Correlation of serum vitamin D, adipose tissue vitamin D receptor, and peroxisome proliferator-activated receptor γ in women with gestational diabetes mellitus

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Abstract

Background: Gestational diabetes mellitus (GDM) is a common complication during pregnancy. Obesity and overweight are closely related to metabolic diseases and diabetes. However, the role of adipose tissue in the pathogenesis of GDM remains to be studied. The aim of this study was to investigate the correlation of vitamin D (VD) levels, VD receptor (VDR), and peroxisome proliferator-activated receptor γ (PPARγ) expression with GDM in overweight or obese women.

Methods: One hundred and forty pregnant women with full-term single-birth cesarean-section were selected as the study subjects and grouped (70 GDM women, including 35 non-overweight/non-obese women [group G1] and 35 women with overweight or obesity [group G2]; 70 pregnant women with normal glucose tolerance, including 35 non-overweight/non-obese women [group N1] and 35 overweight/obese women [group N2]). The levels of serum VD, blood biochemistry, and adiponectin were compared in these women. Subcutaneous adipose tissue was isolated from the abdominal wall incision. VDR and PPARγ messenger RNA (mRNA) transcript levels in these adipose tissues were quantified by real-time polymerase chain reaction. The differences between the levels of PPARγ protein and phosphorylated PPARγ Ser273 were detected by Western blotting.

Results: The serum VD level of GDM women was lower in comparison to that of women with normal glucose tolerance (G1 *vs.* N1: 20.62 ± 7.87 ng/mL *vs.* 25.85 ± 7.29 ng/mL, G2 *vs.* N2: 17.06 ± 6.74 ng/mL *vs.* 21.62 ± 7.18 ng/mL, $P < 0.05$), and the lowest in overweight/obese GDM women. VDR and PPARγ mRNA expression was higher in the adipose tissues of GDM women in comparison to that of women with normal glucose tolerance (VDR mRNA: G1 *vs.* N1: 210.00 [90.58–311.46] *vs.* 89.34 [63.74–159.92], G2 *vs.* N2: 298.67 [170.84–451.25] *vs.* 198.28 [119.46–261.23], PPARγ mRNA: G1 *vs.* N1: 100.72 [88.61–123.87] *vs.* 87.52 [66.37–100.04], G2 *vs.* N2: 117.33 [100.08–149.00] *vs.* 89.90 [76.95–109.09], $P < 0.05$), and their expression was the highest in GDM + overweight/obese women. VDR mRNA levels positively correlated with the pre-pregnancy body mass index (BMI), pre-delivery BMI, fasting blood glucose (FBG), homeostasis model assessment of insulin resistance (HOMA-IR), and PPARγ mRNA while it negatively correlated with the VD and the adiponectin levels ($r = 0.395, 0.336, 0.240, 0.190, 0.235, -0.350, -0.294$, respectively, $P < 0.05$). The degree of PPARγ Ser273 phosphorylation increased in obese and GDM pregnant women. PPARγ mRNA levels positively correlated with pre-pregnancy BMI, pre-delivery BMI, FBG, HOMA-IR, serum total cholesterol, triglyceride, free fatty acid, and VDR mRNA, while it negatively correlated with the VD and adiponectin levels ($r = 0.276, 0.199, 0.210, 0.230, 0.182, 0.214, 0.270, 0.235, -0.232, -0.199$, respectively, $P < 0.05$).

Conclusions: Both GDM and overweight/obese women had decreased serum VD levels and up-regulated VDR and PPARγ mRNA expression in adipose tissue, which was further higher in the overweight or obese women with GDM. VD may regulate the formation and differentiation of adipocytes through the VDR and PPARγ pathways and participate in the occurrence of GDM.

Keywords: Gestational diabetes mellitus; Overweight; Obesity; Serum vitamin D; Vitamin D receptor; Peroxisome proliferator-activated receptor gamma

Introduction

Gestational diabetes mellitus (GDM) is associated with normal glucose metabolism before pregnancy while diabetes-associated symptoms are exhibited during pregnancy. The incidence of GDM in China is on the rise, and has reached 17.5% according to the latest statistics.[1] GDM is a disease caused by multiple factors which seriously threatens maternal and child safety. Women with GDM are at an increased risk of developing metabolic syndrome, type 2 diabetes mellitus (T2DM), and cardiovascular disease.[2] Women with GDM

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were more likely to undergo cesarean deliveries, and their newborns had a higher birth weight.[3] GDM is associated with increased risks of depression,[4] significantly and independently associated with childhood impaired glucose tolerance.[5] Infants born to mothers with GDM also have a higher risk of developing T2DM in their teens or early adulthood.[6] Insulin resistance (IR) and decreased islet β cell secretion are considered to be important links in the pathogenesis of GDM.[7] Most studies have shown that low vitamin D (VD) level is also associated with GDM,[8] the risk of GDM increases with the increase of pre-pregnancy body mass index (BMI).[9] A meta-analysis of obesity and GDM relationship in pregnant women showed that the unadjusted odds ratio (OR) of GDM occurrence in women with overweight, obesity, and severe obesity, when compared with normal-weight pregnant women, were 2.14 (95% confidence interval [CI]: 1.82–2.53), 3.56 (95% CI: 3.05–4.21), and 8.56 (95% CI: 5.07–16.04), respectively.[10] Overweight or obesity refers to a condition of excess amount of adipose in the body and is caused by various reasons. The adipose tissue is an active endocrine organ that secretes various kinds of proteins and peptides,[11] including the adipocytokines such as leptin, adiponectin, and tumor-associated cytokines that are involved in insulin signal transduction, glycolipid metabolism, etc. Recent research supports that the adipose tissue is the origin of IR,[12] and VD interacts with adipose tissue and affects each other. A variety of enzymes that can synthesize $25(OH)D$, $1,25\text{-}(OH)_2D_3$ are expressed in the adipose tissue. Large amounts of adipocytes in obese individuals can inhibit the expression of 25 hydroxylase (CYP2J2) and 1α-hydroxylase (the expression of CYP27B1), thus leading to metabolic impairment of VD.[13] Fat can also be used as a reservoir of VD and limits its biological activity.[14] However, $1,25\text{-}(OH)_2D_3$ affects adipocytes, and adipokine formation and cytokine secretion through the VD receptor (VDR) of adipocytes.[15] Peroxisome proliferator-activated receptor γ (PPARγ) is an adipocyte-specific nuclear transcription factor belonging to the nuclear receptor family. It is abundant in the adipose tissue and is a key factor in controlling the production of adipocytes and fatty acid metabolism. It is closely associated to the metabolic syndrome. PPARγ is necessary for the lipid formation both *in vivo* and *in vitro*.[16]

In the low vitamin status, obesity is closely related to GDM. Whether the alteration of serum VD in women with GDM is caused by the VDR and PPARγ in the adipose tissues is not clear. Here, we studied the changes in serum VD, VDR levels, and PPARγ expression in the subcutaneous adipose tissue of overweight or obese women with GDM, aiming to investigate if there is a correlation between VDR levels and PPARγ expression in the subcutaneous adipose tissue of overweight/obese and diabetic pregnant women and their effects on GDM.

Methods

Ethical approval

This study was conducted in accordance with the *Declaration of Helsinki*. This study was conducted with approval from the Ethics Committee of Nanjing Medical University. Written informed consent was obtained from all participants.

General information

Pregnant women admitted to the Changzhou Woman and Children Health-Care Hospital Affiliated to Nanjing Medical University from January 2015 to April 2017 for full-term delivery were selected as the subjects. Oral glucose tolerance test (75 g glucose) was performed at 24 to 28 weeks of gestation as it is a diagnostic criterion for GDM recognized by The International Association of Diabetes and Pregnancy Study Groups. Normal fasting blood glucose (FBG) and blood glucose levels at 1 and 2 h after glucose administration are less than 5.1, 10.0, and 8.5 mmol/L, respectively. Any individual with blood glucose levels higher than the reference range for any one of the above time points can be diagnosed as having GDM.[17] Seventy full-term single-birth women diagnosed with GDM, including 35 women with normal BMI (18.5–24.9 kg/m^2) (group G1) and 35 women with overweight or obesity (BMI ≥25.0 kg/m^2) (group G2). Another 70 normal glucose tolerance pregnant women with cesarean section due to malposition or scar uterus were selected as group non-GDM, among whom 35 had normal BMI (group N1) and 35 were women with overweight or obesity (group N2). Exclusion criteria were patients with a history of diabetes or hypertension before pregnancy or other pregnancy-related complications.

Sample conditions and data collection

The height and weight of each pregnant woman were measured and BMI was calculated by the assigned nurse after hospital admission. A face-to-face questionnaire was also performed. The questionnaire included questions related to the general health conditions during pregnancy, weight before pregnancy, and weight during childbirth. Specialists were assigned for the inquiry of VD and calcium tablets taken during the pregnancy and gestational weeks. The dosage of taken tablets was then investigated and converted to VD$_3$ content (U) and calcium content (mg) according to the instructions. The product of supplemented oral VD (U) multiplied by the supplement gestational weeks was used to represent the oral supplemental dose of VD during pregnancy. The product of supplemental calcium content (mg) during pregnancy multiplied by the supplement gestational weeks was used to represent the supplemental dose of calcium during pregnancy. If no ectogenic oral VD or calcium were administered at 4 weeks before delivery, it was considered as no recent intake of VD and calcium. The daily sun exposure of each pregnant woman was also asked in detail, based on which the pregnant women were divided into four different grades: women with <0.5 h of sun exposure per week was considered as 0, ≥0.5 to <1 h of sun exposure per week was considered as 1; ≥1 to <2 h of sun exposure per week was considered as 2, and ≥2 h of sun exposure per week was considered as 3.

Detection of VD, biochemical indicators, and adiponectin

Four milliliters of fasting elbow venous blood anticoagulated by ethylenediaminetetraacetic acid was sampled pre-operatively, followed by centrifugation at 1500 r/min for isolating the plasma and cryopreservation at −70°C. FBG

levels were determined by the glucose oxidase method. Serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were determined by one automatic biochemical analyzer. Fasting insulin (FINS) was measured by the electrochemiluminescence method and free fatty acid (FFA) levels were measured by the enzyme colorimetry method (Beckman reagent, Shenzhen, China). The homeostasis model assessment of insulin resistance (HOMA-IR) = FBG × FINS/22.5. The serum VD level was detected by the electrochemiluminescence method (Roche Diagnostics GmbH, Mannheim, Germany), which was the sum of 25-OH-D_2 and 25-OH-D_3; according to the kit standards <26 ng/mL indicated VD deficiency. Adiponectin levels were detected by the enzyme-linked immunosorbent assay (R&D System, Minneapolis, MN, USA).

Collection of adipose tissue specimen

After having obtained the informed consent, we sampled two pieces of subcutaneous fat tissue at the abdominal wall during cesarean-section, with the size as approximately 1.0 cm × 1.0 cm × 1.0 cm; after saline rinsing, one piece of the sample was fixed in 10% formaldehyde, followed by paraffin embedding within 24 h. The other piece of the sample was suspended in the RNA later solution and stored at –20°C for 24 h and then cryopreserved at –70°C for later use. Immunohistochemistry was used to observe the location of VDR and PPARγ in the adipose tissue of the four groups, as well as to semi-quantitatively detect the expression level. The transcription level of VDR messenger RNA (mRNA) and PPARγ mRNA in each group was detected by quantitative real-time polymerase chain reaction (PCR). Western blotting was used to detect the expression of PPARγ protein and PPARγ Ser273 phosphorylation.

Immunohistochemistry

The paraffin-embedded specimens were serially sliced at 4 μm, de-waxed until water was removed, re-suspended in citrate saline bath for 20 min, incubated for 10 min, closed in freshly prepared 3% H_2O_2 for 5 min, washed with phosphate buffer saline (PBS), and then incubated with 1:100 mouse anti-human VDR (PPARγ) (Thermo Fischer Scientific, former Savant, MA, USA) overnight at 4°C. After PBS washing, the horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin G (IgG) (1:100, Thermo Scientific) was added for 30-min incubation at room temperature, followed by PBS washing, diaminobenzidine coloration, and hematoxylin counterstaining. The nuclei of positively stained cells showed brownish-yellow particles, and the negative control replaced the primary antibody with PBS. The cells were counted by the double-blind method with the microscopic brownish-yellow particles as the positive result. According to the percentage of positively stained cells, <5% was scored as 0 point, 5% to 25% was scored as 1 point, 26% to 50% was scored as 2 points, 51% to 75% was scored as 3 points, and >75% was scored as 4 points. The score was combined with the staining intensity: no coloration was scored as 0, weakly positive was scored as 1 point, positive was scored as 2 points, and strongly positive was scored as 3 points. The product of the positive rate score and the

intensity score was finally calculated as the final score of the related group.

Quantitative real-time polymerase chain reaction

The total RNA of adipose tissue was extracted using the Trizol Reagent (InvivoGen, San Diego, CA, USA). After identifying the RNA integrity by electrophoresis, complementary DNA (cDNA) was synthesized by reverse transcription of 2 μg of total RNA. The primer reaction system: 10× PCR 2.5 μL, $MgCl_2$ (25 mmol/L) 2.5 μL, deoxy-ribonucleoside triphosphate (10 mmol/L), 0.5 μL, Taq polymerase (5 U/μL) 0.25 μL, VDR (PPARγ) forward primer (F) (100 μmol/L) 0.04 μL, VDR (PPARγ) reverse primer (R) (100 μmol/L) 0.04 μL, VDR (PPARγ) probe (P) (100 μmol/L) 0.04 μL; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (F) (100 μmol/L) 0.04 μL, GAPDH (R) (100 μmol/L) 0.04 μL, GAPDH (P) (100 μmol/L) 0.04 μL, sterilized double distilled water supplement 17.01 μL, cDNA 2 μL, a total reaction volume of 25 μL. The amplification conditions of the PCR instrument (Roche 480II) were: pre-denaturation at 95°C for 3 min; 95°C for 5 s, 60°C for 15 s (temperature conversion rate is 20°C/s), and amplification rounds of 40 cycles at 40°C for 1 min per cycle. The fluorescence signals were acquired during the period of 60°C extension. The results were expressed as the ratio of the relative expression of the gene of interest to the expression of the internal reference gene GAPDH. Primer probe sequences in reaction system of real-time PCR are shown in Table 1.

Western blotting

Protein was extracted at low temperature, quantified by Bradford method, then transferred to nitrocellulose filter (NC) membrane by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and washed with Tris buffered saline

Table 1: The primer probe sequences in reaction system of real-time polymerase chain reaction.

Primers	Sequence (5′–3′)
PPARγ	
Forward	GAC CAA AGC AAA GGC GAG G
Reverse	TTG ATT TTA TCT TCT CCC ATC ATT AAG
Probe	FAM-CTT GAC AGG AAA GAC AAC AGA CAA ATC ACC-BHQ1
VDR	
Forward	GCT AAG ATG ATA CCA GGA TTC AGA GAC
Reverse	AAG GAC TCA TTG GAG CGC AAC
Probe	FAM-ACC TCT GAG GAC CAG ATC GTA CTG CTG A-BHQ1
GAPDH	
Forward	GGA AGG TGA AGG TCG GAG TC
Reverse	CGT TCT CAG CCT TGA CGG T
Probe	Cy5-TTT GGT CGT ATT GGG CGC CTG-BHQ2

PPARγ: Peroxisome proliferator-activated receptor γ; VDR: Vitamin D receptor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Tween 20 (TBST) for 5 min ×3 times. NC film was placed in a dish covered by sealing liquid (5% skimmed milk powder) and shaken for 2 h, then TBST was used to wash the membrane for 5 min ×3 times. The membrane was incubated overnight in a shaking bed at 4°C in a dish containing 1:10,000 rabbit anti-GAPDH (Jiangsu Kaiji Biotechnology Co., Ltd., China), 1:200 mouse anti-PPARγ (Santa Cruz Biotechnology, Dallas, TX, USA), 1:200 rabbit anti-p-PPARγ (Beijing Boosen Biotechnology Co., Ltd., China). The next day, it was taken out and shaken at room temperature for 30 min. The primary antibody was absorbed and washed with TBST for 10 min ×3 times. The secondary antibody was diluted with the diluent of the second antibody (sheep anti-mouse IgG-HRP, sheep anti-rabbit IgG-HRP; Jiangsu Kaiji Biotechnology Co., Ltd.) and the shaking reaction at room temperature was 2 h. After the secondary antibody reaction, the secondary antibody was recovered. Thereafter, TBST was used to wash the film for 5 min ×3 times. The liquid A and B in the electrochemiluminescence kit were mixed in equal volume of 1:1 to form a working liquid for reserve. NC film was removed from TBST, and the relative content of the target protein was calculated with GADPH as internal parameter, by using G: BOX chemiXR5 (Syngene company, UK) to image the blot.

Observation indexes

The difference of VD level, biochemical indexes, and VDR and PPARγ expression between groups N1 and N2, groups G1 and G2 were compared and the difference between pregnant women with or without overweight/obesity were explored. Different study groups were weight matched, and the difference of VD level, biochemical indexes, VDR and PPARγ expression between group N1 and G1, as well as between group N2 and G2, were compared to explore the difference between diabetic and non-diabetic pregnant women with different BMI. Western blotting was used to detect the PPARγ protein and phosphorylation of PPARγ Ser273 in adipose tissue of the four groups. The correlation of serum VD and VDR and PPARγ with overweight/obesity in women with GDM were explored.

Statistical analysis

All the data were entered into Excel and analyzed using SPSS19.0 (SPSS Inc., Chicago, IL, USA). The data with normal distribution (age, gravidity, parity, gestational age, pre-pregnancy BMI, pre-delivery BMI, neonatal birth weight, VD, TC, LDL-C, adiponectin, VDR, PPARγ, and p-PPARγ) were expressed as mean ± standard deviation, and the median (M) and interquartile range (P_{25}–P_{75}) were used to describe the data with non-normal distribution (VD supplement, calcium supplement, FBG, FINS, HOMA-IR, TG, HDL-C, FFA, VDR mRNA, and PPARγ mRNA). The difference between women with GDM and women with group non-GDM was compared statistically by the t test or the Wilcoxon rank-sum test. The count data was expressed by n (%). The comparison between groups was performed by the Chi-square test, the spearman rank correlation analysis was used to analyze the correlation between indicators. Binary logistic regression was used to evaluate the relative risk (OR) and 95% CI. The test level was $\alpha = 0.05$, and $P < 0.05$ was considered as statistical significant.

Results

Comparison of general conditions

The comparison of the age, gravidity and parity, gestational age, VD supplement, calcium supplement, recent VD supplement rate, calcium supplement rate, average sun exposure time, and neonatal birth weight showed no statistical difference between the group G1 and group G2, the same results were found between group N1 and group N2 ($P > 0.05$). The pre-pregnancy BMI of group N2 was higher than that of group N1, and the pre-pregnancy BMI of group G2 was higher than that of group G1, whereas the pre-pregnancy BMI of the overweight/obese sub-group were higher than the non-overweight/non-obese sub-group ($P < 0.05$), there was no significant difference in the BMI between the two sub-groups of the non-overweight/non-obese group. While compared in pre-delivery BMI, the same results were found. The neonatal birth weight in the group G2 was significantly higher than that in the group N2 (3892.6 ± 497.7 g $vs.$ 3624.9 ± 332.1 g, $P < 0.05$). There were no significant differences in other indexes of sub-groups of the overweight/obese group ($P > 0.05$) [Table 2].

Detection of biochemical indexes during pregnancy

The levels of serum VD, HDL-C, and adiponectin decreased, while the FBG and FFA levels increased in group G1 when compared to the group N1 ($P < 0.05$). The levels of serum VD, HDL-C, and adiponectin decreased, while the FBG, FINS, HOMA-IR, and FFA levels increased in group G2 when compared to the group N2 ($P < 0.05$). The levels of serum VD and adiponectin decreased, while the FINS and HOMA-IR increased in group G2 when compared to the group G1 ($P < 0.05$). The levels of serum VD and adiponectin decreased while the FINS and HOMA-IR levels increased in group N2 when compared to the group N1 ($P < 0.05$).

In this study, 110 pregnant women with VD deficiency in the third trimester showed a VD deficiency rate of 78.6%, but there was no significant difference between overweight/obese group and non-overweight/non-obese group, or between group GDM and group non-GDM; the data in group G2 were significantly higher than that in the group N1 (32/35 $vs.$ 22/35, $\chi^2 = 8.102$, $P = 0.004$), suggesting that GDM women with overweight/obesity showed more obvious VD deficiency than non-overweight/non-obesity pregnant women with normal glucose tolerance [Table 3].

Immunohistochemistry

VDR and PPARγ are the brown-yellow particles in the nuclei of adipocytes in each group [Figure 1]. VDR expression in group GDM increased significantly, and its expression in the group G1 was higher than its expression in the group N1. Similarly, its expression in the group G2 was higher than its expression in the group N2 ($P < 0.05$). PPARγ expression in group GDM was higher than that in group non-GDM, and its expression in the group G1 was

Table 2: Clinical characteristics of pregnant women with full-term single-birth cesarean-section ($n = 140$).

Indexes	Non-overweight and non-obesity				Overweight and obesity				GDM (G1 vs. G2)		Non-GDM (N1 vs. N2)	
	Group N1 ($n = 35$)	Group G1 ($n = 35$)	$t/Z/\chi^2$	P	Group N2 ($n = 35$)	Group G2 ($n = 35$)	$t/Z/\chi^2$	P	$t/Z/\chi^2$	P	$t/Z/\chi^2$	P
Age (years)	28.8 ± 3.1	30.1 ± 4.0	−1.535	0.130	28.7 ± 3.2	29.4 ± 5.1	−0.728	0.469	0.624	0.534	0.152	0.880
Gravidity (times)	2.03 ± 0.95	2.51 ± 1.12	−1.952	0.055	2.14 ± 1.22	2.06 ± 1.19	0.563	0.766	1.657	0.102	−0.437	0.663
Parity (times)	1.26 ± 0.44	1.37 ± 0.55	−0.960	0.340	1.20 ± 0.41	1.23 ± 0.60	−0.234	0.816	1.043	0.301	0.562	0.576
Gestational age (weeks)	38.95 ± 0.78	38.79 ± 1.08	0.686	0.495	38.83 ± 0.71	38.60 ± 0.88	1.226	0.224	0.471	0.412	0.643	0.523
VD supplement (mg × gestational age)	6000 (0–10,400)	6600 (2375–11,000)	−0.513	0.608	3000 (0–14,400)	6000 (1750–16,000)	−0.691	0.489	−0.212	0.832	−0.273	0.785
Ca supplement (mg × gestational week)	6000 (0–10,800)	5760 (2640–11,400)	−0.431	0.667	7200 (0–11,400)	7800 (0–12,000)	−0.457	0.648	−0.410	0.967	−0.202	0.840
Recent VD supplement rate, n (%)	20 (57.1)	25 (71.4)	1.556	0.212	18 (51.4)	21 (60.0)	0.521	0.470	1.014	0.314	0.230	0.631
Recent Ca supplement rate, n (%)	23 (65.7)	26 (74.3)	0.612	0.434	20 (57.100)	24 (68.6)	0.979	0.322	0.280	0.597	0.543	0.461
Average sun exposure time	1.77 ± 1.06	1.69 ± 1.32	0.299	0.766	1.69 ± 1.21	2.00 ± 1.06	−1.159	0.251	−1.098	0.276	0.316	0.753
Pre-pregnancy BMI (kg/m^2)	21.10 ± 2.29	21.89 ± 1.97	−1.537	0.129	29.15 ± 2.88	29.35 ± 3.84	−0.253	0.801	−10.229	<0.010	−12.949	<0.010
Pre-delivery BMI (kg/m^2)	27.93 ± 2.79	28.76 ± 3.27	−1.140	0.258	35.22 ± 3.44	35.11 ± 4.30	−0.123	0.903	−6.956	<0.010	−9.736	<0.010
Neonatal birth weight (g)	3500.9 ± 402.1	3682.6 ± 518.0	−1.639	0.106	3624.9 ± 332.1	3892.6 ± 497.7	−2.647	0.010	−1.729	0.088	−1.407	0.164

Data are expressed as mean ± standard deviation or median (P_{25}–P_{75}). The statistical results of age, gravidity, parity, gestational age, average sun exposure time, pre-pregnancy BMI, pre-delivery BMI, neonatal birth weight are expressed by t value. The statistical results of VD supplement, Ca supplement were expressed by Z value. The statistical results of recent VD supplement rate, recent Ca supplement rate were expressed by χ^2 value. GDM: Gestational diabetes mellitus; Group G1: Non-overweight and non-obese pregnant women with GDM; Group G2: Overweight/obese pregnant women with GDM; Group N1: Non-overweight and non-obese pregnant women with normal glucose tolerance; Group N2: Overweight/obese pregnant women with normal glucose tolerance; Ca: Calcium; VD: Vitamin D; BMI: Body mass index.

Table 3: Biochemical variables in pregnant women with full-term single-birth cesarean-section ($n = 140$).

Indexes	Non-overweight and non-obesity				Overweight and obesity				GDM (G1 vs. G2)		Non-GDM (N1 vs. N2)	
	N1 ($n = 35$)	G1 ($n = 35$)	$t/Z/\chi^2$	P	N2 ($n = 35$)	G2 ($n = 35$)	$t/Z/\chi^2$	P	$t/Z/\chi^2$	P	$t/Z/\chi^2$	P
VD (ng/mL)	25.85 ± 7.29	20.62 ± 7.87	2.886	0.005	21.62 ± 7.18	17.06 ± 6.74	2.738	0.008	2.029	0.046	2.448	0.017
VD deficiency rate, n (%)	22 (62.9)	27 (77.1)	1.701	0.192	29 (82.90)	32 (91.4)	1.148	0.284	2.696	0.101	3.540	0.060
FBG (mmol/L)	4.27 (3.99–4.52)	4.64 (4.30–5.12)	−2.896	0.004	4.52 (4.21–4.73)	4.72 (4.40–5.36)	−2.702	0.007	−1.192	0.233	−1.803	0.071
FINS (mmol/L)	12.04 (10.41–13.27)	12.02 (10.08–15.77)	−0.781	0.435	13.32 (12.31–14.52)	15.29 (12.26–19.46)	−2.355	0.019	−2.537	0.011	−2.825	0.005
HOMA-IR	2.23 (1.98–2.68)	2.56 (2.08–3.16)	−1.909	0.056	2.66 (2.43–2.99)	3.44 (2.55–4.13)	−3.189	0.001	−2.931	0.003	−3.154	0.002
TC (mmol/L)	5.58 ± 0.71	5.91 ± 1.02	−1.556	0.124	5.92 ± 0.73	6.22 ± 1.07	−1.348	0.182	−1.250	0.216	−1.998	0.051
TG (mmol/L)	3.23 (2.66–3.76)	3.69 (2.99–4.40)	−1.398	0.162	3.55 (2.93–4.13)	3.89 (2.93–5.36)	−1.386	0.166	−1.022	0.307	−0.987	0.324
HDL-C (mmol/L)	2.06 (1.63–2.40)	1.73 (1.58–1.93)	−2.590	0.010	1.91 (1.66–2.23)	1.61 (1.43–1.83)	−2.949	0.003	−1.692	0.091	−0.993	0.321
LDL-C (mmol/L)	3.05 ± 0.56	3.07 ± 0.65	0.473	0.870	2.99 ± 0.65	3.16 ± 0.74	−0.993	0.324	−0.511	0.611	0.410	0.700
FFA (mmol/L)	0.52 (0.42–0.64)	0.66 (0.49–0.80)	−2.385	0.017	0.49 (0.38–0.67)	0.74 (0.58–1.07)	−3.748	<0.001	−1.850	0.064	−0.188	0.851
Adiponectin (ng/mL)	74.90 ± 29.29	62.10 ± 23.17	2.028	0.047	61.76 ± 20.42	50.32 ± 22.32	2.238	0.028	2.167	0.034	0.580	0.033

Data are expressed as mean ± standard deviation or median (P_{25}–P_{75}). The statistical results of VD, TG, LDL-C, adiponectin are expressed by t value. The statistical results of FGB, FINS, HOMA-IR, TC, HDL-C, FFA are expressed by Z value. The statistical results of recent VD deficiency rate are expressed by χ^2 value. GDM: Gestational diabetes mellitus; Group G1: Non-overweight and non-obese pregnant women with GDM; Group G2: Overweight/obese pregnant women with GDM; Group N1: Non-overweight and non-obese pregnant women with normal glucose tolerance; Group N2: Overweight/obese pregnant women with normal glucose tolerance; VD: Vitamin D; FBG: Fasting blood glucose; FINS: fasting insulin; HOMA-IR: Homeostasis model assessment of insulin resistance; TC: Total cholesterol; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; FFA: Free fatty acid.

higher than that in the group N1, and its expression in the group G2 was higher than that in the group N2 ($P < 0.05$).

Quantitative real-time polymerase chain reaction

The transcript levels of VDR mRNA in the group GDM was higher than that in the group non-GDM (the level of VDR mRNA in the group G1 was higher than that in the group N1, while its expression in the group G2 was higher than that in the group N2, $P < 0.05$). The VDR mRNA level of pregnant women with overweight/obesity was higher than that of non-overweight/non-obese pregnant women (the level of VDR mRNA in group N2 was higher than that in group N1, which its expression in group G2 was higher than that in group G1, $P < 0.05$). And the GDM pregnant women with overweight/obesity showed the statistically highest level of VDR mRNA.

Quantitative PCR analysis of adipose tissue showed that the expression level of PPARγ mRNA in group GDM was higher than that in group non-GDM (group G1 vs. group N1, group G2 vs. group N2, $P < 0.05$). The expression level of PPARγ mRNA in the group G2 was higher than that in the group G1 ($P < 0.05$). The GDM pregnant women with obesity showed the statistically highest level of PPARγ mRNA [Table 4 and Figure 2].

Western blotting

PPARγ levels in group GDM was higher than that in group non-GDM (group G1 vs. group N1, group G2 vs. group N2, $P < 0.05$). The expression levels of PPARγ in pregnant women with overweight/obesity was higher than that in the non-overweight/non-obese pregnant women (group N2 vs. group N1, group G2 vs. group G1, $P < 0.05$). The
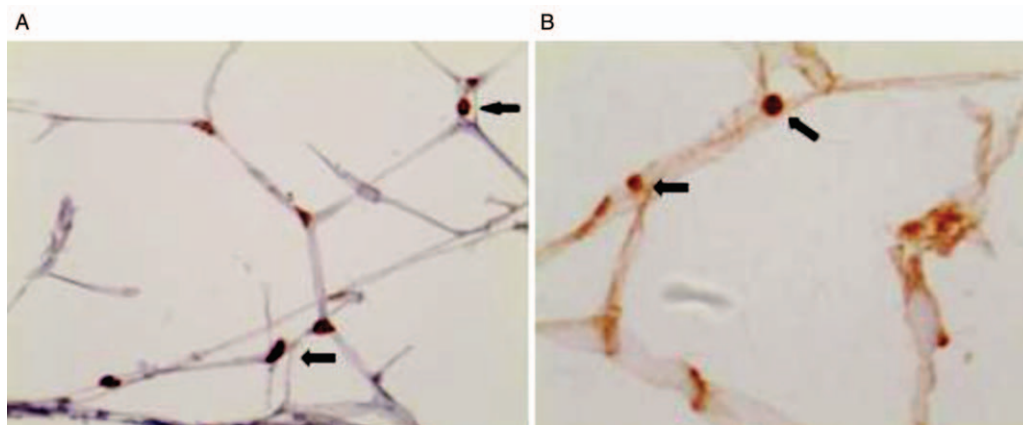
Figure 1: Immunohistochemistry was used to observe the location of VDR and PPARγ in adipose tissue (Streptomyces antibiotic protein-peroxidase ligation staining, original magnification ×400). Brown-yellow granules (arrows) were seen in the adipose tissue nucleus, indicating the location of VDR (A) and PPARγ (B) in the adipose tissue nucleus. PPARγ: Peroxisome proliferator-activated receptor γ; VDR: Vitamin D receptor.

Table 4: Immunohistochemical semi-quantitative expression intensity and qPCR expression intensity of VDR and PPARγ in the four groups.

	Non-overweight and obesity				Overweight and obesity				GDM (G1 vs. G2)		Non-GDM (N1 vs. N2)	
Indexes	N1	G1	t/Z	P	N2	G2	t/Z	P	t/Z	P	t/Z	P
Immunohistochemistry												
VDR	1.34 ± 0.59	1.80 ± 0.79	2.76	0.007	1.46 ± 0.56	2.06 ± 0.76	3.760	<0.001	1.403	0.166	0.872	0.386
PPARγ	1.40 ± 0.49	1.89 ± 0.58	3.818	<0.001	1.63 ± 0.69	2.09 ± 0.82	2.539	0.013	1.178	0.243	1.608	0.112
qPCR												
VDR mRNA	89.34 (63.74–159.92)	210.00 (90.58–311.46)	−3.101	0.002	198.28 (119.46–261.23)	298.67 (170.84–451.25)	−2.672	0.008	−2.249	0.024	−3.894	<0.001
PPARγ mRNA	87.52 (66.37–100.04)	100.72 (88.61–123.87)	−2.907	0.004	89.90 (76.95–109.09)	117.33 (100.08–149.00)	−3.518	<0.001	−1.973	0.048	−1.774	0.076
Western blotting												
PPARγ	0.05 ± 0.09	0.07 ± 0.01	7.691	<0.001	0.07 ± 0.01	0.09 ± 0.02	4.902	<0.001	2.156	0.035	4.026	0.001
p-PPARγ	0.38 ± 0.09	0.65 ± 0.19	7.551	<0.001	0.55 ± 0.11	0.74 ± 0.10	7.421	<0.001	2.325	0.023	7.178	<0.001

Data are expressed as mean ± standard deviation or median (P_{25}–P_{75}). The statistical results of immunohistochemistry, Western blotting are expressed by t value. The results of qPCR are expressed by Z value. qPCR: Quantitative real-time polymerase chain reaction; VDR: Vitamin D receptor; PPARγ: Peroxisome proliferator-activated receptor γ; GDM: Gestational diabetes mellitus; Group G1: Non-overweight and non-obese pregnant women with GDM; Group G2: Overweight/obese pregnant women with GDM; Group N1: Non-overweight and non-obese pregnant women with normal glucose tolerance; Group N2: Overweight/obese pregnant women with normal glucose tolerance; p-PPARγ: PPARγ Ser273 phosphorylation.
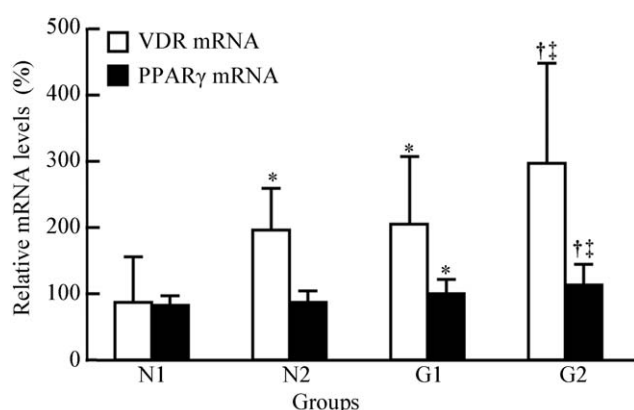

Figure 2: Adipose tissue VDR/GAPDH and PPARγ/GAPDH expression in the four groups. *$P < 0.05$, compared with group N1; †$P < 0.05$, compared with group N2; ‡$P < 0.05$, compared with group G1. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GDM: Gestational diabetes mellitus; Group G1: Non-overweight and non-obesity pregnant women with GDM; Group G2: Overweight/obesity pregnant women with GDM; Group N1: Non-overweight and non-obesity pregnant women with normal glucose tolerance; Group N2: Overweight/obesity pregnant women with normal glucose tolerance; PPARγ: Peroxisome proliferator-activated receptor γ; VDR: Vitamin D receptor.

same changes were observed in the levels of Ser273 phosphorylated PPARγ [Table 4 and Figure 3].

Correlation test

VDR mRNA levels positively correlated with the pre-pregnancy BMI, pre-delivery BMI, FBG, HOMA-IR, and PPARγ mRNA while negatively correlated with the VD and adiponectin levels ($r = 0.395, 0.336, 0.240, 0.190, 0.235, −0.350, −0.294, P < 0.05$) [Table 5], the PPARγ mRNA levels positively correlated with the pre-pregnancy BMI, pre-delivery BMI, FBG, HOMA-IR, TC, TG, FFA, and VDR mRNA while it negatively correlated with the VD and adiponectin levels ($r = 0.276, 0.199, 0.210, 0.230, 0.182, 0.214, 0.270, 0.235, −0.232, −0.199, P < 0.05$).

Logistic regression analysis

In the overweight/obese women, univariate logistic regression analysis showed that VD, adiponectin, VDR mRNA, PPAR mRNA, HDL-C, FFA, FINS, HOMA-IR levels correlated with GDM ($P < 0.05$). Multivariate logistic

regression analysis showed that HDL-C (OR = 0.065, 95% CI: 0.007–0.592) and FFA (OR = 39.853, 95% CI: 2.363–672.154) correlated with GDM.

In the non-overweight/non-obese women, univariate logistic regression analysis showed that VD, VDR mRNA, PPAR mRNA, HDL-C, FFA, and HOMA-IR levels correlated to GDM, $P < 0.05$. Multivariate logistic regression analysis showed that VDR mRNA (OR = 1.007, 95% CI: 1.000–1.013), PPAR mRNA (OR = 1.029, 95% CI: 1.003–1.057), HOMA-IR (OR = 2.568, 95% CI: 1.004–6.564) levels correlated to GDM.

Discussion

We revealed that VD deficiency exists in pregnant women in their third trimester, and under the same conditions, such as the age of pregnancy, gravidity and parity, gestational weeks, calcium and VD supplement, and sun exposure, the VD level in GDM patients with overweight/obesity decreased significantly, but the VDR and PPARγ mRNA levels in the adipose tissue were up-regulated. Studies have shown that higher the BMI of obese people, lower the concentration of VD.[18] High BMI[19] and low vitamin status were closely related to GDM. Active VD bind to the VDR, which is a ligand-dependent nuclear transcription factor and has roles in regulating the metabolism of calcium and phosphorus, cell proliferation and differentiation, and immune function together with

Figure 3: Western blotting of PPARγ/p-PPARγ in the four groups. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GDM: Gestational diabetes mellitus; Group G1: Non-overweight and non-obesity pregnant women with GDM; Group G2: Overweight/obesity pregnant women with GDM; Group N1: Non-overweight and non-obesity pregnant women with normal glucose tolerance; Group N2: Overweight/obesity pregnant women with normal glucose tolerance; PPARγ: Peroxisome proliferator-activated receptor γ; p-PPARγ: PPARγ Ser273 phosphorylation.

1,25(OH)$_2$D$_3$.[20] Knabl et al[21] has reported that the maternal VD level in GDM patients is lower, and low-dose exogenous VD can up-regulate VDR in the trophoblasts. Specific transfer of VDR to VDR-knockout mice can promote the growth of adipose tissue and induce fat accumulation in the body,[22] thus leading to obesity. BMI and HOMA-IR were independent positive predictors of subcutaneous fat VDR gene expression.[23] This study is consistent with previous reports.

PPARγ is a member of the nuclear receptor superfamily. The binding of 1,25-(OH)$_2$D$_3$ to VDR and inhibition of adipogenesis are closely related to the activity of PPARγ. The 1,25-(OH)$_2$D$_3$ regulates lipogenesis mainly by reducing the formation of PPARγ ligand in early stages of adipocyte differentiation,[24] decreasing the transcriptional activity of PPARγ,[25] or directly regulating its upstream factors.[26] Studies have shown that the expression level of PPARγ gene positively correlated with the size of adipocyte volume and its differentiation degree,[27] and that the excessive activation of PPARγ is involved in the occurrence of obesity. Phosphorylation is the most common post-transcriptional modification of PPARγ. CDK5-mediated phosphorylation of PPARγ Ser273 in adipose tissue is considered to be associated with obesity. CDK5-mediated phosphorylation of PPARγ may be involved in the pathogenesis of insulin-resistance, and present an opportunity for development of an improved generation of anti-diabetic drugs through PPARγ.[28] The PPARγ ligands include polyunsaturated fatty acids, thiazolidinedione (TZD), etc, of which TZD has been used as an insulin sensitizer for the treatment of T2DM. Belenchia et al[29] studied the filial generation in pregnant mice with VD deficiency during perinatal period and their studies suggested that VD deficiency can directly affect the development of adipose tissue in the non-obese offspring, and the VD deficient progeny has stronger fat regulation genes that could regulate the expression of PPARγ and VDR. Studies by Nobre et al[30] suggested that CCAAT/enhancer binding protein beta (C/EBPβ) and PPARγ are highly expressed in the adipose tissue of obese animals. The cyp27b1-1 hydroxylase and VDR expression is decreased in prosome adipocyte 3T3L1 incubated with 1,25(OH)$_2$D. C/EBPβ and PPARγ are decreased. The level of PPARγ in the plasma of GDM pregnant women is significantly higher than that of other groups. With the increase of PPARγ concentration, the cytoplasmic lipid uptake increases suggesting that PPARγ may participate in the regulation
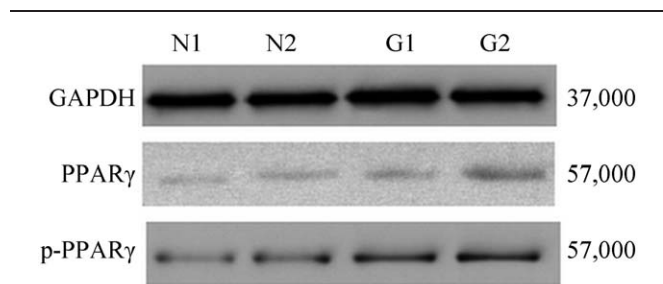
Items	Pre-pregnancy BMI	Pre-delivery BMI	FBG	FINS	HMOA-IR	TG	TC	HDL-C	FFA	VD	VDR mRNA	PPARγ mRNA	Adiponectin
r	0.395	0.336	0.240	0.101	0.190	0.005	<0.001	−0.157	0.162	−0.350	–	0.235	−0.294
P	<0.001	<0.001	0.004	0.234	0.025	0.951	0.999	0.064	0.056	<0.001	–	0.005	<0.001
r*	0.276	0.199	0.210	0.161	0.230	0.182	0.214	−0.080	0.270	−0.232	0.235	–	−0.199
P*	0.001	0.019	0.013	0.057	0.006	0.031	0.011	0.348	0.001	0.006	0.005	–	0.018

Table 5: Spearman correlations between expression of VDR and PPARγ mRNA with BMI and biochemical parameters in all the pregnant women.

r and P is the value of correlation analysis of VDR mRNA; r* and P* is the value of correlation analysis of PPARγ mRNA. VDR: Vitamin D receptor; PPARγ: Peroxisome proliferator-activated receptor γ; BMI: Body mass index; FBG: Fasting blood glucose; FINS: Fasting insulin; HOMA-IR: Homeostasis model assessment of insulin resistance; TC: Total cholesterol; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol; FFA: Free fatty; VD: vitamin D.

of lipid transport among the maternal-fetal interface cells and might have a role in the lipid dysmetabolism in GDM patients.[31]

In this study, the expression of FINS and HOMA-IR increased in patients with diabetes and overweight/obesity, whereas that of HDL-C and adiponectin decreased; the FFA levels in patients with GDM increased. In the non-overweight/obese women, VDR mRNA, PPAR mRNA, and HOMA-IR were related to GDM, while in the women with overweight/obesity, HDL-C and FFA levels were related to GDM. VDR/PPARγ expression correlated to the glucose levels and lipid metabolism. Herrera and Desoye[32] has shown that lipid metabolism is abnormal in diabetic patients, IR exists in the adipose tissue of obese and diabetic pregnant women, and adipose tissue plays an important role in the pathogenesis of diabetes.[12] VDR levels positively correlate with IR,[33] Pregnant women with high pre-pregnancy BMI or GDM have impaired FFA transport at the mother-fetal interface,[34]GDM and FFA levels also correlate with IR,[35] adiponectin is an adipokine and an endogenous insulin sensitizer that reduces the circulating level of insulin in patients obesity and diabetes. Mousa et al[36] showed that the baseline concentration of 25(OH)D negatively correlated with TC /TG and positively correlated with adiponectin in 102 high-risk women with overweight or obesity. Adiponectin can up-regulate the PPARγ expression through by regulating the insulin content and insulin secretion, and decreased levels of adiponectin in the circulation of obese individuals may be directly associated with the β-cell dysfunction in T2DM.[37]

PPARγ and VDR are the members of transcription factor and nuclear receptor superfamily, which regulates the signaling cascade by interacting with other nuclear receptors and transcription factors. Transcription factors, VDR, and PPARγ regulate the gene transcription by acting as VD's reactive elements or peroxisome proliferator response elements in the promoter of the target genes.[38,39] VDR and PPARγ also interact with nuclear receptors, and the corresponding heterodimers formed by their binding with the retinol X receptor regulate the activation of the target genes.[38,39] In this study, we found that the serum VD level is lower in patients with GDM and obese women, the transcription level of VDR and PPARγ in the adipose tissue increased, which may be caused by the negative feedback initiated by low VD. Increased VDR can result in lipopexia, increase IR, and lipid metabolism disorder; FFA, as a ligand of PPARγ, increases significantly, which can increase the transcriptional activity of PPARγ, thus further leading to lipopexia and obesity. Obese patients then suffer from lipid dysmetabolism, significant adiponectin decrease, β-cell dysfunction, and insulin sensitivity decrease, which further leads to diabetes and thus forms a vicious circle.

Therefore, our studies suggest a possibility that obese women with VD deficiency are at high risk of GDM. VD may regulate the formation and differentiation of fat cells through the nuclear receptor VDR and PPARγ pathways and participate in the occurrence of GDM. Adipokines play certain roles in the pathogenesis of GDM.

This study explored the correlation between VD and VDR levels, and PPARγ expression, in subcutaneous adipose tissue of overweight/obese diabetic pregnant women. However, the study had small sample size and did not investigate the molecular mechanisms of the VDR and PPARγ pathway. So the specific mechanism of how adipose tissue and adipokines contributes to the pathogenesis of GDM remains to be further studied. The roles of VD supplement in clinical work and weight control during pregnancy in reducing the incidence of GDM remains to be further studied.

Conflicts of interest

None.

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