at 95°C for 10 s, the amplifications were carried out with 40 cycles at a melting temperature of 95°C for 5 s and an annealing temperature of 60°C for 34 s, followed by a melting curve analysis (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s). The experiment was conducted with three technical replicates for each sample. The relative expression level of *FPR2* was normalized to *GAPDH* and calculated using the $2^{-\Delta\Delta T}$ method.

ELISA

The expressions of FPR2, IL-33, IL-1β, TNF-α, and CRP in the blood were determined by ELISA according to the manufacturer's instructions. Samples, including standards, control specimens, or unknown specimens, were added into 96-well plate pre-coated with respective antibodies. During the first incubation, the standards or samples were simultaneously incubated with a biotinylated monoclonal specific antibody. The plate was then washed, followed by addition of horse radish peroxidase-conjugated streptavidin. A tetramethylbenzidine substrate solution was added to trigger the production of a colored product. The intensity of such colored product directly and proportionally reflected the concentration of the target protein present in the samples.

Immunohistochemistry

The FPR2 antibody (NLS1878, USA) was used for immunohistochemistry. Formalin-fixed, paraffin-embedded consecutive sections (4-μm thick) were heated at 65°C for 4 h and then cooled at room temperature for 20 min. The slides were immersed in dimethylbenzene for deparaffinage and then consecutively hydrated in ethanol. Slides were heated at 125°C in 2% EDTA-citrate antigen retrieval solution in a pressure cooker. Subsequently, the slides were rinsed with PBS and then immersed in hydrogen peroxide at room temperature to block endogenous peroxidase, followed by incubation with 3% bovine serum albumin at 37°C to block nonspecific binding. Next, the slides were incubated with FPR2 antibody (1:500 diluted using antibody diluent) at 4°C for overnight, followed by incubation with corresponding secondary antibody according to the manufacturer's instructions. A DAB substrate kit was employed, and its staining process was carried out according to the manufacturer's instructions. After staining, the sections were counterstained using hematoxylin, followed by dehydration through ethanol and xylene.

Statistical analysis

Data were expressed as the mean and SD. SPSS software (Statistical Analysis System, version 22.0) was employed to perform the statistical analyses. Normally distributed data were analyzed by Student's *t* test. Logistic regression model will be considered for the occurrence of gestational diabetes as a discrete and binary response variable, and factors such as CRP, FPR2, IL-1B, IL-33, and TNFα are explanatory variables. Odds ratio and 95% confidence interval

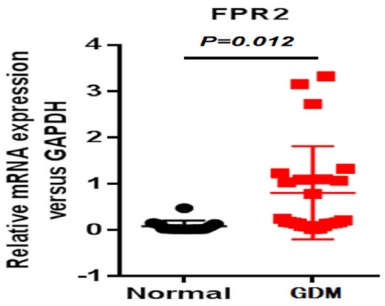

Figure 1 FPR2 expression with PCR is shown. Normal: placenta from healthy pregnant women. GDM: placenta from GDM women. $p < 0.05$

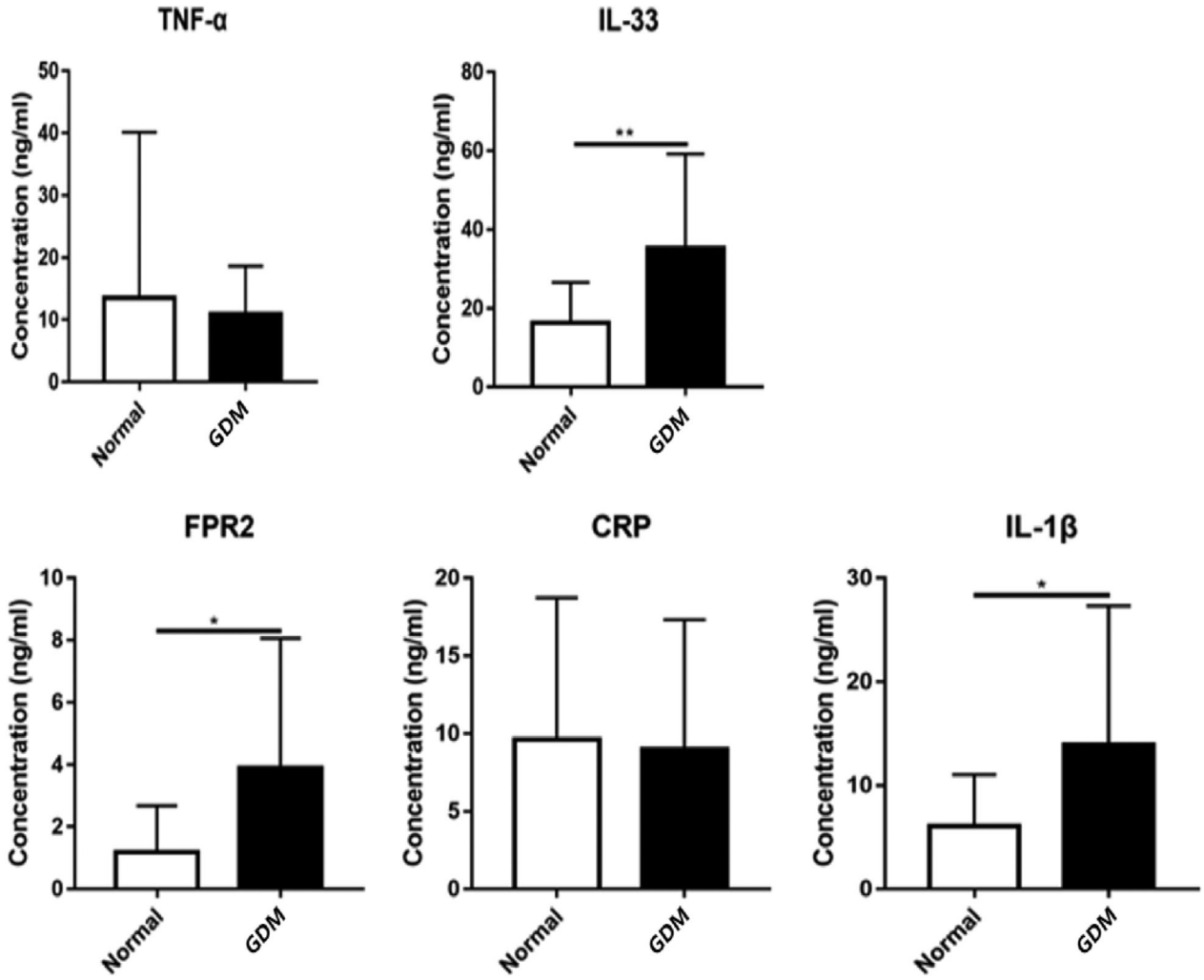

Figure 2 FPR2 and inflammatory mediators in the maternal peripheral blood with ELISA are shown. Results are presented as mean ± SD. Normal = 30, GDM = 50,*$p < 0.05$, **$p < 0.01$ (Student's t test)

were calculated. A $p < 0.05$ was considered as statistically significant.

Results

Clinical characteristics of the study population

Table 1 summarizes the clinical features of the two groups. We found that the maternal age, maternal birth weight, fetal birth weight, rate of vaginal delivery, and BMI were not significantly different between the GDM group and control group. In contrast, there were remarkable differences in blood sugar, including fasting sugar, 1-h sugar and 2-h sugar, HbA1C, and family history of DM between the GDM group and control group.

The expression of *FPR2* at the mRNA level is upregulated in placenta of GDM subjects

We examined the expression of *FPR2* in placenta in 37 specimens, including 23 GDM samples and 14 controls. The expression of *FPR2/ALX* at the mRNA level was significantly increased in the GDM group compared with the control group ($p < 0.05$, Figure 1).

The expressions of FPR2 and some inflammatory mediators are increased in the maternal peripheral blood of the GDM group

The expressions of FPR2 and inflammatory markers at the protein level in maternal peripheral blood were analyzed (Figure 2). It shows that the FPR2 protein

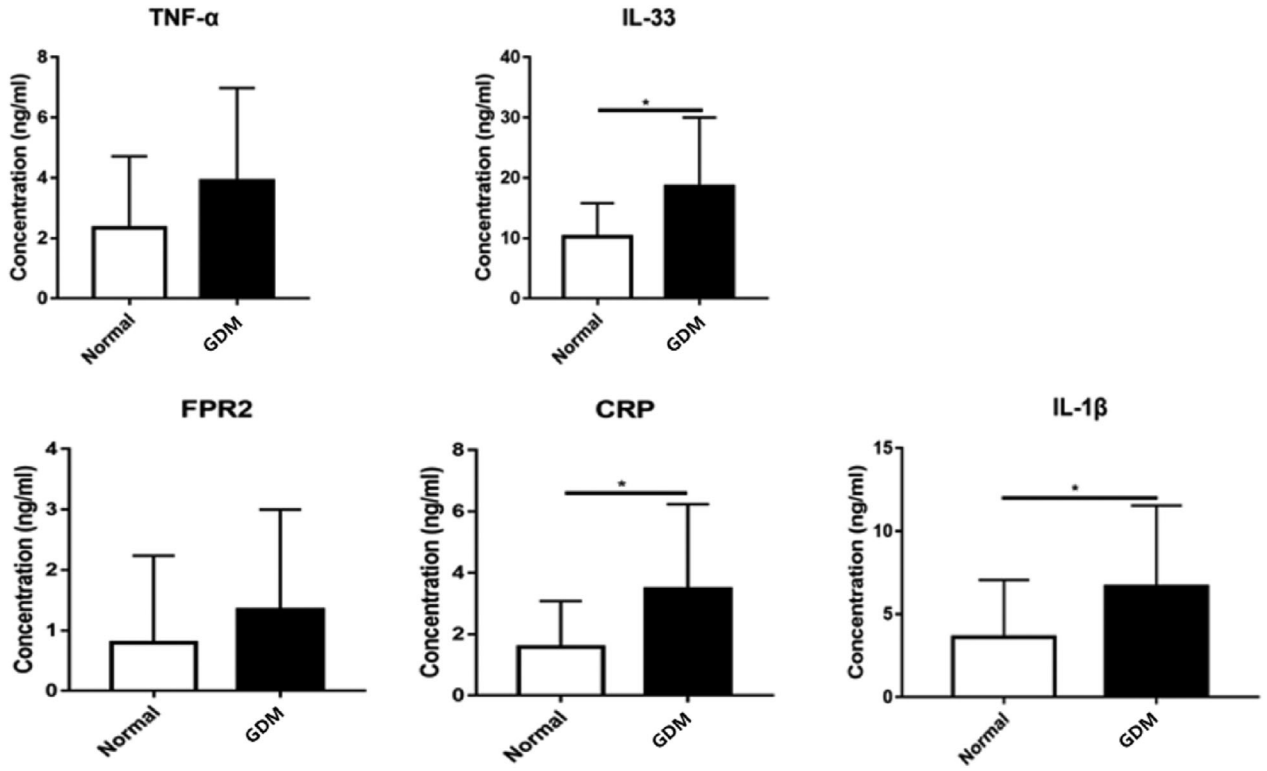

Figure 3 FPR2 and inflammatory mediators in the umbilical blood with ELISA are shown. Results are presented as mean ± SD. Normal = 30, GDM = 5O, *$p < 0.05$, **$p < 0.01$ (Student's t test)

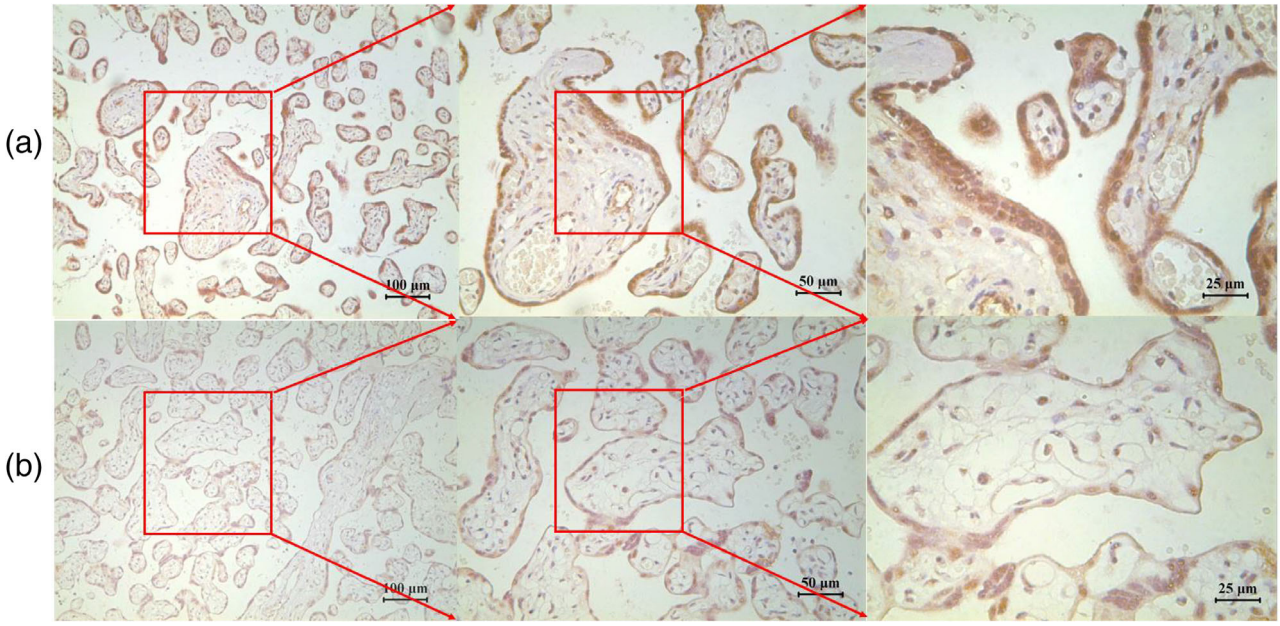

Figure 4 FPR2 immunohistochemical staining. (a) GDM; (b) normal pregnancy

Table 2 Logistic regression analysis for prediction of gestational diabetes

Variation	OR value	95%CI	*p*-Value
CRP (maternal)	0.991	0.919 ~ 1.069	0.823
FPR2 (maternal)	1.591	1.049 ~ 2.412	0.029
IL-1B (maternal)	1.093	0.999 ~ 1.195	0.051
IL-33 (maternal)	1.077	1.015 ~ 1.143	0.014
TNFa (maternal)	0.991	0.955 ~ 1.029	0.650

level in maternal peripheral blood of the GDM group was increased compared with the control group ($p < 0.05$). Moreover, the expressions of inflammatory markers, such as IL-1β and IL-33, were significantly upregulated in the GDM group compared with the control group ($p < 0.05$). In addition, there were no differences of TNF-α and CRP between the two groups (Figure 2).

There is no significant difference in FPR2 level in the cord blood between the two groups

We assessed the levels of FPR2 and inflammatory markers in the umbilical blood. The FPR2 level was higher in the GDM group compared with the control group, but no significant difference. Moreover, the levels of inflammatory markers, such as IL-1β, CRP, and IL-33, were greater in the GDM group compared with the control group ($p < 0.05$). In addition, there was no difference in TNF-α between the two groups (Figure 3).

The expression of FPR2 protein is upregulated in placenta of GDM subjects

FPR2 immunohistochemistry staining was observed in various proportions of placental villous trophoblast. It was localized in the cytoplasm of placental villous trophoblast (brown granular). The expression of FPR2 protein level was increased in the GDM group compared with the control group ($p < 0.05$, Figure 4).

Logistic regression analysis

After adjusting for confounding factors such as maternal age, time of gravidity and parity, BMI during pregnancy, dietary intake and admission BMI, logistic regression analysis was used to estimate the probability and odds ration (OR) of FPR2 and other inflammatory markers for GDM. Table 2 shows the results of logistic regression. FPR2 (OR 1.591 [1.049–2.412], $p = 0.029$) and IL-33 (OR 1.077 [1.015–1.143], $p = 0.014$) in the maternal blood were independent risk factors for GDM (Table 2).

Discussion

GDM is a type of complex disease, and its predisposition has not been fully elucidated. Two main mechanisms, including IR and decreased insulin secretion, play an important role in the pathogenesis of GDM. These elements may be affected by pro-inflammatory cytokines before or during pregnancy. Therefore, increased chronic inflammation can play a critical role in the pathophysiology of GDM directly or indirectly, leading to aggravated IR and promoted occurrence of GDM. Therefore, it is necessary to explore the molecular mechanisms underlying the occurrence of GDM using pro-inflammatory cytokines.

As a remarkably versatile transmembrane protein, FPR2 belongs to the G-protein-coupled receptor (GPCR) family. FPR2 can be activated by a wide range of ligands, such as serum amyloid A (SAA), glucocorticoid-induced annexin 1, urokinase, and lipoxin A4, indicating that the FPR2 activation can cause potent pro- or anti-inflammatory response.[21,22] In our study, we found that not only the FPR2 expression in the placenta but also the serum FPR2 level in the maternal blood from the GDM patients were higher compared with the healthy controls. Moreover, the expressions of other maternal inflammatory cytokines, including IL-33 and IL-1β, in the GDM group were higher compared with the control group. However, the levels of TNF-α and CRP in the maternal blood were not significantly different between the two groups. Meanwhile, we found that the expressions of inflammatory cytokines, including IL-33, CRP, and IL-1β, in the umbilical blood from GDM women were higher compared with the healthy controls. There were no differences in TNF-α and FPR2 expressions in the umbilical blood between the two groups. In addition, the expressions of CRP and TNF-α were not consistent between the maternal blood and umbilical blood. Previous studies have shown that the TNF-α level is increased in GDM women compared with the healthy controls,[23,24] which is not consistent with our findings.[25,26] Such discrepancy could be

attributed to the small sample size and some possible confounding factors.

In our study, we found that the expressions of several inflammatory biomarkers were altered in GDM cases, indicating that the occurrence of GDM might be attributed to the synergistic effect of various factors on the inhibition of insulin production. In summary, FPR2, IL-33, and IL-1β protein may contribute to the development of GDM. Cytokines, mainly be produced in the immune system and adipose tissue, also be synthesized in human placenta so the placental cytokines are thought to participate inflammatory reaction process in the late pregnancy. In patients with gestational diabetes, increasing inflammatory cytokine means the deterioration of inflammation in the cell. Because most patients with gestational diabetes do not show signs of GDM in early pregnancy, the reason for the increased expression of FPR2 in maternal blood and placenta in GDM patients is not certain whether the increase of FPR2 in maternal blood cause the placental change, or the change of oxidation caused by placental inflammation that causes the change of maternal blood. So we could use the character of the marks to improve the outcomes of GDM cases in the future, but it is not certain for using these marks to screen GDM in the early. However, our finding should be validated in more cases and in more Chinese districts with more prospective investigations. In addition, the conflicting data on TNF-αand CRP suggested that these mediators had other functions besides the development of GDM. The equilibrium between pro-inflammatory and anti-inflammatory cytokines played a central role in distinguishing GDM subjects from healthy population.

Considering that these markers are interrelated and involved in the development of GDM, further studies should investigate the diagnostic capabilities of abovementioned markers as a combinatorial set.

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Conflict of Interest

All authors declare no conflict of interest for this work.

Data Availability Statement

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

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