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ORIGINAL ARTICLE

The Expression and Clinical Value of miR-221 and miR-320 in the Plasma of Women with Gestational Diabetes Mellitus

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SUMMARY

Background: **The goal was to investigate the expression of plasma miR-221 and miR-320 in gestational diabetes mellitus (GDM) and to further explore the relationship between miRNA and risk factors for GDM.**
Methods: **This study included 85 GDM and 85 age-matched normal pregnant women who visited our hospital from January 2019 to January 2020. Real-time polymerase chain reaction (RT-qPCR) was used to determine the expression of miR-221 and miR-320 in the plasma of pregnant women. The correlation analysis was used to detect the relationship between miR-221, miR-320, and risk factors of GDM, including homeostatic model assessment for insulin resistance (HOMA-IR), percentage of glycosylated hemoglobin (HbA1c), and the pre-pregnancy BMI. The receiver operating characteristic curve (ROC) was used to determine the diagnostic value of miR-320 and miR-221 in GDM.**
Results: **Compared with normal pregnant women, the expression of miR-221 and miR-320 in GDM was significantly higher ($p < 0.05$). The results also demonstrate that the expression of miR-221 and miR-320 increases gradually with the development of pregnancy in GDM at 24 weeks, 28 weeks, and 32 weeks ($p < 0.05$). Spearman's correlation analysis confirmed that the expression level of miR-221 and miR-320 in the plasma of GDM is positively correlated with the HOMA-IR and HbA1c, but has no significant correlation with pre-pregnancy BMI. The area under the curve (AUC) values of miR-221 and miR-320 were 0.862 and 0.853, respectively. Meanwhile, the area under the combined detection curve is 0.904.**
Conclusions: **Plasma miR-221 and miR-320 are significantly elevated in GDM, and are positively correlated with HbA1c and HOMA-IR. The high expression of miR-221 and miR-320 in the peripheral plasma of pregnant women may directly or indirectly participate in the occurrence and development of GDM or may become a new target for the diagnosis, treatment, and prognosis of GDM.**
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KEY WORDS

miR-221, miR-320, gestational diabetes mellitus, homeostatic model assessment for insulin resistance, percentage of glycosylated hemoglobin

INTRODUCTION

Gestational diabetes mellitus (GDM) is a common disease in pregnant women, which seriously threatens the health of mothers and babies [1]. During the past decade, GDM has become a major public health issue and

one of the most discussed topics in modern obstetrics and has gained worldwide popularity [2]. It is generally believed that the pathogenesis of GDM is complicated, which may be the result of multiple factors such as inflammatory factors, insulin resistance, genetics, and environment [3].

MicroRNA (miRNA) is a small non-coding RNA with 18 - 22 nucleotides [4]. Exploring the function of miRNAs may improve understanding of the etiology and pathophysiology of GDM, and may lead to the diagnosis of GDM earlier than currently available methods. Dai et al. demonstrated that the level of miR-2467 in the serum and placenta of pregnant women with GDM was higher than that in the control group, suggesting that the increase in serum miR-2467 may be involved in the occurrence of GDM [5]. Interestingly, the down-regulation of miR-185 expression in serum and placenta of pregnant women with GDM is correlated with HOMA-IR, suggesting that the reduction of miR-185 may play an important role in the occurrence and development of GDM [6]. In terms of glucose and lipid metabolism, many studies have found that the expression level of miR-221 and miR-320 in the blood or tissues of patients with metabolic syndrome, diabetes, and its complications or animal disease models has changed [7-9]. MiR-221 and miR-320 also have been shown to be associated with insulin resistance [9,10], but the expression and role of miR-221 and miR-320 in plasma of gestational diabetes have not been explicitly well studied.

Therefore, by analyzing the differences in the expression of miR-221 and miR-320 in the plasma of GDM and normal pregnant women, this paper discussed the possible regulatory relationship between miRNAs and HOMA-IR, HbA1c, and pre-pregnancy BMI, and revealed that miRNA-221 and miRNA-320 may be used as a prediction and therapeutic target for the pathological process of GDM.

MATERIALS AND METHODS

Patient samples

We collected 85 pregnant women with GDM in our hospital from January 2019 to January 2020, and 85 normal pregnant women in the same period were collected by age-matched method. Diagnosis criteria for gestational diabetes: carry out a glucose tolerance test at 24 weeks of gestation, fast for 8 hours, check the blood glucose indicators in the fasting state, then take 75 g of glucose. Diagnosis points: (1) fasting blood glucose ≥ 5.0 mmol/L; (2) 1-hour postprandial blood glucose ≥ 10 mmol/L; (3) 2-hour postprandial blood glucose ≥ 8.5 mmol/L. As long as one of the requirements are met, gestational diabetes can be diagnosed. The exclusion criteria mainly include: (1) pre-pregnancy diabetes or family history of diabetes; (2) age < 18 years and ≥ 35 years; (3) family with abnormal pregnancy history; (4) family history of fetal chromosomes abnormalities or major malformations; (5) serious systemic diseases. All participants signed an informed consent form. This study was approved by the obstetrics and gynecology health and human research ethics committee of Bozhou People's Hospital.

Sample collection

In this study, we collected 2 mL of peripheral blood of normal pregnant women and those diagnosed with GDM at 24 weeks and also 2 mL of peripheral blood of the same pregnant women with confirmed gestational diabetes and the same normal pregnant women at 28 and 32 weeks. The pre-pregnancy BMI, HOMA-IR, and HbA1c of pregnant women with confirmed GDM at 24 weeks were calculated. The homeostasis model assessment of insulin resistance (HOMA-IR) is calculated as: (empty abdominal blood glucose x fasting insulin)/22.5. BMI = weight (kg)/height^2 (m).

MiRNA extraction and RT-qPCR

The blood sample was collected in a blood collection tube containing EDTA, and the plasma was separated by centrifugation at 3,000 g at 4°C for 5 minutes. Total RNA was isolated from 200 μL plasma using RNeasy plus mini kit (Qiagen). NanoVue plus (GE Healthcare, Piscataway, NJ, USA) measures total RNA concentration and purity. According to GeneCopoeia's "All-in-One^{TM} miRNA First-Strand cDNA Synthesis Kit" operating instructions, take 1 - 3 μg RNA to establish a 20 μg reverse transcription system to synthesize cDNA. Gene-Copoeia's "miRNA qPCR Kit" kit and ABI7500 Fast Real-time PCR amplification instrument for fluorescence amplification. U6 was used as an internal reference, and ΔCt (Ct purpose-Ct internal reference) method was used for relative quantitative analysis. The $2^{-\Delta\Delta Ct}$ was used as the relative expression of target RNA. MiR-221, miR-320, and U6 upstream primers were purchased from Guangzhou Funeng Company. The sequences were as follows: miR-221 forward,
5′- CAGCATACATGATTCCTTGTGA-3′;
and reverse
5′- CTT TGGTGTTTGAGATGT TTGG-3′;
miR-320 forward,
5′- ACACTCCAGCTGGGAAAAGCTGGGTTGAGA -3′;
and reverse
5′-TGGTGTCGTGGAGTCG-3′;
U6 forward,
5′-AGAGCCTGTGG TGTCC-3′;
and reverse
5′-CATCTTCAAAGCACTTCCCT-3′.

Statistical methods

The SPSS20.0 software was used for statistical processing and analysis. The measurement data were tested for normality and homogeneity of variance. Data were expressed as mean ± standard deviation. The statistics of the mean between the two groups were analyzed by LSD *t*-test. Pearson correlation analysis was used to evaluate the correlation between microRNA and risk

Table 1. The clinicopathological factors of healthy pregnancies and GDM patients.

Characteristics	No-GDM (n = 85)	GDM (n = 85)	t	p-value
Age (years)	26.35 ± 3.37	26.87 ± 3.40	1.001	0.318
Pregnancy (times)	2.35 ± 0.92	2.47 ± 0.97	0.826	0.409
Delivery (times)	1.36 ± 0.57	1.26 ± 0.54	1.174	0.242
Pregnant week	38.26 ± 1.66	38.66 ± 1.70	1.552	0.123
FPG (mmol/L)	4.26 ± 0.56	5.76 ± 0.43	19.200	< 0.001
HbA1c (%)	3.25 ± 0.74	5.51 ± 0.96	17.190	< 0.001
HOMA-IR	3.13 ± 0.68	5.41 ± 0.88	18.900	< 0.001
BMI (kg/m^2)	24.15 ± 2.48	29.82 ± 2.40	15.150	< 0.001
Neonatal weight (g)	3,352.94 ± 403.14	3,404.71 ± 420.29	0.819	0.413

GDM - gestational diabetes mellitus, No-GDM - pregnant women with no GDM diagnosis, HbA1c - percentage of glycosylated hemoglobin, HOMA-IR - Homeostatic Model Assessment for Insulin Resistance, BMI - pre-pregnancy body mass index, FPG - fasting plasma glucose.

Table 2. The diagnostic value of miR-221 and miR-320 for GDM.

Biomarker	Cutoff value	Sensitivity %	Specificity %	Youden's index	AUC	95% CI
miR-320	2.125	84.00	80.22	64.22	0.862	0.780 ~ 0.944
miR-221	1.723	81.26	79.23	60.49	0.853	0.773 ~ 0.932
miR-221 + miR-320	-	87.69	85.38	73.07	0.904	0.852 ~ 0.953

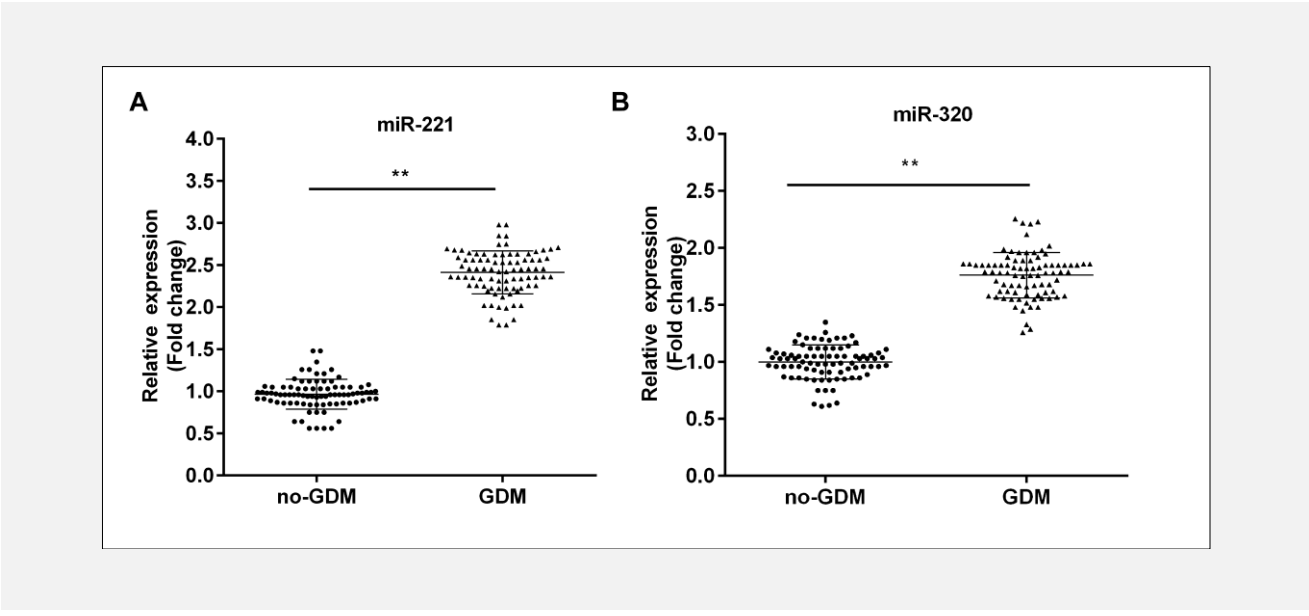

Figure 1. The expression of miR-221 and mR-320 in the plasma from no-GDM pregnant women and GDM patients.

(A) The expression of miR-221 in the plasma of 170 samples from no-GDM pregnant women and GDM patients. (B) The expression of miR-320 in the plasma of 170 samples from no-GDM pregnant women and GDM patients. GDM-gestational diabetes mellitus, no-GDM-pregnant women with no GDM diagnosis. **p < 0.001.

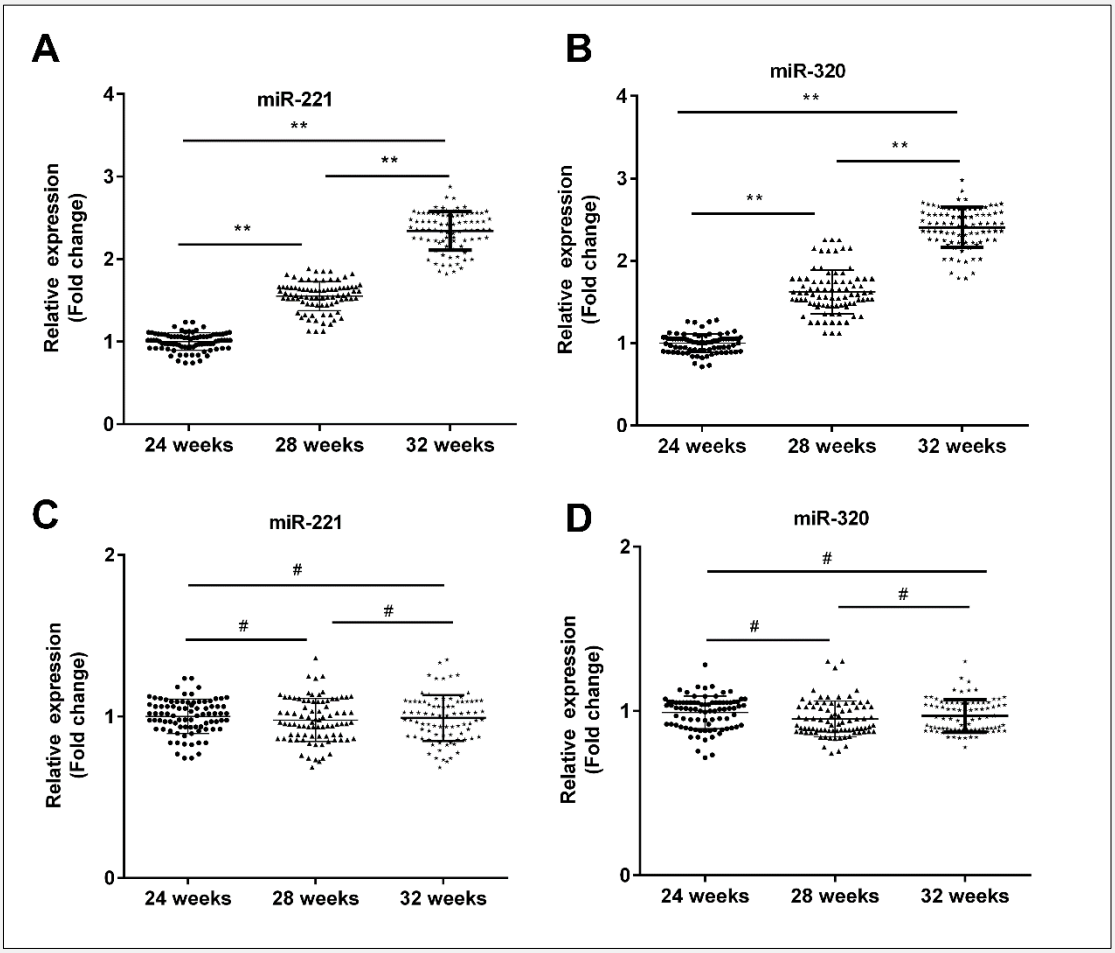

Figure 2. The expression of miR-221 and miR-320 in pregnant women with GDM (A and B) and no-GDM (C and D) in different pregnancy periods.

GDM - gestational diabetes mellitus, no-GDM - pregnant women with no GDM diagnosis. ** $p < 0.001$, # $p > 0.05$.

factors. The diagnostic value of miR-221 and miR-320 in GDM were calculated using ROC analysis. The result is considered statistically significant if $p < 0.05$.

RESULTS

Clinical Features

The clinical characteristics of 85 GDM pregnant women and 85 non-GDM pregnant women are shown in Table 1. GDM pregnant women have higher FPG (fasting plasma glucose), HbA1c, HOMA-IR and pre-pregnancy BMI than normal pregnant women at 24 weeks, and the difference is statistically significant.

The expression of miR-221 and miR-320 is increased in the plasma of pregnant women with GDM

The RT-qPCR method detects the expression of miR-221 and miR-320 in the plasma of pregnant women with GDM at 24 weeks, 28 weeks, and 32 weeks, as shown in Figure 1 and 2.

The expression of miR-221 and miR 320 in the plasma of pregnant women with GDM increases progressively

The expression of miR-221 and miR-320 in plasma of pregnant women with GDM at 32 weeks of gestation was about 2.3 times and 2.4 times, respectively, higher than that at 24 weeks of gestation. Interestingly, the expression of miR-221 and miR-320 increases progressively with the prolonged pregnancy time in GDM preg-

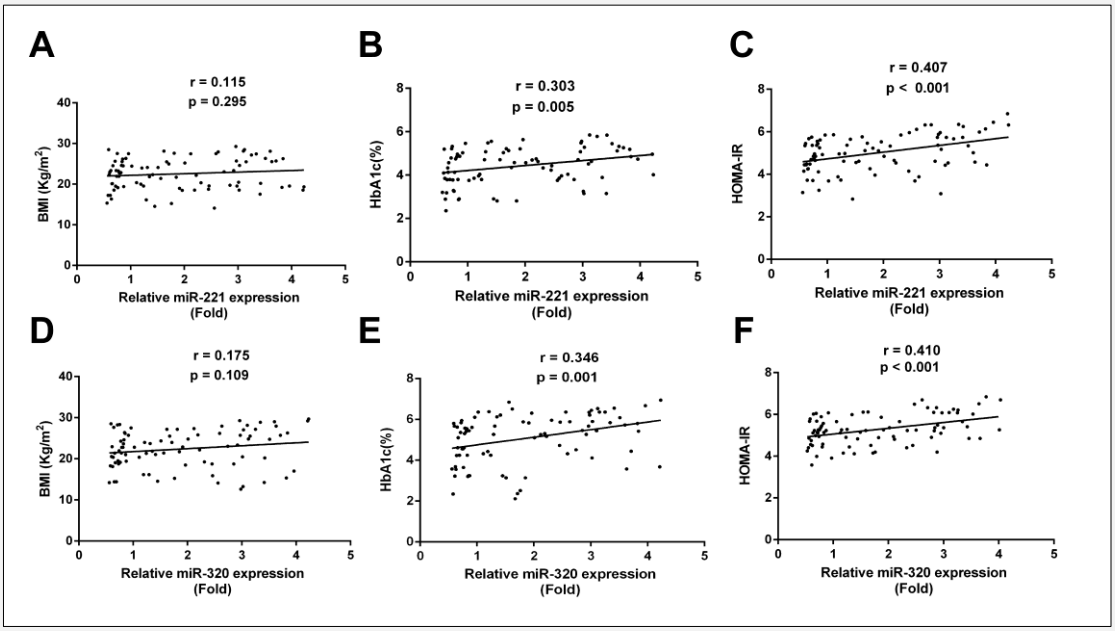

Figure 3. The correlation analysis of plasma miR-221 and miR-320 with clinical data (BMI, HbA1c and HOMA-IR) in GDM patients.

(A) and (D) There has no significant correlation with the pre-pregnancy BMI. The present study demonstrated a positive correlation between plasma miR-221 and HbA1c (B) and HOMA-IR (E). A positive correlation was also found between plasma miR-320 and HbA1c (C) and HOMA-IR (F). HbA1c - percentage of glycosylated hemoglobin, HOMA-IR - Homeostatic Model Assessment for Insulin Resistance, BMI - pre-pregnancy body mass index.

nant women. However, the expression levels of miR-221 and miR-320 in the plasma of normal pregnant women did not change significantly with the prolonged pregnancy time.

Pearson correlation analysis for GDM pregnant women miR-221 and miR-320 expression

We analyzed the correlation between the expression of miR-221 and miR-320 in the plasma of pregnant women with GDM at 24 weeks of gestation and the pre-pregnancy BMI, HbA1c, and HOMA-IR. The results are shown in Figure 3. The expression of miR-221 and miR-320 in plasma is positively correlated with HOMA-IR ($r = 0.407$, $p < 0.001$; $r = 0.410$, $p < 0.001$) and HbA1c ($r = 0.303$, $p = 0.005$; $r = 0.346$, $p = 0.001$), but has no significant correlation with the pre-pregnancy BMI ($r = 0.115$, $p = 0.295$; $r = 0.175$, $p = 0.109$).

The diagnosis value of miR-221 and miR-320 in GDM

In the present study, the ROC curve was used to measure the diagnostic value of miR-320 and miR-221 in GDM. As shown in Table 2. The area under the curve (AUC) values of miR-320 and miR-221 were 0.862 (84.00% sensitivity and 80.22% specificity) and 0.853 (81.26% sensitivity and 79.23% specificity). Meanwhile, the area under the combined detection curve is 0.904 (87.69% sensitivity and 85.38% specificity).

DISCUSSION

GDM refers to diabetes occurring in pregnancy in women who did not have diabetes prior to pregnancy. Diabetes during pregnancy is one of the common diseases in obstetrics and gynecology, which seriously threatens the health of mothers and babies. For pregnant women, the main risks are cesarean section, pre-eclampsia, and increased risk of type 2 diabetes [10]. Macrosomia, hypoglycemia, dystocia, respiratory distress syndrome, and death are serious threats to the fetus and baby health [11]. Current main treatment methods for gestational diabetes are exercise therapy, diet control and insulin replacement therapy to reduce diabetes-related complications and try to improve the prognosis of pregnant women [12]. Presently, the clinical diagnosis of gestational diabetes uses the American Diabetes Association (ADA) and International Association of Diabetes and

Pregnancy Study Groups (IADPSG) standard diagnosis [13]. Diagnosis is usually carried out at 24 to 28 weeks of pregnancy and can sometimes be delayed to 32 weeks. Therefore, there is an urgent need to accurately and quickly predict and diagnose gestational diabetes, and early intervention for pregnant women with gestational diabetes to improve the prognosis of pregnant women.

Many studies have tried to find potential biological markers that may predict GDM, including changes in gene expression early and second trimester, epigenetic modifications, and non-coding RNA changes [14]. However, these biomarkers have some limitations. miRNA is a small non-coding RNA, which regulates the expression of target genes at the post-transcriptional level and participates in the occurrence and development of many diseases [15]. Related studies have confirmed that miRNAs in peripheral blood are similar to microRNAs in tissues [16]. Because miRNAs are stable in peripheral blood and easy to collect, miRNAs have been used as biological markers for diagnosis and prognosis in a variety of diseases, including tumors, cardiovascular diseases and diabetes [17,18]. However, when GDM is diagnosed at 24 to 28 weeks of gestation, whether plasma microRNAs can be used as diagnostic biomarkers for GDM is unclear.

Zhao et al. confirmed that the expression of miR-29a, miR-132, and miR-222 decreased in the peripheral blood of gestational diabetic patients such as early diagnosis [19]. Cao et al. also found that miRNA in the peripheral blood of patients with gestational diabetes at 24 to 28 weeks the expression of miR-16-5p, miR-17-5p and miR-20a-5p is significantly increased, and may be related to high-risk factors of gestational diabetes [20]. This study found that, compared with normal pregnant women, plasma miR-221 and miR-320 expressions in GDM pregnant women were significantly up-regulated at 24 weeks of gestation, and the expressions of the two miRNAs increased as pregnancy progressed. It suggests that miR-221 and miR-320 may play an important role in the occurrence and progression of gestational diabetes.

Some studies have shown that pregnant women with GDM have a higher BMI, HbA1c, and HOMA-IR than healthy pregnant women, and these indicators have been identified as risk factors for GDM [21,22]. Therefore, in this study, our team further analyzed whether plasma miR-221 and miR-320 are related to risk factors for GDM. Ours results showed that both miR-221 and miR-320 were positively correlated with HbA1c and HOMA-IR. Hence, these observations suggested that miR-221 and miR-320 were closely related to glucose metabolism disorders in GDM. It has been found that overexpression of miR-221 reduced the protein abundance of SIRT1 and caused inflammation and HOMA-IR in differentiated 3T3-L1 cells [9]. Similarly, miR-320 could regulate HOMA-IR in adipocytes by improving the insulin PI3-K signaling pathway [10]. Correspondingly, miR-27a promotes HOMA-IR and mediates glucose metabolism by targeting PPAR-γ-mediated PI3K/AKT signaling [23]. MiR-873 also has a role to play in the HOMA-IR and myocardial injury of GDM, and the low expression of miR-873 can slow down the progression of GDM [24]. Lately, a few studies demonstrated that miRNAs such as let-7c-5p and let-7a-5p and miR-124a expression were correlated with HbA1c levels in diabetes [25,26]. These findings provide information about potential new treatment strategies to control HOMA-IR and HbA1c levels. Therefore, it is speculated that miR-221 and miR-320 may be involved in GDM by HOMA-IR and HbA1c levels. However, its mechanism of action in GDM needs to be further studied.

In terms of the area under the ROC curve (AUC) analysis results show that both miR-221 (84.00% sensitivity and 80.22% specificity) and miR-320 (81.26% sensitivity and 79.23% specificity) have a certain diagnostic value for GDM, but both have the disadvantage of low sensitivity. Combined detection (87.69% sensitivity and 85.38% specificity) can effectively reduce the missed diagnosis and misdiagnosis rate of GDM diagnosis, and can provide more reliable information for clinical diagnosis and treatment.

To sum up, miR-221 and miR-320 may be used as an important target for the diagnosis, treatment, and prognosis of GDM. The shortcomings of this research are that the sample size is small and only the level of miRNA in plasma was detected. Therefore, the experimental conclusions and specific mechanisms of this study still need to be confirmed by further large samples and in-depth studies. In follow-up experiments, research also will be conducted before 24 weeks of pregnancy to provide a certain reference basis for early clinical diagnosis of GDM.

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Declaration of Interest:

The authors declare that they have no conflict of interest.

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