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Association of abnormal Pref-1 level with gestational diabetes mellitus

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Abstract

Objective: To analyze the abnormalities of Pref-1 levels in women with gestational diabetes mellitus (GDM) and fetuses, and to explore the molecular mechanism.

Methods: Fifty normal pregnant women and another 50 women with GDM were selected. Their biochemical indices, including serum insulin, fasting blood glucose, postprandial 2 h blood glucose, glycosylated hemoglobin, C-reactive protein (CRP), triglyceride, total cholesterol, high-density lipoprotein and low-density lipoprotein levels, were detected. Serum Pref-1 and FN levels were measured by ELISA. Serum FAS, IL-6, Sox9 and ERK1 expressions were detected by RT-PCR. The correlations of FAS expression with IL-6 expression and insulin resistance index of GDM patients were subjected to logistic regression analysis.

Results: The two groups had significantly different levels of insulin, fasting blood glucose, postprandial 2 h blood glucose and CRP ($P<0.05$). The serum Pref-1 levels of observation and control groups were (1.22 ± 0.49) μg/L and (1.75 ± 0.58) μg/L respectively, with a significant difference ($P<0.05$). The two groups had similar FN expression levels ($P>0.05$). The serum expression levels of FAS and IL-6 in the observation group were significantly higher than those of the control group ($P<0.05$). The expression levels of Sox9 and ERK1 in the observation group were significantly lower than those of the control group ($P<0.05$). Pref-1 level was negatively correlated with FAS and IL-6 expressions, whereas positively correlated with Sox9 and ERK1 expression ($P<0.05$). Rank sum analysis revealed that FAS expression was positively correlated with IL-6 expression and insulin resistance index in GDM patients.

Conclusion: Abnormal Pref-1 level was closely related to the onset and progression of GDM. It may not be able to bind FN after expression reduction, thereby inhibiting the ERK/MAPK signaling pathway, resulting in excessive secretion of adipocytes, forming an inflammatory environment, causing insulin resistance and finally participating in the onset and progression of GDM.

Keywords: pregnant diabetes mellitus, Pref-1, ERK/MAPK signaling pathway, insulin resistance.

1. Introduction

Gestational diabetes mellitus (GDM) refers to different degrees of abnormal glucose metabolism firstly discovered or during pregnancy (Shaat N. & Groop L., 2007). GDM causes not only serious maternal metabolic disorders, but also increase in perinatal mortality and morbidity. Besides, elevated blood glucose level of pregnant woman increases the weight of fetus, thus raising the incidence rates of giant neonates and poor pregnancy outcomes such as shoulder dystocia, premature delivery and

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neonatal hypoglycemia (Yang H. et al., 2009). The risks of obesity, diabetes, hypertension and metabolic syndrome in the children of GDM pregnant woman are significantly increased in their adulthood (Leng J. et al., 2015). The children of GDM pregnant women are prone to abnormal development of the nervous system, mental and motor developmental disorders, as well as possible decline in language function and social ability (Ijäs H. et al., 2015)(Malcolm J., 2012). Therefore, GDM is hazardous to both pregnant women and their fetuses.

Pref-1 is a transmembrane protein containing six epidermal growth factor-like repetitive sequences (Hudak CS. & Sul HS., 2003)(Hudak CS. et al., 2014). It can inhibit the differentiation of adipocytes and human skeletal stem cells, and promote the development of thymocytes (Wang Y. & Sul HS., 2006)(Raeth S. et al., 2014)(Sul HS., 2009). During addition of insulin, dexamethasone and fetal calf serum to promote lipogenesis, a rapid but transient activation of MEK1 can be detected, accompanied by appearance of considerable ERK1 and its phosphorylation. Afterwards, adding a reagent that inhibits the MAPKs signaling pathway can significantly suppress the differentiation of adipocytes, suggesting that the differentiation and maturation of adipocytes are evidently related with this pathway. It has previously been reported that pref-1 played an important role in GDM onset and progression. However, the underlying molecular mechanism has seldom been referred.

On this basis, we herein studied the clinical value of Pref-1 inhibition of the ERK/MAPK signaling pathway in the onset and progression of GDM, aiming to clarify the abnormalities of Pref-1 levels in GDM pregnant women and their fetuses, and to explore the decrease of Pref-1 which no longer bound FN. As a result, GDM may occur and further induce high-risk metabolic diseases in the children of GDM pregnant woman in their adulthood. The findings provide a probable molecular mechanism for this process.

2. Materials and methods

2.1. Subjects

This study has been approved by the ethics committee of our hospital, and written consent has been obtained from all patients. Fifty GDM patients in the late pregnancy, who were hospitalized from March 2018 to June 2018, were selected as an observation group. Meanwhile, 50 normal pregnant women were selected as a control group. Diagnostic criteria: GDM was diagnosed by the 75 g oral glucose tolerance test (OGTT) between 24 and 28 weeks of gestation during routine pregnancy check-up. The subjects were given a normal diet 3 d before the test. After fasting for 8-12 h, 50 g of glucose powders were dissolved in 200 ml of water, which was drunk up by the subjects within 5 min. Peripheral venous blood was taken to measure blood glucose level 1 h later (timing was started from water drinking). The patients with blood glucose levels of $\geq$7.8 mmol/L or 7.1-7.8 mmol/L complicated with high risk factors were subjected to 75 g OGTT within one week.

High risk factors: 1) Age >30 years old; 2) obesity (BMI>24 kg/m^2); 3) polycystic ovary syndrome before pregnancy; 4) irregular menstruation; 5) family history of diabetes mellitus (parents, sibling, etc.); 6) fasting urine glucose positive during early pregnancy; 7) abnormal obstetric history (GDM history, RDS, deformed neonates, intrauterine death and history of giant neonates); 8) long-term use of glucocorticoids, β receptor blockers, etc.; 9) suspicious fetal macrosomia and polyhydramnios in this pregnancy.

75 g OGTT: The subjects were given a normal diet 3 d before the test. After fasting for 8-12 h, peripheral venous blood was taken to measure fasting blood glucose level. Then 75g of glucose powders were dissolved in 300 ml of water, which was drunk up by the subjects within 10 min. Peripheral venous blood was collected to measure blood glucose levels 1, 2, and 3 h later.

Inclusion criteria: 1) In accordance with GDM diagnostic criteria; 2) without severe liver or kidney diseases; 3) cooperation with this study.

Exclusion criteria: 1) Diagnosis of type 2 diabetes mellitus before pregnancy; 2) with severe acute or chronic infectious diseases; 3) with severe liver and kidney diseases; 4) with endocrine diseases such as hyperthyroidism, hypothyroidism and polycystic ovary syndrome; 5) failure to cooperate with this study.

2.2. Methods

Baseline clinical data, such as age, gestational age, height, body weight, BMI, number of pregnancies and history of smoking or alcohol drinking, were recorded.

Fasting venous blood was collected and centrifuged at 1000 r/min for 15 min within 30 min to obtain the serum which was then stored at -80°C

prior to use. Serum insulin, fasting blood glucose, postprandial 2 h blood glucose, glycosylated hemoglobin, C-reactive protein (CRP), triglyceride, total cholesterol, high-density lipoprotein and low-density lipoprotein levels were detected by an automatic biochemical analyzer.

The homeostatic model assessment for insulin resistance (HOMA-IR) was assessed by HOMA-IR = FBS × FINS/22.5, where FBS is fasting blood sugar and FINS is fasting insulin.

Serum Pref-1 and FN levels were measured by ELISA with corresponding kits (RandD, USA).

2.3. ELISA

Diluted sample (0.1 ml) was placed in a coated reaction well, and incubated at 37°C for 1 h. Meanwhile, blank, negative control and positive control wells were set. The wells were washed with PBST five times, 30 s each time. Freshly diluted enzyme-labeled antibody (0.1 ml) was added into each well, and incubated at 37°C for 0.5-1 h. Subsequently, the wells were washed with PBST five times, 30 s each time, and added 0.1 ml of freshly prepared TMB substrate solution for color development at 37°C for 10-30 min. The reaction was terminated by adding 0.05 ml of 2 M sulfuric acid to each well. The optical density of each well was measured at 450 nm with a microplate reader.

2.4. Detection of serum FAS, IL-6, Sox9 and ERK1 expression levels by RT-PCR

Cell suspension isolated from the serum was inoculated into a 6 cm culture dish (5×10^5 each well), incubated at 37°C with 5% CO_2 and photographed. After 48 h, total RNA was extracted and reverse-transcribed into cDNA. Reaction system (20 μl): 1 μg of RNA, 11 μl of DEPC·H_2O, 4.1 μl of 5× reaction buffer, 0.6 μl of RiboLock™ Ribonuclease inhibitor (20 U/μl), 2.1 μl of dNTP mix (10 mM), 1.1 μl of ReverTra Ace® and 1.1 μl of Oligo(dT) primer (0.5 μg/μl).

PCR conditions: Predenaturation at 95.0°C for 2 min, and then reaction at 95°C for 15 s, 60°C for 20 s and 72°C for 2 min, 40 cycles in total.

2.5. Statistical analysis

Intergroup comparisons were performed by the t test. Correlations between FAS, IL-6, Sox9, ERK1 and Pref-1 were explored by logistic regression analysis. Correlations of FAS with IL-6 expression and HOMA-IR of GDM patients were subjected to the rank sum test. P<0.05 was considered statistically significant.

3. Results

3.1. Baseline clinical data

There were no significant differences in age, gestational age, height, body weight, BMI, frequency of pregnancy, and history of smoking and alcohol drinking between the two groups (P>0.05) (Table 1).

3.2. Blood glucose, CRP and insulin levels

The two groups had significantly different levels of insulin, fasting blood glucose, postprandial 2 h blood glucose and CRP (P<0.05) (Table 2).

3.3. Serum Pref-1 and FN expression levels

The serum Pref-1 levels of observation and control groups were (1.22 ± 0.49) μg/L and (1.75 ± 0.58) μg/L respectively, with a significant difference (P<0.05). The two groups had similar FN expression levels (P>0.05) (Table 3).

Table 1: **Baseline clinical data**

Group	Observation (n=50)	Control (n=50)	t/χ2	P
Age (year)	30.23±4.78	29.45±4.36	1.243	0.227
BMI	23.23±3.12	23.14±3.23	3.123	0.013
Height (cm)	160.58±4.56	159.97±3.96	1.431	0.064
Body weight (kg)	67.45±10.56	67.56±11.12	0.550	0.567
Educational level (n, %)			1.765	0.214
Junior high school or below	5 (10.00)	14 (28.00)		
Senior high school or polytechnic school	28 (56.00)	23 (46.00)		
Junior college	13 (26.00)	10 (20.00)		
Undergraduate or above	4 (8.00)	3 (6.00)		
Residence (n, %)			1.176	0.342
Urban	33 (66.00)	35 (70.00)		
Rural	17 (34.00)	15 (30.00)		
Occupation (n, %)			2.343	0.453
Worker/farmer	6 (12.00)	8 (16.00)		
Medicine, education, culture or public health	6 (12.00)	7 (14.00)		

Business or service	30 (60.00)	28 (56.00)		
Others	8 (16.00)	7 (14.00)		
Household monthly income per capita (n, %)			0.945	0.712
<2500 CNY	10 (20.00)	12 (24.00)		
2500~4000 CNY	25 (50.00)	26 (52.00)		
>4000 CNY	15 (30.00)	12 (24.00)		
Number of pregnancies (n, %)			1.543	0.411
Once	28 (56.00)	26 (52.00)		
Twice	20 (40.00)	20 (40.00)		
≥Three times	2 (4.00)	4 (8.00)		
History of smoking (n, %)			0.034	0.988
Yes	2 (4.00)	3 (6.00)		
No	48 (96.00)	47 (94.00)		
History if alcohol drinking			2.123	0.342
Often	0	1 (2.00)		
Occasionally	10 (20.00)	13 (26.00)		
No	40 (80.00)	36 (72.00)		
Family history			2.564	0.061
Yes	10 (20.00)	12 (24.00)		
No	40 (80.00)	38 (19.00)		

Table 2: **Blood glucose, CRP and insulin levels**

	Observation group	Control group	
Fasting blood sugar (mol/L)	12.1±3.5	4.9±2.1	t=-3.046, P=0.003
CRP (mol/L)	16.0±3.3	6.9±1.5	t=-4.453, P=0.002
Insulin (U)	2.2±2.1	4.0±1.1	t=-3.433, P=0.032

Table 3: **Serum Pref-1 and FN expression levels**

	Observation group	Control group	
Pref-1 (μg/L)	1.22±0.49	1.75±0.58	t=-3.046, P=0.003
FN (μg/L)	1.75±0.58	22.09±9.14	t=-1.453, P=0.102

3.4. Serum FAS, IL-6, Sox9 and ERK1 expression levels

The serum expression levels of FAS and IL-6 in the observation group were significantly higher than those of the control group (P<0.05). The expression levels of Sox9 and ERK1 in the observation group were significantly lower than those of the control group (P<0.05) (Table 4).

Logistic regression analysis revealed that Pref-1 level was negatively correlated with FAS and IL-6 expressions, whereas positively correlated with Sox9 and ERK1 expression (P<0.05) (Table 5).

Table 4: **Serum FAS, IL-6, Sox9 and ERK1 expression levels**

Group	Observation group	Control group	$\chi 2$	P
FAS (nmol/ml)	2.12±0.76	0.65±0.23	3.452	0.032
IL-6 (nmol/ml)	1.58±0.49	0.34±0.13	6.453	0.046
Sox9 (nmol/ml)	0.07±0.02	3.56±1.23	14.354	0.0012
ERK1 (nmol/ml)	0.87±0.24	3.55±1.24	13.354	0.0013

Table 5: **Logistic regression analysis for correlations between Pref-1 as well as FAS, IL-6, Sox9 and ERK1 expression levels**

Variable	β	SE	Wald	P	OR	95%CI
FAS	0.01	0.045	-6.036	0.028	1.023	0.912-1.112
IL-6	0.30	0.342	-9.472	0.036	1.342	0.560-3.233
ERK1	2.23	0.487	20.23	0.000	9.333	3.453-24.563
Sox9	0.165	0.075	4.353	0.023	1.134	1.014-1.453

3.5. Correlation between serum FAS expression, IL-6 expression and HOMA-IR in GDM patients

Rank sum analysis revealed that FAS expression was positively correlated with IL-6 expression (R=0.231, P=0.172) and HOMA-IR (R=0.107, P=0.259) in GDM patients.

4. Discussion

Pref-1 gene is an imprinted gene of paternal expression, which is located on human chromosome 14q32 and widely expressed in embryonic tissues. Pref-1 protein is a transmembrane protein consisting of 385 amino acids, including 6 tandem epidermal growth factor-like repeats (Kim YJ. et al., 2015). After prof-1 proteolysis, a 50 kDa large fragment soluble form capable of inhibiting lipogenesis is obtained. As to the mechanism of action, after the decomposition of Pref-1 by TACE, the 50 kDa soluble form acts as an active ingredient in an autocrine or paracrine manner, and activates the binding of Sox9 to C/EBPβ through the ERK/MAPK signaling pathway, thereby blocking adipocyte differentiation (Wang Y. et al., 2010)(Wang Y. et al., 2010)). Besides, Pref-1 may also function via the Notch pathway. Pref-1 is a negative regulator of pre-adipocyte differentiation and maturation, and its abnormal expression can cause abnormal size, number and function of adipocytes, leading to the occurrence of obesity and obesity-related diseases (Ferrante SC. et al., 2015). In this study, the serum Pref-1 levels of the observation group and control group were (1.22 ± 0.49) μg/L and (1.75 ± 0.58) μg/L, respectively, between which the former was significantly lower than the latter (P<0.05). With the expression levels of FAS, IL-6, Sox9 and ERK1 in the serum of GDM patients as independent variables, and the expression level of pref-1 as the dependent variable, multivariate logistic regression analysis was conducted, indicating that there is a negative correlation between Pref-1 and FAS, IL-6, and a positive correlation between Pref-1 and Sox9, ERK1 (P>0.05). As evidenced by the Western blot results, the function of pref-1 was related to whether the ERK/MAPK signaling pathway was activated. Pref-1 may block the production of Sox9 protein and significantly reduce the expression of phosphorylated ERK1 protein in cells (P=0.002), finally hindering inhibition of the ERK/MAPK pathway on adipocyte differentiation.

Many pathways regulate the expression of intracellular inflammatory mediators after ERK1/2 is activated (Ban K. et al., 2013). The ERK1/2 pathway, which exists in microglia, is activated by multiple stimuli from thrombin, radiation and lipopolysaccharide, and plays a mediating role in the expressions of inflammatory factors (Sun J. & Nan G., 2017). Moreover, for intracranial infections and strokes, many drugs have been proven effective in the treatment of neuroinflammation by suppressing ERK1/2 activation (Schreckinger M. & Marion DW., 2009)[17]. IL-6 and IL-1 can synergistically promote T cell proliferation, which is partly related to up-regulation of T cell IL-2 receptor. They are involved in inflammatory responses. Some human tumors cells, myeloma cells in particular, secrete IL-6 as a growth factor to stimulate their growth. Furthermore, IL-6 has a synergistic effect on the stimulation of multilineage progenitor cells with IL-3, which also promotes the differentiation of B cells (Groblewska M. et al., 2012). This study showed that there were significant differences in levels of insulin, fasting blood glucose, postprandial 2 h blood glucose and CRP (P<0.05). Also, there was a positive correlation between FAS and the IL-6 expression and HOMA-IR of GDM patients.

5. Conclusion

In summary, the abnormality of Pref-1 level is closely related to the onset and progression of GDM. It may not be able to bind FN after expression down-regulation, which thus inhibits the ERK/MAPK signaling pathway, results in excessive secretion of adipocytes, forms an inflammatory environment, causes IR and finally participates in the progression of GDM.

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