Received: 31 March 2022 | Revised: 7 May 2022 | Accepted: 25 May 2022

DOI: 10.1002/jcla.24557

RESEARCH ARTICLE

WILEY

The downregulation of miR-22 and miR-372 may contribute to gestational diabetes mellitus through regulating glucose metabolism via the PI3K/AKT/GLUT4 pathway

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Funding information
This project was supported by Science and Technology Innovation Strategy of Guangdong Province (2018A030313556) from the Guangdong Science and Technology Department.

Abstract

Background: Identifying effective regulatory mechanisms will be significant for Gestational diabetes mellitus (GDM) diagnosis and treatment.

Methods: The expressions of miR-22 and miR-372 in placenta tissues from 75 pregnant women with GDM and 75 matched healthy controls and HRT8/SVneo cells (a model of insulin resistance) were analyzed by qPCR. The expressions of PI3K, AKT, IRS, and GLUT4 in high glucose-treated HRT8/SVneo cells transfected with miR-22 or miR-372 mimics or inhibitors was assessed by Western blot. A luciferase gene reporter assay was employed to verify miRNAs' target genes.

Results: The expressions of miR-22 and miR-372 in placental tissues from GDM patients and HRT8/SVneo cells were significantly decreased compared with the respective controls. The GLUT4 expression was significantly decreased in the placenta tissues of GDM and HRT8/SVneo cells with high glucose transfected with miR-22 and miR-372 inhibitors. We confirmed that *SLC2A4*, the gene encoding GLUT4, was a direct target of miR-22 and miR-372. In this study, we report that the lower expressions of miR-22 and miR-372 in placental tissue from GDM patients.

Conclusion: Our results further suggested that the downregulations of miR-22 and miR-372 may contribute to GDM through regulating the PI3K/GLUT4 pathway.

KEYWORDS
gestational diabetes mellitus, glucose metabolism, GLUT4, miR-22, miR-372

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1 | INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as any degree of insulin resistance and glucose intolerance identified with onset or first recognition during pregnancy.[1,2] GDM is one of the most frequently diagnosed pregnancy complications, with a prevalence that ranges from 4% to 15% depending on the diagnostic criteria used and the ethnic population evaluated.[3] GDM patients are at increased risk of developing a wide range of chronic diseases after delivery, including type 2 diabetes, independently of other clinical risk factors.[4–6] Additionally, pregnant women with GDM are more susceptible to adverse pregnancy events, such as polyhydramnios, dystocia, fetal death, stillbirth, and fetal macrosomia, while infants with GDM display an increased incidence of neonatal disease.[7–10] The GDM epidemic, along with its related metabolic disorders, is rapidly spreading worldwide, and poses a health and life-threatening risk for pregnant women and their newborns, risks that may last into adulthood [11,12.] However, the mechanisms underlying the pathogenesis of GDM remain poorly understood.[13,14] Most women have no diabetes or present with only mild symptoms before pregnancy. Aggravated diabetes occurs only during pregnancy, but blood sugar levels and other physiological changes return to normal once women have given birth, which is the main clinical feature of GDM.[15] This suggests that the placenta, which appears briefly during pregnancy, is the main effector organ for this condition. During pregnancy, the levels of glucocorticoids, prolactin, progesterone, placental growth hormone, and other hormones increase and may further promote the occurrence of excessive insulin resistance and GDM.[16] Consequently, we mainly focused on the placental tissues of pregnant women for our investigation. The PI3K/AKT signaling pathway plays a key role in insulin resistance, and it is commonly investigated when analyzing the pathogenesis of diabetes.[17,18] Glucose transporters (GLUTs) are important components of the PI3K/AKT pathway. To date, six GLUT proteins have been identified, of which GLUT4 is the key glucose transporter into skeletal muscle.[19,20]GLUT4, encoded by *SLC2A4* (Chromosome 17p13),[21] can translocate from intracellular compartments to the cell surface membrane under insulin signaling stimulation, and is a major player in glucose homeostasis and the pathogenesis of diabetes.[22] Together, these observations suggest that the placenta may also be a target of the PI3K/AKT/GLUT4 signaling pathway. Accordingly, the aim of this study was to elucidate how this pathway regulates the placental changes that lead to GDM.

MicroRNAs (miRNAs) comprise a class of small noncoding RNA molecules 19–24 nucleotides long.[23] Despite being highly conserved, they have a wide diversity of expression profiles, and are involved in numerous biological processes by regulating target gene expression at the post-transcriptional level.[24,25] Several studies have shown that miRNAs contribute to the pathogenesis of GDM and type 2 diabetes.[26–29] For instance, the levels of miR-29a, miR-222, and miR-132 were reported to be significantly downregulated in women with GDM.[30] In addition, the human placenta exhibits a highly dynamic mRNA expression pattern during pregnancy.[31–33] We have previously shown that the levels of multiple miRNAs were significantly increased in placental tissues, some of which were involved in the regulation of the insulin signaling pathway.[34] However, the roles of miRNAs in glucose metabolism mediated by the insulin pathway during GMD remain unclear. Some studies have shown that miR-22 can regulate the occurrences and progressions of type 1 and 2 diabetes mellitus,[35,36]while few studies have shown the relationship between miR22 and GDM. Huan Zhang's study only showed the relationship between miR-22 and insulin resistance in the mouse model of GDM.[37] Up to now, its expression change of miR-22 in real placenta and its regulatory role in GDM cases are not clear. Moreover, the existing studies have reported that miR-372 plays a regulatory role in various diseases, such as tumor and ulcerative colitis,[38,39] but its role in diabetes and gestational diabetes has not been found. The regulatory role and internal mechanism of the both miRNAs in patients with GDM are worth exploring. Our results highlighted that miR-22 and miR-372 could improve glucose metabolic disorders in GDM and may serve as diagnostic biomarkers for GDM as well as promising therapeutic targets for the treatment of this condition.

2 | METHODS

2.1 | Study subjects

A total of 150 participants were recruited from the Armed Police Hospital Affiliated to Guangzhou Medical University between January 2018 and January 2019. All participants of the study agreed with written informed consent. The participants comprised 75 pregnant women with GDM (GDM group) and 75 healthy pregnant controls (HC group). The inclusion criteria used in this study were based on the guidelines of the World Health Organization and the International Diabetes Federation. The exclusion criteria included pregnant women with hypertensive disorder, congenital heart disease, twin pregnancy, hepatitis, thyroid dysfunction, acute inflammation, as well as those who gave premature birth.

2.2 | The acquisition of placental tissue

Tissues were obtained within 15 min of delivery. A placental lobule (cotyledon) was removed from the central region of the placenta. Tissue fragments were cut into $1cm^3$ pieces (100–200 mg wet weight). The basal plate and chorionic surface were removed from the cotyledon, and villous tissue was collected. The placental tissue was dissected to remove visible connective tissue and calcium deposits, blotted dry on filter paper, immediately snap-frozen in liquid nitrogen, and stored at −80°C until further analysis.

2.3 | Cell culture

HTR8/SVneo cells (Zhongyuan Heju Biotechnology Co.), a human chorionic trophoblast, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin) at 37°C with 5% CO_2. The medium was

changed every day. Cells were washed twice with PBS, and then inoculated into 6-well cell culture plates at a density of 3×10^5 cells per well. The cells were divided into a glucose free group and three high-glucose groups that were, respectively, treated with 10 mmol/L glucose (HG1 group), 30 mmol/L glucose (HG2), and 50 mmol/L glucose (HG3) and cultivated at 37°C with 5% CO_2 for 24 h.

2.4 | RNA extraction and preparation of miRNAs

Total RNA was extracted from 140 mg of placental tissue or 5×10^6 cells using TRIzol reagent (Magen Biotechnology Co.) according to the manufacturer's protocol. The quantity and quality of the obtained total RNA were evaluated using a spectrophotometer (NanoDrop). Small RNA molecules less than 35 nucleotides long were defined as miRNAs and separated from the extracted total RNA using an RNAmisi Kit (Aidlab).

2.5 | RT-qPCR

Total RNAs from all the placental tissues and cells were reverse transcribed using a TaKaRa PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa). qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using a miScript SYBR Green PCR Kit (TaKaRa). *U6* snoRNA was used as an endogenous control to normalize miRNA expression and the relative expression of miR-22 and miR-372 target genes was measured using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: hsa-miR-372-3p AAAGUGCUGCGACAUUUGAGCGU and hsa-miR-22-3p: GAAGCUGCCAGUUGAAG. The experiment results from cell sample source were representative from three independent experiments.

2.6 | Transfection of miR-22 or miR-372 mimics or antisense oligonucleotides

The miR-22 and miR-372 inhibitors and mimics were synthesized by Shanghai Integrated Biotech Solutions Co.,Ltd (SIBS Co.). The sequences of the mimics, inhibitors, and controls were showed in the Table 1. On the day before transfection, HTR8/SVneo cells were seeded at 2×10^4 /well in 24-well microplates with 50 mM glucose concentration. Lipofectamine RNAiMAX (Thermo Fisher Scientific) was prepared according to the manufacturer's instructions. MiR-22 or miR-372 mimics/inhibitors/negative control (NC) was diluted in 200 µl of Opti-MEM serum-free medium and added to the cells with the final concentration of 50 nM miRNA mimics and 100 nM inhibitors/NC. The cells were cultured at 37°C in 5% CO_2 for 24 h.

2.7 | Western blotting

During the Western Blotting (WB) experiment of placental tissues, tissue proteins were extracted by T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific) following the instructions. As for WB experiment of cell samples, cells were collected by centrifugation at 1006 *g* for 5 min after transfection. Total protein was extracted by M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). The protein concentration was identified by BCA protein assay kit (Beyotime Biotechnology) according to the specification. Equal amount of proteins (10 µg, 15 µl) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) at 80 V for 0.5 h and then120V for 1 h, which were electrotransferred onto PVDF membranes (Millipore) at 80 mA for 3 h. The membrane is cut to the matching destination strip area in advance. The membranes were incubated with primary antibodies (rabbit anti-human) targeting IRS1(1:500, Abcam), PI3K (p85-Y607) (1:500,Abcam), AKT(1:400,Abcam), GLUT4(1:500,Abcam) and beta-actin(1:1000,Abcam) at 4°C overnight. After washing with TBST, the membranes were incubated with the corresponding horseradish peroxidase-labeled secondary antibodies (goat anti-rabbit, 1:10000, Abcam) for 2 h. Drop the luminous liquid on the film and put it into the chemiluminescence instrument iBright FL1500 (Invitrogen Co.) for automatic exposure imaging. After exposure, the target blots area was intercepted in the imaging system to save the picture with absence of images of adequate length. The optical density of the target proteins was quantified using a gel image processing system, with beta-actin

TABLE 1 The sequences of the mimics, inhibitors, and controls

	Name		Sequences
1	hsa-miR-372-3p Mimics	Sense	5′-AAAGUGCUGCGACAUUUGAGCGU-3′
		Anti-sense	5′-GCUCAAAUGUCGCAGCACUUUUU-3′
2	hsa-miR-22-3p Mimics	Sense	5′-AAGCUGCCAGUUGAAGAACUGU-3′
		Anti-sense	5′-AGUUCUUCAACUGGCAGCUUUU-3′
3	hsa-miR-372-3p inhibitors		5′-ACGCUCAAAUGUCGCAGCACUUU-3′
4	hsa-miR-22-3p inhibitors		5′-ACAGUUCUUCAACUGGCAGCUU-3′
5	miRNA Mimic NC (control)	Sense	5′-UCACAACCUCCUAGAAAGAGUAGA-3′
		Anti-sense	5′-UACUCUUUCUAGGAGGUUGUGAUU-3′
6	miRNA inhibitor NC (control)		5′-UCUACUCUUUCUAGGAGGUUGUGA-3′

serving as the internal control. The experiments were repeated at least three times under the same conditions.

2.8 | Dual-luciferase gene reporter assay

The online tool RNA22 v2 microRNA target detection (https://cm.jefferson.edu/rna22/Interactive) was utilized to predict and analyze miRNA-22 and miRNA-372 target genes. A database query predicted the *SLC2A4* gene (also known as *GLUT4*) in the insulin pathway as being regulated by miR-22 and miR-372. The tool predicted that miR-22 and miR-372 had two binding sites at the 3'UTR of the *SLC2A4*: miR-22 may be targeted to 333-345 bp of *SLC2A4* 3'UTR and miR-372 targeted to its 68-74 bp (Data S1). According to these two binding sites, wild type and mutant plasmids were designed and synthesized by SIBS Co. The vector of the plasmid is psiCHECK-2 vector, and the sequence information is shown in Table 2. The reporter plasmids were co-transfected with the miR-22 or miR-372 mimic or the miR-22 or miR-372 NC into HTR8/SVneo cells using Lipofectamine 2000. After 48 h, luciferase activity was assessed using the dual-luciferase reporter assay system (Promega Corporation) according to the manufacturer's instructions. This results were representative from at least three independent experiments with three parallel well per sample.

2.9 | Statistical analysis

Data were presented as means ± standard deviation (SD). Comparisons were calculated using *t* tests between two groups or one-way analysis of variance (ANOVA) among groups (>two groups), respectively. *p*-values < 0.05 was considered to be statistically significant. Data were analyzed by using GraphPad Prism 8.0.2 software (GraphPad Software) or SPSS 22.0 software (IBM).

3 | RESULTS

3.1 | General features of the HC and GDM groups

The characteristics of the two groups data are shown in Table 3. No significant differences were found between the GDM and control groups in gestational age ($p = 0.160$), maternal body mass index (BMI) before pregnancy ($p = 0.750$), and fetal gender ($p = 0.833$). Therefore, the gestational age, BMI, and fetal gender were not considered to be contributing factors for GDM onset.

3.2 | The expressions of miR-22 and miR-372 in placental tissues

To explore the changes in miRNA levels in placental tissues, we first performed a qPCR assay on placental tissues derived from the GDM and HC groups. As shown in Figure 1, the expressions of miR-22 (Figure 1A) and miR-372 (Figure 1B) in placental tissues from participants in the GDM group were significantly lower than that of the HC group ($p < 0.001$). Specifically, the changes of two miRNA varied greatly, and the biggest change was observed with miR-22 treatment, which displayed a greater than 5000-fold difference.

3.3 | The expressions of miR-22 and miR-372 in HRT8/SVneo cells

To investigate the mechanisms underlying the miR-22- and miR-372-mediated regulation of target proteins in GDM, we established HRT8/SVneo cell model of glucose tolerance (Figure 2A). HRT8/SVneo cells were stimulated via a high-glucose gradient (10, 30, and 50 mM). We measured the expression of miR-22 and miR-372 in HRT8/SVneo cells, and found that the expressions of both miRNAs gradually decreased with increasing glucose concentrations (Figure 2B). These results were consistent with those obtained for the GDM and HC tissues, and suggested that high-glucose treatment was negatively correlated with miR-22 and miR-372 expression.

3.4 | PI3K/AKT/GLUT4 expression of insulin signaling pathway in placentas

As the insulin signaling pathway is the key mediator of GDM occurrence, we first sought to identify which proteins in this pathway were differentially expressed in placentas between GDM and HC groups by Western Blot. As shown in Figure 3A,B, the expressions of IRS,PI3K, AKT and GLUT4 were significantly downregulated in placenta tissue obtained from women in GDM group when compared with that in HCs: this trend was consistent with the changes

TABLE 2 Plasmid Construction Information

Serial number	Name	Target gene	Length (bp)	Plasmid	Cloning site
IG200017-1	psiCHECK-2-SLC2A4-wt1	SLC2A4-wt1	224	psiCHECK-2	XhoI, NotI
IG200017-2	psiCHECK-2-SLC2A4-mut1	SLC2A4-mut1	224	psiCHECK-2	XhoI, NotI
IG200017-3	psiCHECK-2-SLC2A4-wt2	SLC2A4-wt2	187	psiCHECK-2	XhoI, NotI
IG200017-4	psiCHECK-2-SLC2A4-mut2	SLC2A4-mut2	187	psiCHECK-2	XhoI, NotI

in miR-22 and miR-372 expression in placental tissues. It can be seen that the pathological state of GDM can inhibit the IRS/PI3K/AKT pathway and the low expression of both miRNAs occurs simultaneously. This results suggested that there may be a relationship between the low expressions of miR-22 and miR-372 and the regulation of this signal pathway in GDM.

TABLE 3 General features of the study participants

Variable	HC group	GDM group
Sample size	75	75
Gestational age	26.46 ± 2.1	27.54 ± 2.8
BMI before pregnancy	24.09 ± 2.31	25.17 ± 1.65
Fetal gender (M/F)	34/41	37/38

Abbreviations: BMI, body mass index; GDM, gestational diabetes mellitus; HC, healthy controls.

3.5 | MiR-22 and miR-372 regulated the PI3K/AKT/GLUT4 signaling pathway

Through the previous experimental results, we found that high-glucose exposure was negatively correlated with miR-22 and miR-372 expressions. For further study on the relationship between proteins changing in insulin signaling pathway and both miRNAs expression differences in GDM, we explored the regulatory effect of miR-22 and miR-372 on proteins of the PI3K/AKT/GLUT4 pathway in HRT8/SVneo cells transfected with miR-22 or miR-372 mimics or inhibitors. As shown in Figure 4A,B, the expression of GLUT4 was significantly increased in high-glucose-cultured HRT8/SVneo cells transfected with miR-22 or miR-372 mimics; in contrast, the expression of GLUT4 was significantly decreased in cells treated with miR-22 or miR-372 inhibitors (Figure 4C,D). Meanwhile, the expressions of IRS, PI3K, AKT and GLUT4 were all significantly decreased in cells

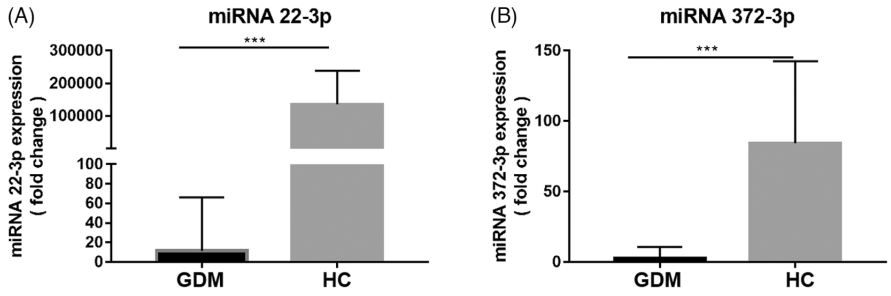


FIGURE 1 Expression levels of miR-22 and miR-372 in placental tissue. MiR-22 and miR-372 were isolated from placental tissue and quantified by RT-qPCR. (A) The miR-22-3p expression level in placental tissue of women with gestational diabetes mellitus (GDM) and in that of healthy controls (HCs). (B) The miR-372-3p expression level in placental tissue of women with GDM and in that of HCs. Student's *t* test, $^{***}p < 0.001$, $n = 75$

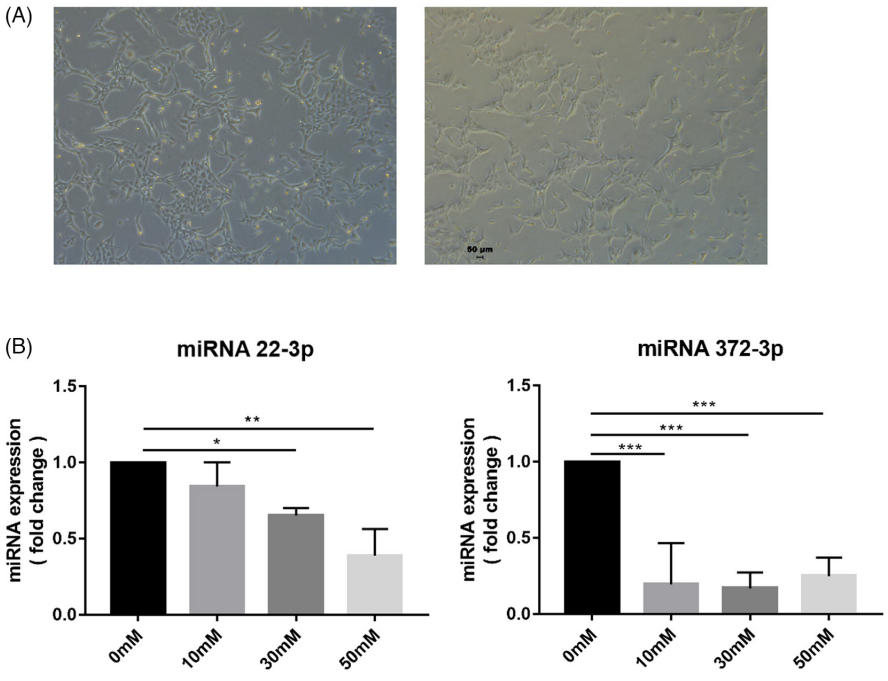


FIGURE 2 Expression levels of miR-22 and miR-372 in HRT8/SVneo (human chorionic trophoblast) cells. HRT8/SVneo cells were treated with glucose of 0,10, 30 and 50 mM concentration gradients, respectively, and the levels of miRNA-22 and miRNA-372 in HRT8/SVneo were detected by qPCR assay. (A) Representative images of HRT8/SVneo cells. (B) The expression level of miR-22 and miR-372 in HRT8/SVneo cells cultured with different glucose concentrations. The results were representative from at least three independent experiments. Left panel: miRNA-22; right panel: miRNA-372 (one-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$)

treated with miR-22 or miR-372 mimics or inhibitors. Interestingly, miR-372 exerted stronger regulatory effects on GLUT4 expression than miR-22.

3.6 | *SLC2A4* is a target gene for both miR-22 and miR-372

To further verify whether miR-22 and miR-372 directly interact with genes involved in the insulin signaling pathway, we established a dual-luciferase gene reporter assay and evaluated the correlation between these miRNAs and their putative target genes. A database query (using the RNA22 webtool) led to the prediction of a gene with role in the insulin signaling pathway—*SLC2A4* (the gene encoding GLUT4)—that was clearly regulated by miR-22 and miR-372 (Supplementary 1 and 2). Consequently, we designed and constructed plasmids containing either the wild-type or mutant forms of the two putative miR-22 and miR-372 target 3'UTRs of *SLC2A4* (Table S1). As shown in Figure 5, the results of the Dual-luciferase assay showed that miR-22 significantly decreased in both wild type and mutant type (Figure 5A,B), whereas miR-372 increased (Figure 5C), wild-type *SLC2A4* 3'UTR-driven luciferase activity, without statistical difference in the mutant type (Figure 5D). However, neither miRNA exerted a significant effect on *AKT1* 3'UTR-driven luciferase activity, or on that driven by their respective mutated 3'UTRs, suggesting that miR-22 and miR-372 directly target the 3'UTR of *SLC2A4* and regulate its expression.

4 | DISCUSSION

Studies have shown that changes in miRNA expression profiles in human plasma derived from GDM patients play a significant role in the progression of the disease.[30,32] However, because these miRNAs were derived from different tissues and cells, the differential miRNA expression profiles in the circulation did not fully and accurately

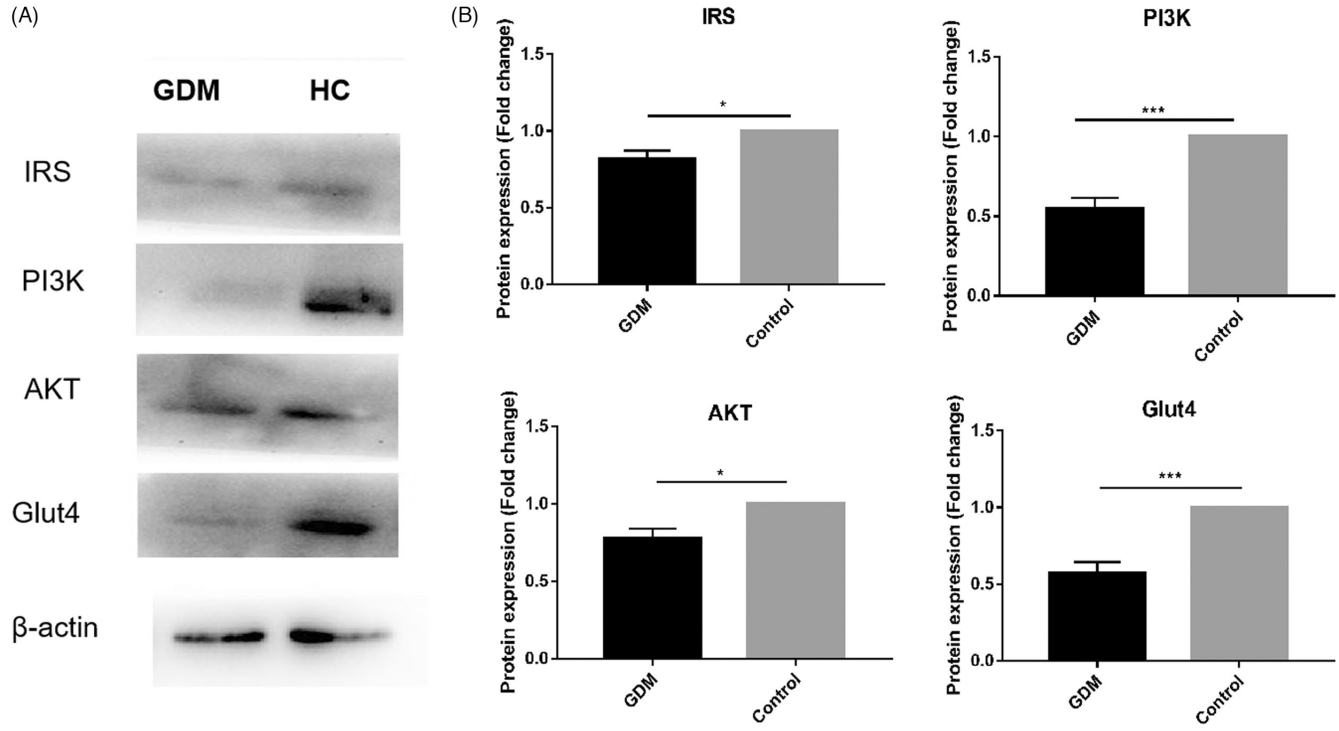
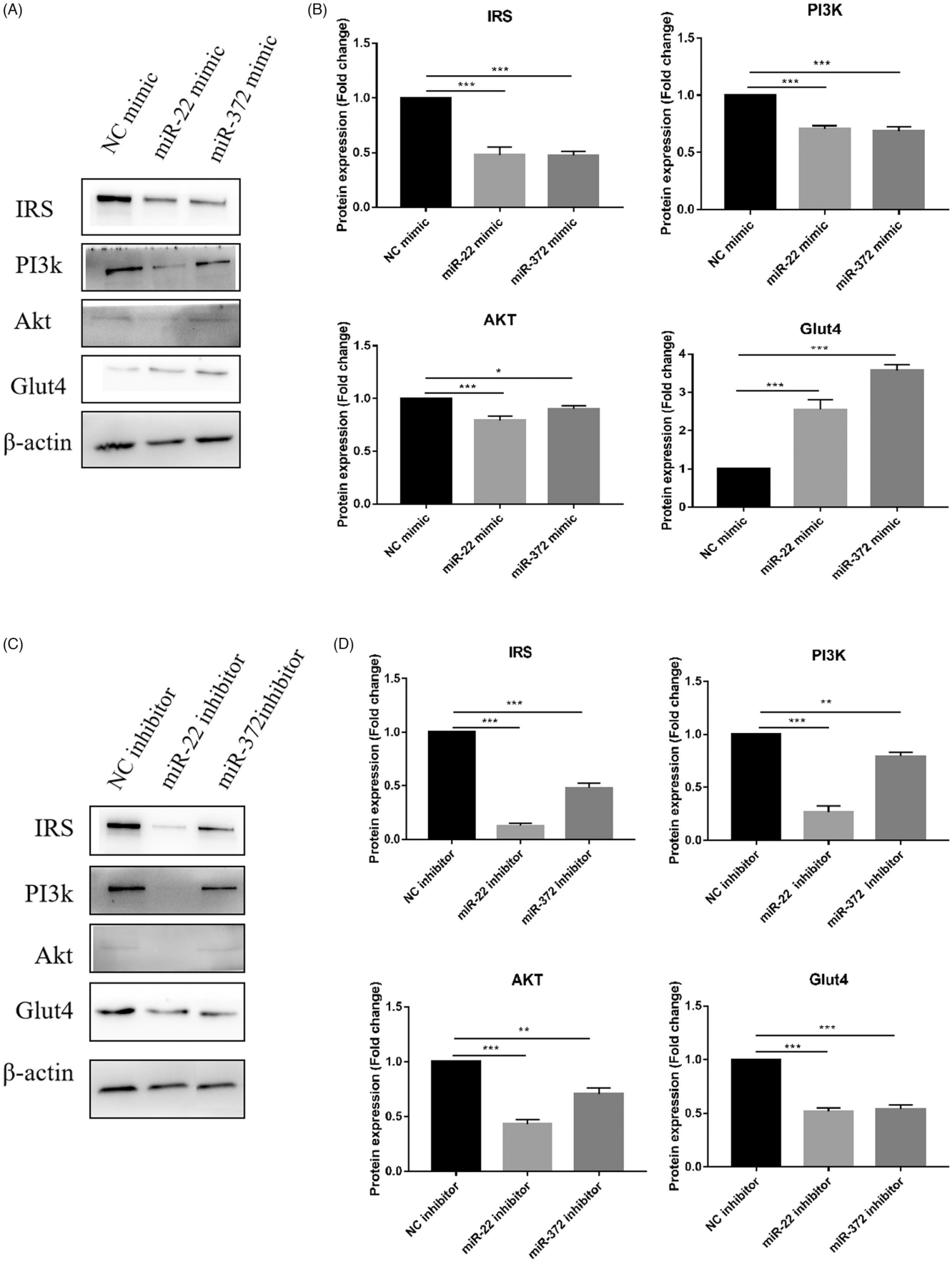


FIGURE 3 IRS/PI3K/AKT expression of insulin signaling pathway in placentas tissues. The expression of proteins in insulin signaling in placental tissue from women with gestational diabetes mellitus (GDM) and in that of healthy controls (HCs) as determined by western blotting. (A) Representative images of IRS, PI3K, AKT, and GLUT4 expression in placental tissues of the two groups. (B) Semi-quantitative analysis of protein expression. Student's *t* test, $^{*}p < 0.05$, $^{***}p < 0.001$. The IRS protein detected is the IRS1 subtype and the PI3K protein is the p85-Y607 subtype

FIGURE 4 Regulated effects of IRS/PI3K/AKT in insulin signaling pathway induced by miR-22 and miR-372.The expression of proteins involved in insulin signaling in HTR8/SVneo (human chorionic trophoblast) cells transfected with miR-22 or miR-372 mimics or inhibitors as determined by western blotting. (A) The expression of IRS, PI3K, AKT, and GLUT4 in HTR8/SVneo cells treated with the miR-22 or miR-372 mimic. (C) The expression of IRS, PI3K, AKT, and GLUT4 in HTR8/SVneo cells treated with the miR-22 or miR-372 inhibitor. (B and D) Semi-quantitative analysis of these protein expression. The results were representative from at least three independent experiments. (one-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$). The IRS protein detected is the IRS1 subtype and the PI3K protein is the p85-Y607 subtype


(A)
NC mimic
miR-22 mimic
miR-372 mimic
IRS
PI3k
Akt
Glut4
β-actin
(B)
IRS
PI3K
AKT
Glut4
Protein expression (Fold change)
NC mimic
miR-22 mimic
miR-372 mimic
(C)
NC inhibitor
miR-22 inhibitor
miR-372inhibitor
IRS
PI3k
Akt
Glut4
β-actin
(D)
IRS
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AKT
Glut4
Protein expression (Fold change)
NC inhibitor
miR-22 inhibitor
miR-372 inhibitor

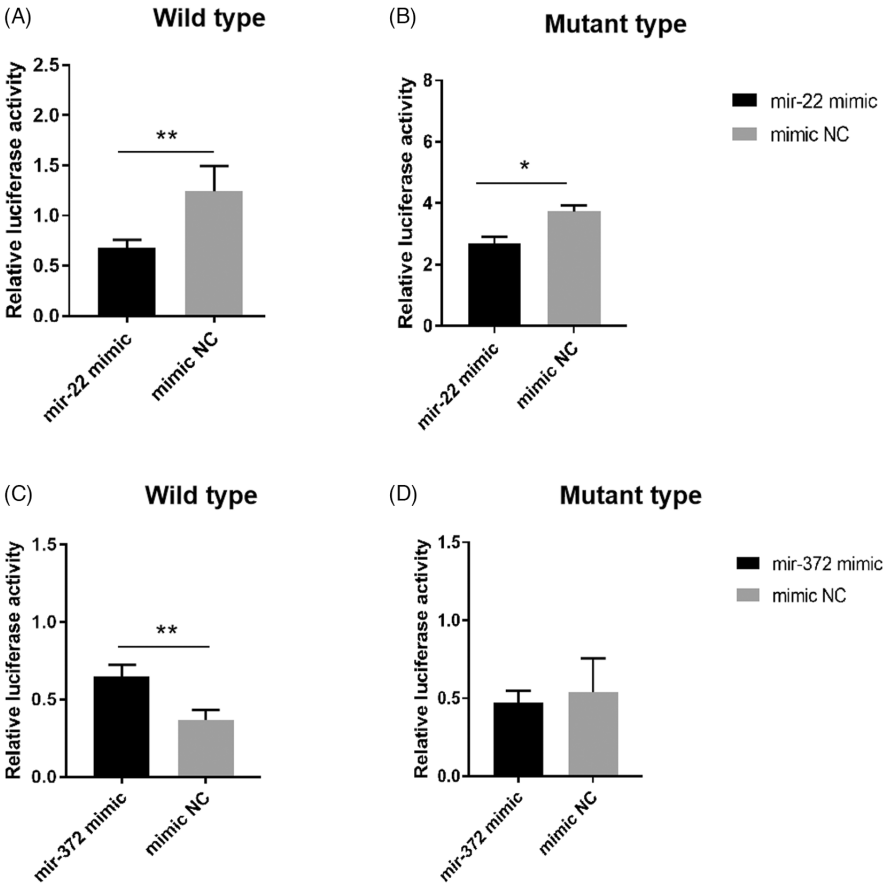

FIGURE 5 Dual-luciferase gene reporter assay depicting the regulation of SLC2A4 in HTR8/SVneo cells treated with miR-22 or miR-372 mimics or inhibitors. (A and B) SLC2A4 3′UTR-driven luciferase activity of miR-22-treated HTR8/SVneo cells in wild type and mutant type. (C and D) SLC2A4 3′UTR-driven luciferase activity of miR-372-treated HTR8/SVneo cells in wild type and mutant type. The results were representative from at least three independent experiments. Student's *t* test, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$

explain their role in GDM occurrence. Placental tissue plays an important part in GDM development, and changes in this tissue likely represent the mechanism underlying GDM occurrence, at least in part.[40] Relatively few studies have investigated the effects of specific miRNAs on insulin resistance. In the present study, we explored how miR-22 and miR-372 influence the insulin signaling pathway in GDM to identify the proteins involved and elucidate the underlying mechanism.

To investigate the roles of miR-22 and miR-372 in the pathobiology of GDM, we first compared the placental tissue expression profiles of miR-22 and miR-372 between GDM patients and HCs by RT-qPCR. The results showed that the expressions of miR-22 and miR-372 were lower in placental tissue of women in the GDM group than in that of women in the HC group. In vitro, we found that high-glucose-cultured HTR8/SVneo cells exhibited lower expressions of miR-22 and miR-372 when compared with cells cultured under normal-glucose conditions. These results suggested that miR-22 and miR-372 might act as modulators of the insulin signaling pathway. To further investigate the regulatory function of miR-22 and miR-372, we evaluated the expression levels of proteins involved in the insulin signaling pathway in cells transfected with miR-22 and miR-372 mimics or inhibitors. Western blot results showed that the expression of GLUT4 was significantly increased in high glucose-cultured cells transfected with miR-22 or miR-372 mimics, whereas the opposite result was obtained when the cells were transfected with miR-22 or miR-372 inhibitors. However, the expression of PI3K, AKT, and IRS were all significantly decreased in cells treated with either mimics or inhibitors of miR-22 or miR-372. This suggested that GLUT4 is the main target for miR-22 and miR-372 in the insulin pathway. When GLUT4 expression level is increased, glucose uptake and utilization can be enhanced.[19] As miR-22 and miR-372 exert distinct, positive regulatory effects on GLUT4, both miRNAs can reduce glucose accumulation and improve hyperglycemia in GDM, highlighting their potential as therapeutic targets for the treatment of GDM. Importantly, this is the first study to report that the expression of miR-22 is significantly downregulated in the placental tissue of patients with GDM, and that miR-22 targets the GLUT4-encoding gene, *SLC2A4*, in the insulin signal pathway.

Impaired insulin signaling transduction is an important factor in insulin resistance and is a key characteristic of diabetes. Insulin signaling activation involves insulin binding to the insulin receptor (IR), autophosphorylation of receptor tyrosine residues in the cytoplasm, and tyrosine phosphorylation of insulin receptor substrate (IRS) proteins. Activated IRS then associates with downstream effectors such as PI3K. PI3K is a heterodimeric lipase containing a regulatory unit (p85) and a catalytic unit (p110). In the resting state, p85 inhibits P110. However, following insulin stimulation, a specific tyrosine residue on IRS-1 can bind to the p85 subunit of PI3K, leading to the activation of the P110 subunit. PI3K activation leads to the phosphorylation of a serine residue on PKB/AKT, which induces GLUT4 translocation from intracellular compartments to the cell membrane, thereby promoting glucose utilization.[41] In GDM, insulin resistance is

reported to be significantly increased, while the expression of insulin signaling pathway-related proteins is dysregulated.[42] MiR-372 is the product of the miR-371-372 gene cluster located on Chromosome 19. Chen et al.[43] found that miR-372 regulates the PI3K/AKT pathway by inhibiting the phosphorylation of AKT, mTOR, and P70S6K. Gjorgjieva et al.[44] reported that miR-22 knockout mice bred under standard conditions developed glucose intolerance, and the authors proposed that miR-22-3p was a master regulator of lipid and glucose metabolism. Additionally, the results of an oral glucose tolerance test indicated that miR-22-3p was likely to be involved in restoring glucose homeostasis after the test.[45] We believe that miR-372 and miR-22 are involved in GDM pathogenesis through the regulation of proteins involved in the insulin pathway, including IRS, PI3K, AKT, and, importantly, GLUT4 (Figure 6).

Interestingly, we found that the regulatory effects of miR-22 and miR-372 on the same target gene are of great difference. This result reflects the diverse regulatory roles of miRNAs, including in the post-translational modification of proteins and the diversity of regulation. There may be a variety of miRNA interactions in the miRNA regulation mode, forming sponges to adsorb on the target gene 3'UTR, we speculate that in addition to miR-372 and miR-22, there are other miRNAs acting on the target gene *slc2a4* to form a balance. Mir-22 plays a negative regulatory role on *slc2a4*. At the same time, miR-22 inhibits other miRNAs, which also act on the target gene *slc2a4*. When miR-22 is inhibited, the inhibition of other miRNAs is enhanced, and the luciferase activity of *slc2a4* (transcription GLUT4) is reduced. Similar results were recently reported, namely, that miR-351 can ameliorate GDM by downregulating PI3K and AKT expression and upregulating that of GLUT2.[46] Bioinformatic analysis and dual-luciferase gene reporter assays indicated that *SLC2A4* was a target gene for miR-22 and miR-372, and that *SLC2A4* was positively regulated by miR-372 but negatively regulated by miR-22. It was traditionally believed that miRNAs inhibit post-transcriptional translation mainly by binding to target mRNAs. However, growing evidence indicates that other regulatory modes may exist in addition to the canonical mechanism, including directly silencing or activating target genes.[25] Similarly, one study reported a positive correlation between miRNA and protein expression level.[47] In a recent study, Li et al.[48] found that miR-320 was highly expressed in heart tissue of mice with diabetes mellitus-triggered cardiac dysfunction and induced the expression of its target gene *CD36*. The authors further showed that miR-320 acts as a small activating RNA in the nucleus at the level of transcription. In addition, miRNA-22 and 372 mimics and inhibitors both decreased PI3K, AKT and IRS, but inhibitors more obviously in our research. The possible reason of this phenomenon was that the mimic and inhibitor of miRNA were not one-to-one counterparts, one mimic may have multiple inhibitors, and also the inhibitor did not completely inhibit the role of miRNA. Similar findings were made by Temo Barwari et al who reported that when cultured cells were transfected with a miR-21 mimic or inhibitor, target proteins expression has all increased.[49]

In this study, we found that miR-22 negatively regulated the expression of *SLC2A4*, which encodes GLUT4, but positively regulated that of GLUT4 protein. There may be two possible explanations for this. Firstly, the gene that regulates the GLUT4 is not only *SLC2A4*, and the other gene that may regulate GLUT4 is not found so far. Secondly, *SLC2A4* may be positively regulated by other miRNAs or by long noncoding RNAs, which may increase GLUT4 protein expression independently of miR-22 and miR-372. Nevertheless, the above results suggest that miR-22 and miR-372 may regulate the transcription of *SLC2A4* or play a protective role by stabilizing GLUT4 translation or the degradation of its transcripts.

So far, we have only shown that miR-22 and miR-372 regulate GLUT4 through in vitro experiments. Next, we sought to clarify the expression profiles of miR-22 and miR-372 and whether the two miRNAs interact to form a co-regulatory gene expression network. However, further studies are needed to verify that miR-22 or

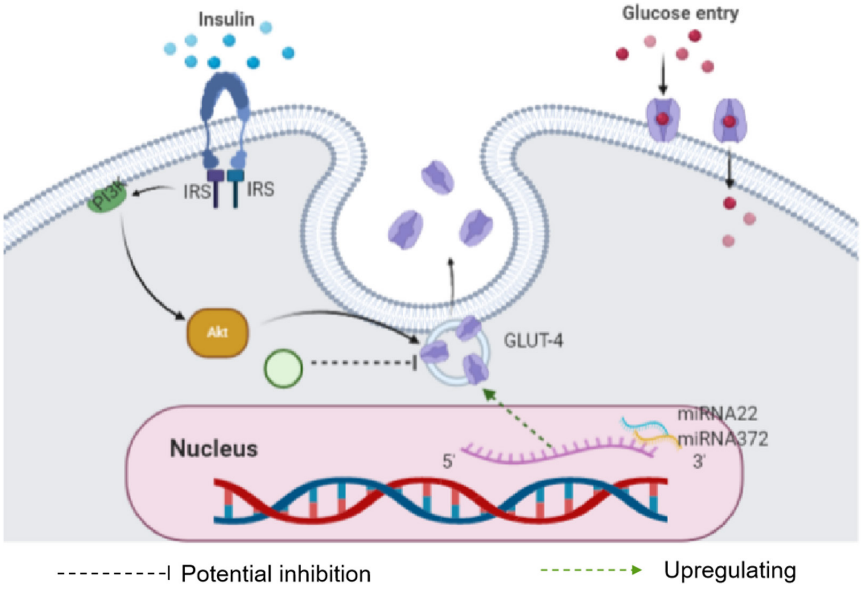

FIGURE 6 Predicted mechanism underlying the miR-22- and miR-372-mediated regulation of target genes. The schematic diagram is created online through an open website:app.biorender.com

miR-372 indeed regulate the insulin pathway using animal models or other in vivo methods.

In conclusion, we elucidated the expression patterns of miR-22 and miR-372 in placental tissue from healthy women and those with GDM, as well as in chorionic trophoblast (HRT8/SVneo) cells cultured under high-glucose conditions. The activation or inhibition of miR-22 and miR-372 in high glucose-stimulated cells was achieved via the transfection of miR-22 and miR-372 mimics or antisense oligonucleotides, following that the expressions of PI3K, AKT, IRS, and GLUT4 in the cells was measured by western blot. A dual-luciferase gene reporter assay was employed for the verification of miR-22 and miR-372 target genes. Our data revealed that the expressions of miR-22 and miR-372 were downregulated in GDM, and that miR-22 and miR-372 target genes acting in the insulin pathway have a role in GDM occurrence and progression. In this study, our analysis of a large number of human tissue samples revealed that the downregulation of miR-22 and miR-372 expression may closely related to GDM through the regulation of the PI3K/GLUT4 pathway, while increased miR-22 and miR-372 expression can counteract these effects.

AUTHOR CONTRIBUTIONS

W.L. involved in conceptualization; W.L. and X.Y. involved in methodology and formal analysis; W.L., Y.W., X.H., and L.Y. involved in resourcing; W.L., X.H., Y.W., L.Y., and X.Y. wrote the original draft; Y.Z., K.H., and B.T. wrote, reviewed, and edited the manuscript; X.Y. and B.T. involved in supervision; K.H., L.Y., and X.Y. administered the project; W.L. involved in funding acquisition. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGEMENT

We are grateful to all the patients and volunteers who participated in the present study, the medical personnel of the Armed Police Hospital Affiliated to Guangzhou Medical University and Zhujiang Hospital for their kindly assistance in collecting questionnaires and tissue samples.

CONFLICT OF INTEREST

The authors declare that the research was conducted without any financial and non-financial interests that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Li W, Yuan X, He X, et al.. The downregulation of miR-22 and miR-372 may contribute to gestational diabetes mellitus through regulating glucose metabolism via the PI3K/AKT/GLUT4 pathway. *J Clin Lab Anal*. 2022;36:e24557. doi: 10.1002/jcla.24557