Analysis of Serum Circulating MicroRNAs Level in Malaysian Patients with Gestational Diabetes Mellitus

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Abstract

Gestational diabetes mellitus is a serious global problem and needs urgent attention. Aberrant microRNAs expression is potentially disease-specific and may contribute to GDM pathological processes. Even though GDM is diagnosed at the end of the second or beginning of the third trimester, there is no way to prevent pathological changes that may occur during the first and second trimesters. Therefore, to identify a specific miRNAs expression and their predicted target genes in maternal serum subjected with GDM in especially early stage, we performed miRNA expression profiling using miRNA PCR Array and *in-silico* analysis. In this study, demographic data and miRNAs expression levels and their specific potential as biomarkers were investigated. The findings showed that the expression levels of *hsa-miR-193a, hsa-miR-21, hsa-miR-23a, and hsa-miR-361* are significantly upregulated while *miR-130a* is significantly downregulated in GDM patients. The ROC curve analysis revealed that *hsa-miR-193a* (AUC = 0.8906 ± 0.04470, P < 0.0001), *hsa-miR-21(*0.8950 ± 0.04411, P < 0.0001) and *miR-130a* (0.7222 ± 0.07450, P = 0.0083) have a potential biomarker characteristics in GDM. Furthermore, the *in-silico* analysis revealed that *KLF, ZNF25, AFF4, C1orf143*, *SRSF2*, and *ZNF655* were prominent genes targeted by the common nodes of *miR23, miR130, miR193, miR21, and miR361*. Our findings imply that circulating microRNAs in the first trimester might be used as indicators for GDM.

Introduction

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance discovered during pregnancy[1]. Furthermore, GDM is a risk factor for maternal and fetal morbidity during pregnancy[1]. Babies born to women with GDM are more likely to have an excessive birth weight, known as macrosomia (weighing more than 4 kg), which can lead to juvenile obesity, type 2 diabetes, and/or cardiovascular disease later in life[2,3]. Globally, 21.3 million pregnancies are associated with hyperglycemia, with 18.4 million pregnancies related to GDM[4].It has been shown that GDM is a complex disease with multiple etiologies[5]. Studies have shown that the development of GDM may be the result of a combined genetic and environmental effect, but the exact cause is still unknown [5].GDM is diagnosed at the end of the second or early third trimester, depending on the physiological findings, and the generally accepted time for screening is the end of the second trimester, that is, between 24 and 28 weeks of pregnancy [6]. Over the past few decades, several studies have shown that aberrant expression of miRNAs is associated with pregnancy complications and the progression of GDM[7]. miRNAs also function in blood glucose homeostasis and insulin production and secretion [8]. Furthermore, miRNAs have been reported to be present in biological fluids such as plasma/serum of diabetic patients and are highly stable there which can be easily detected and measured[9]. MicroRNA are short, single stranded RNA that post-transcriptionally regulate gene expression[10]. miRNAs produced in the nucleus are pri-miRNA and they are processed into pre-miRNA that is exported to the cytoplasm by exportin-5 and converted to a mature miRNA by Dicer complex and exert their action by targeting 3' untranslated region (3'-UTR) of mRNA resulting in inhibition of protein synthesis[11]. Although GDM is diagnosed at the end of the second or beginning of the third trimester, using this diagnostic threshold, there is no opportunity to prevent pathological changes (accumulated damage) that may occur during first and second trimester (Undiagnosed Period). Furthermore, the implementation of screening tests such as microRNAs evaluation during early pregnancy affords opportunity to identify women at risk of disease and to evaluate intervention strategies on pregnancy outcome and the long-term health of both mother and baby[3]. Therefore, this study aimed to evaluate changes in the expression of circulating miRNAs in the serum of patients with GDM during the first, second and third trimesters of pregnancy and subsequent changes in miRNA expression at postpartum period.

Results

Participant characteristics

The results demonstrated that except miscarriage, other demographics and clinical data in Table 1 were significant different (p<0.05).

MiRNA expression profiles in GDM patients

The results of the miScript miRNA PCR Array revealed a significant dysregulation pattern of miRNAs in the first, second, third and postpartum periods (results presented in table2). In the first trimester, four miRNAs, namely *hsa-miR-193a*, *hsa-miR-21, hsa-miR-23a*, and *hsa-miR-361*, were significantly upregulated while only *has-miR-130a* was significantly downregulated in GDM patients. In the second trimester, *hsa-miR-let7-i*, *hsa-miR-126, hsa-miR-129, were* significantly upregulated and *hsa-miR-125, hsa-miR-129-2, hsa-miR-130, hsa-miR-34 and hsa-miR-375* were significantly downregulated. In the third trimester, upregulation was observed with *hsa-miR-let7e, hsa-miR-107, hsa-miR-361* and *hsa-miR-370* while downregulated miRNAs were *hsa-miR-125, hsa-miR-129* and *hsa-miR-130*. In the postpartum period, data analysis revealed that two microRNAs *hsa-miR-194* and *hsa-miR-24* are significantly upregulated and three miRNAs *hsa-miR-125, hsa-miR-370,* and *hsa-miR-375* are significantly downregulated. A scatter plot and expression diagram of relative fold change showed the dysregulation patterns of miRNAs in a separate phase of GDM and control groups (Figure 1 and 2). Implementation of screening tests such as miRNAs evaluation during early pregnancy, offers an opportunity to identify women at risk of GDM and to evaluate intervention strategies on pregnancy outcome and the long-term health of both mother and baby. Therefore, we focused more on the results from the first trimester. Dysregulated microRNAs in the first trimester are presented in Table 3.

ROC curve analysis

The diagnostic evaluation of ROC curve based on distribution of ΔCt of dysregulated microRNAs in the 1st trimester in GDM patients revealed that the area under the curve (AUC) for *hsa-miR-193a, hsa-miR-21, hsa-miR-23a*, *hsa-miR-361*and *hsa-miR-130* were 0.8906±0.04470 (P<0.0001, 95% CI=0.8-0.9), 0.8950±0.04411(P<0.0001, 95% CI=0.8-0.9),0.6337±0.08262 (P=0.1124, 95% CI=0.4-0.7) ,0.6510±0.08722(P=0.07, 95% CI=0.4-0.8) and 0.7222±0.07450 (P=0.0083, 95% CI=0.5-0.8) respectively. The data indicated that three microRNAs namely, *hsa-miR-193a, hsa-miR-21*and *hsa-miR-130* fit the biomarker role and have a potential diagnosis value for GDM detection in the first trimester of pregnancy (Figure3).

In-silico target analysis

In silico analysis revealed the interaction between miRNAs and their target genes involved in the first trimester of GDM. In this analysis, *KLF, ZNF25, AFF4, SRSF2*, C1orf143 and *ZNF655* were prominent genes targeted by the common nodes *miR23, miR130, miR193, miR21*, and *miR361* (Figure 4).

Discussion

GDM is an increasingly common condition and can cause serious and lifelong harmful complications for both mother and child. There is great interest in the potential role of miRNAs as regulators of biological processes, mediators of tissue crosstalk, and biomarkers of GDM [12]. MiRNAs participate in various mechanisms associated with pregnancy and GDM. In addition, in maternal blood, circulating miRNAs are persistent and detectable. As a result, they are potential biomarker candidates for non-invasive pregnancy problems diagnostic tests.

Studies have shown that dysregulated miRNAs are associated with pregnancy complications, suggesting the potential use as prognostic markers for GDM disease[13,14]. Furthermore, previous studies have reported that serum or plasma miRNAs are differentially expressed between GDM patients and controls.

In this study, we attempted to identify the differentially expressed serum-derived miRNAs and the molecular interactions of the *in-silico* miRtarget genes that may be involved in GDM. Furthermore, the potential characteristics of dysregulated miRNAs were investigated. GDM is diagnosed at the end of the second or beginning of the third trimester[3], leaving no time to prevent pathological changes that could occur during the first and second trimester. Thus, miRNAs detection as early as in the first trimester is crucial and was our main study objective. Our results showed that miR130a is significantly downregulated in the GDM sample, while miR193a, miR21, miR23a and miR361 were significantly upregulated in the first trimester.

MiR130a affects a variety of cellular processes, from inhibition of glucose uptake, mitochondrial function, oxidative stress to fetal development [15,16]. As a consequence of its cellular functions, miR-130a expression is deregulated in a wide range of pathologies, such as oral squamous cell carcinoma [17], ovarian epithelial cell carcinoma, thyroid eye disease [18], cervical cancer[19], and acute myeloid leukemia[20]. It was also shown that upregulated miR-130a reduces intracellular ATP levels in the pancreatic beta cell [21]. Furthermore, previous studies found upregulation of circulating miR-130a has a crucial role in diabetes-related complications[22]. In a study by Meng et al., decreased miR-130a in endothelial progenitor cells from diabetes mellitus was reported to contribute to impaired EPC (Endothelial progenitor cell dysfunction) function via its target RunX3[23].

MiR23a identified from this study, has been proposed as a potential biomarker for early diagnosis of prediabetes and type 2 diabetes and is particularly useful in distinguishing between undiagnosed and prediabetes [24]. Our finding was in an agreement with Yang et al. in which we found that miR-23a could function as a potential biomarker in the first trimester of GDM. On the other hand, upregulated miR193a has been shown to play a vital role in the pathogenesis of placenta accreta spectrum development mediated by the target in *EFNB2* gene via the EMT signaling pathway [25]. In addition to that, elevated miR193a was reported as a potentially new biomarker for the diagnosis of diabetic nephropathy [26]. Although miR193a was upregulated in the current study, and its biomarker potential was confirmed in other disease conditions, but its etiologic role in GDM is unknown and requires further study.

Literatures proven that miR21 has a significant biological route in a variety of diseases, although, as a molecular diagnostic marker can therapeutically regulate type 2 diabetes and pancreatic cancer[27]. However, it is still unclear how miR21 is linked to GDM. One plausible explanation could be that miR21 promotes glucose uptake through induction of the PPARα gene in GDM patients and inhibits cell proliferation and infiltration[28]. Gestational-adjusted expression of miR21 has been reported to be positively associated with GDM[29]. Sexually dimorphic miRNA expression during pregnancy in the human placenta was reported by Amy E. Flowers et al. They found that miR361 was differentially expressed and upregulated in women of the 1st and 3rd trimesters. [30]. Overall, our study data investigated differentially expressed microRNAs in GDM patients and we showed the potential role of *hsa-miR-193a, hsa-miR-21*and *hsa-miR-130a* as biomarker but the role of these dysregulated microRNAs in the pathogenesis of GDM is still not clearly understood.

Future Directions And Conclusions

The findings obtained in this study showed a significant dysregulation pattern of five miRNAs hsa-*miR-130a, hsa-miR-193a*, *hsa-miR-21, hsa-miR-23a*, and *hsa-miR-361* in the stage of first trimester in GDM patients. However, despite their dysregulated pattern in GDM, there are existing analytical and pre-analytical challenges that need to be addressed before the clinical use of these circulating miRNAs. The current research was conducted only on 24 GDM samples therefore, in addition, large prospective cohort studies should be performed to investigate their biological role in GDM progression and identify whether they may be diagnostic or prognostic candidates.

Methods

Participant characteristics

A case-control study was conducted on a total of 1122 women (267 GDMs and 855 controls) who had spontaneous deliveries at the University of Malaya Medical Center (UMMC) from April 2014 to June 2016. All participants were characterized by pregnancy, were nonsmokers, and did not abuse alcohol. The selection criteria for the study were the age of the mother between the ages of 18 and 45 and the diagnosis of gestational diabetes by a trained doctor. The age of mothers between the ages of 18 and 45 with normal pregnancies served as a control. Abnormal fetal, still giving birth, sickle cell anemia, thalassemia or other hemoglobinosis, and other pregnancies such as lupus, hypertension, thyroid disease, cardiovascular disease, transplantation, kidney disease, asthma or other serious disease Women diagnosed with previous conditions, drug abuse, a history of smoking and depression, and carriers of blood-borne infections were excluded. Screening was performed between the 24th and 28th weeks of gestation using the Modified Oral Glucose Tolerance Test (mOGTT). Universal

screening includes pregnant women with a BMI greater than 27 kg / m2, previous giants weighing 4 kg or more, previous GDM, one-time relatives with diabetes, a history of unexpected prenatal fetal death, and birth defects. It was performed on pregnant women with a medical history Positive. GDM is defined as fasting plasma glucose (FPG) (≥5.1) and 75 g mOGTT plasma glucose (≥7.8). Substantial risk women with normal initial screening results were subjected to a repeat mOGTT at 4–6 weeks later. The control consisted of uncomplicated pregnant mothers who gave birth to a baby between 38 and 41 weeks. Trained personnel assessed the physical health of the mother and gestational age was calculated from the first day of the last menstrual cycle or from the patient's early ultrasound scan results.

Serum RNAs extraction and cDNA synthesis

Twenty-four women with GDM were included. Maternal samples were collected within first, second, third trimesters and 2-6 months postpartum. Twenty-four healthy pregnant women served as controls. Total RNAs were extracted from serum samples using the miRNeasy serum/plasma kit and synthesized into cDNA using the miScript II Rt kit according to the manufactures protocol (Qiagen, Mississauga, Ontario, Canada).

MiRNAs expression profiling

A pathway-focused miScript miRNA PCR Array Human Diabetes (Qiagen, Mississauga, Ontario, Canada) was used in combination with miScript SYBR Green PCR kit (Qiagen) to profile miRNA expression in a 96-well plate using a Step One Plus™ real time PCR detection system (Applied Biosystem, California, USA) following the cycling conditions recommended by the manufacturer's instructions. Amplification conditions were 15 min at 95∘C, followed by 40 cycles of 15s at 94°C, 30s at 55°C, and 30s at 70°C. A total of 37 miRNAs involved in GDM phenotype were selected for the PCR array. Six snoRNA/snRNA, including SNORD61, SNORD68, SNORD72, SNORD95, and SNORD96A were selected as housekeeping genes. Furthermore, miRTC and PPC genes served as reverse transcription control and primer assay positive control, respectively. PCR array reactions were conducted in triplicate repeats. The data were analyzed using online Gene Globe data analysis software version 2.1.0 (https://geneglobe.qiagen.com/). The validity and accuracy of array PCR were confirmed using reverse transcription-qPCR for five randomly selected miRNAs in triplicate.

ROC curve analysis

A receiver operating characteristic (ROC) which is a plot of the true positive rate (Sensitivity) in function of the false positive rate (100-Specificity) for different cut-off points of a parameter was performed to evaluate the potential microRNAs biomarker characteristics.

Insilico Target Genes Identification and Bioinformatic Analysis

Target Scan Human Release 8.0[31] and miRDB [32] were used to obtain predicted or previously experimentally validated miRNA target genes. Interaction network analysis was performed using the Cytoscape 3.7.1 tool to verify all potential interactions between the identified target gene and potential functional mediators.

Declarations

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Author contributions

Conceptualization, SJ and SMZ.; Methodology, SJ,RV, SZM,ZM,TPC,SZO and YFP; Investigation & Statistical analysis, SJ, RV,TPC, AND HK; Writing-original Draft, SJ, and SMZ; Resources for reagents, materials and analysis tools, SJ,SZO and TPC; All authors read and approved the final manuscript.

Data availability statement

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

Competing Interests

The author(s) declare no competing interests.

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Tables

Table 1

Demographics and clinical data of the study population. Data presented as mean±SD and differences in demographical data were evaluated using ANCOVA test. P<0.05 =significant.

Characteristics	GDM (n=267)	Control (n=855)	P-Value
Age	31.31 ± 4.50	29.89 ± 4.42	0.0001
Weight	62.07 ± 12.11	55.57 ± 11.32	0.0001
BMI	26.80 ± 5.44	23.20 ± 7.92	0.0001
FPG	5.00 ± 1.48	4.26 ± 0.36	0.0001
2h FPG/ Random	9.13 ± 1.52	5.86 ± 0.93	0.0001
Previous GDM	Yes 77 (28.8%)	Yes 19 (2.2%)	0.0001
	No 190 (71.2%)	No 836 (97.8%)	
Previous C-Section	Yes 77 (28.8%)	Yes 99 (11.6%)	0.0001
	No 190 (71.2%)	No 756 (88.4%)	
Previous HBP	Yes 16 (6.0%)	Yes 4 (0.5%)	0.0001
	No 251 (94.0%)	No 851 (99.5%)	
Miscarriage	Yes 22 (8.2%)	Yes 55 (6.4%)	**0.332**
	No 245 (91.8%)	No 800 (93.6%)	

Table 2

Dysregulated microRNAs in each trimester and post-partum period of GDM.

Dysregulated miRNAs	Trimesters							
	1st		2nd		3rd		Post-partum	
	Fold Change	P-Value	Fold Change	P-Value	Fold Change	P-Value	Fold Change	P-Value
miR-130	-0.32	0.006	-0.43	0.004	-0.34	0.007		
miR-193	3.1	0.03						
miR-21	7.4	0.007						
miR-23	2.14	0.01						
miR-361	2.9	0.000			1.77	0.001		
miR-let7i			0.71	0.008				
miR-125			-0.047	0.008	-0.42	0.008	-0.21	0.01
miR-126			0.55	0.04				
miR-129-2			-0.42	0.021				
miR-129			0.62	0.01	-0.29	0.002		
miR-34			-0.22	0.003				
miR-375			-0.48	0.009			-0.31	0.021
miR-let7e					0.59	0.004		
miR-107					0.57	0.002		
miR-370					0.61	0.004	-0.33	0.043
miR-194							13.27	0.02
miR-24							4.85	0.04

Table 3

MiRNAs role and their putative and predictive target genes in the first trimester of GDM.

microRNAs symbol	Putative and predictive target genes /Target score98-100)	microRNA's role in GDM	Current study	
			Expression pattern/FC	P-Value
miR-130a	*SRSF2, CLIP1, GJA1, CPEB1, SLAIN1, SKIDA1, ESR1, ACVR1, TSC1, RPS6KA5, MDM4, KLF, IGF1, RAP2C, ACSL4, PIK3CB, ZBTB20, MYBL1, DDX6, FAM155A, KBTBD8, ZBTB18, ZFYVE9,TBL1XR1,LGALSL, ATG16L1, RO60, CHST1, MECP2, FMR1, CNOT6, SOS2, DYNC1LI2, SECISBP2L,AMPKa,RunX3*	Inhibition of glucose uptake[15] Impaired mitochondrial function and oxidative stress which affects fetal development (Jiang, S et al,2017)	Decreased/**-3.12**	0.006548
miR-193a	*SRSF2, MAPK10, PIGA, DCAF7, RAPGEF6, KLF, AFF4,EFNB2*	promote trophoblast migration and invasion (Hong, Yet al.2021)	Increased/**3.1**	0.032241
miR-21	*C1orf143, ZNF25, YOD1, PRDM11, FASLG, ZNF367, VCL, SKP2, TGFBI,PPARα*	Down regulated of miR-21 Inhibit Cell Proliferation and Infiltration (Guan, C. Y et al.2020), increased miR-21 is associated with pregnancy complications (Ibarra, A et al.2018)	Increased/**7.4**	0.007121
miR-23a	*ZNF25, ZNF99, SEMA6D, FAM234B, TRIL, INTU, ZNF138, AUH, TAB3, SEC23IP, ZBTB34, PDE7A, PPARGC1A, PDE4B, TOP1, KLF, TMED5, FUT9, SESN3, DNAJC6, PPM1K, VGLL3, SFT2D1, ZNF716, C2orf69, ATP11C, NEK6, LPP, ARHGAP20, MRC1, ETNK1, WBP2, FAM126B, TMPO, PPP4R4, HEXIM1, CCNT2, PKP4, PTEN, TNRC6A, SLC4A4, MICU3, RAB8B, CACUL1, PPIF, ZNF117, REPS2, CSNK1G3, ZFHX4, SLC1A1, MAP4K4, ROBO2, PRTG, NUFIP2, RPRD2, CTCF, SCG5, SEC24A, CDC40, BORA, CNOT6L, NCOA2, NUP50, NACC2, ZNF655*	potential biomarkers for GDM in the first trimester (Yoffe, L et al.2019)	Increased/**2.14**	0.018739
miR-361	*PIK3CG, TFAP2B, ZMAT3, RANBP17, AFF4, ZNF655, C1orf143*	ultimately is a sex specific early markers of extremely low gestational age (Flowers, A. E et al.2021)	Increased/**2.9**	0.000063

Figures

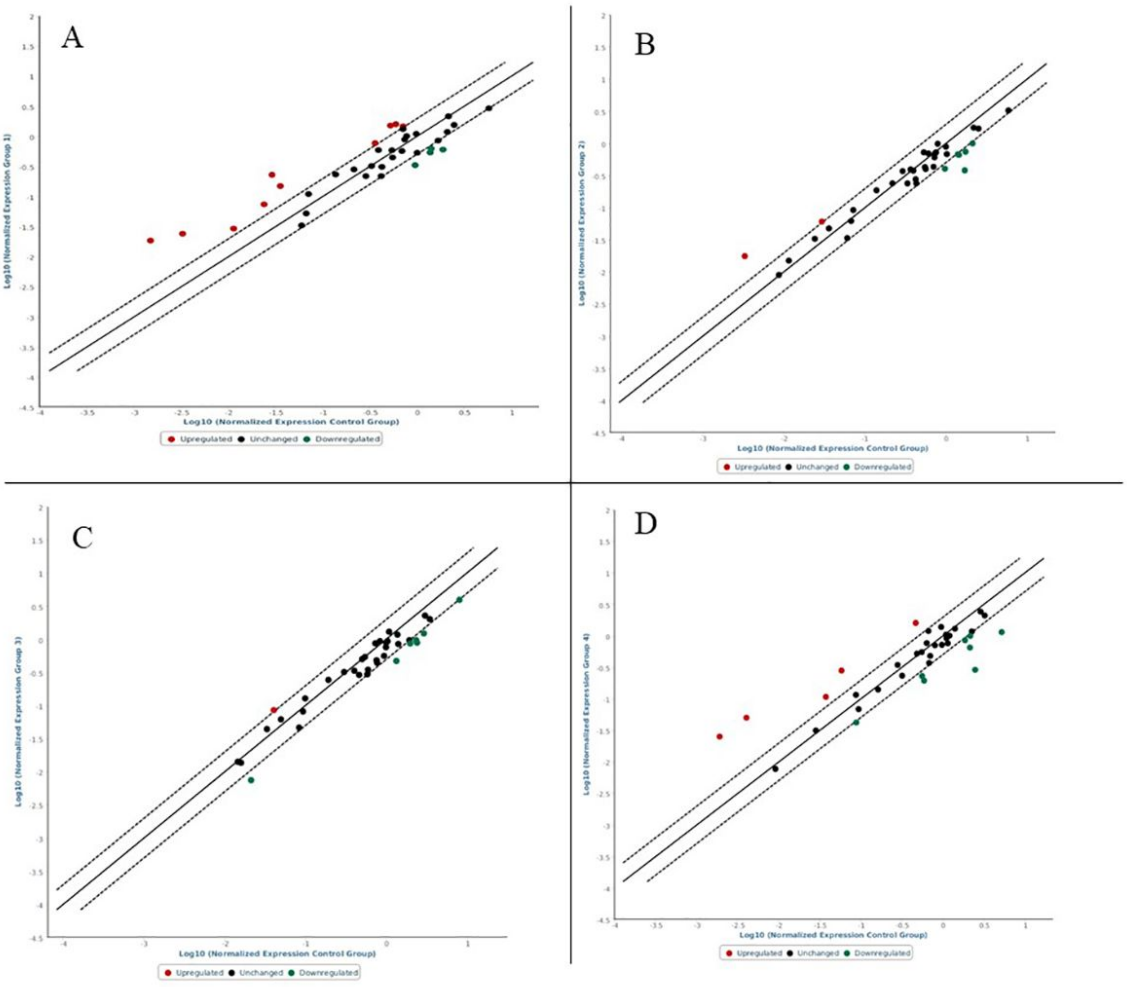

Figure 1

Scatter plot analysis of the relative expression of thirty-seven miRNAs compared between control and GDM patients .A= first trimester, B=second trimester C=third trimester D= Post-partum. **Color scheme:** Red color indicates upregulated miRNAs, while blue indicates downregulated miRNAs. The miRNAs that are significantly altered between the two groups (control and GDM) are located above. The diagonal black line correspond to P<0.05. Analysis was performed using online statistical analysis by Qiagen.

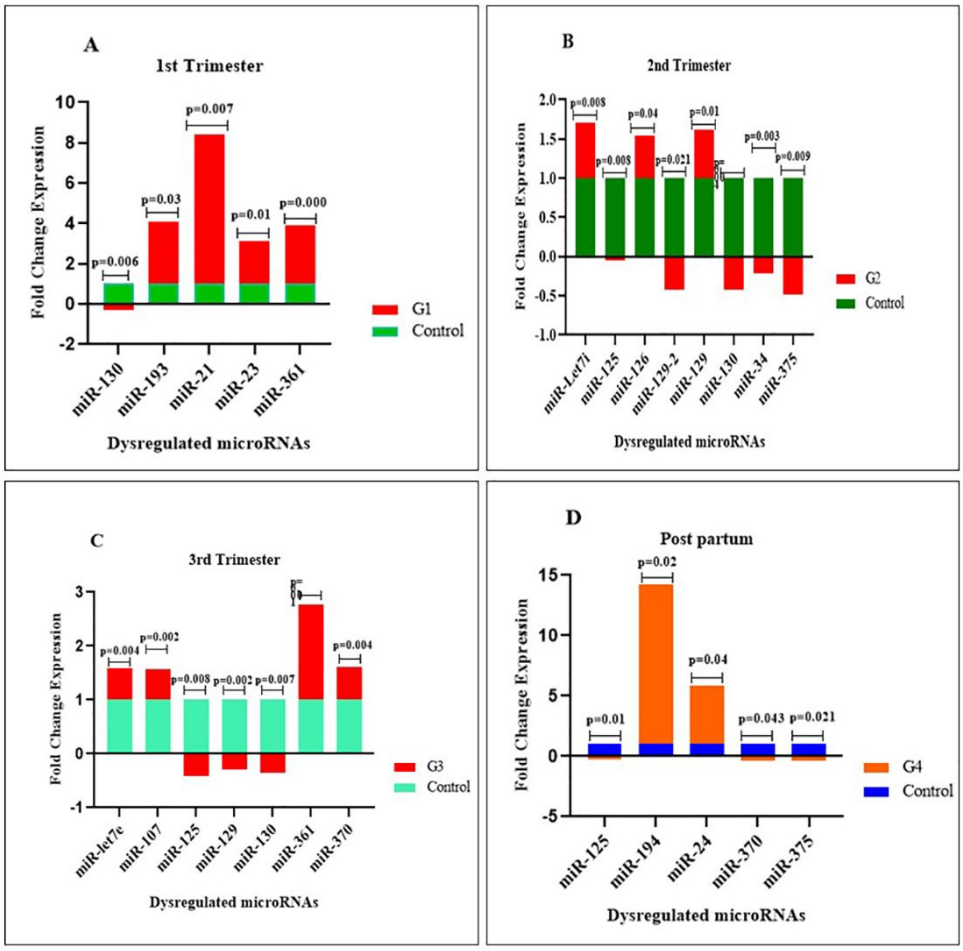

Figure 2

Dysregulated expression pattern of microRNAs in First (A), Second (B), Third (C) and postpartum (D) in GDM patients. Charts were derived from Graf Pad Prism version8. P-value calculated using non-parametric analytical test.

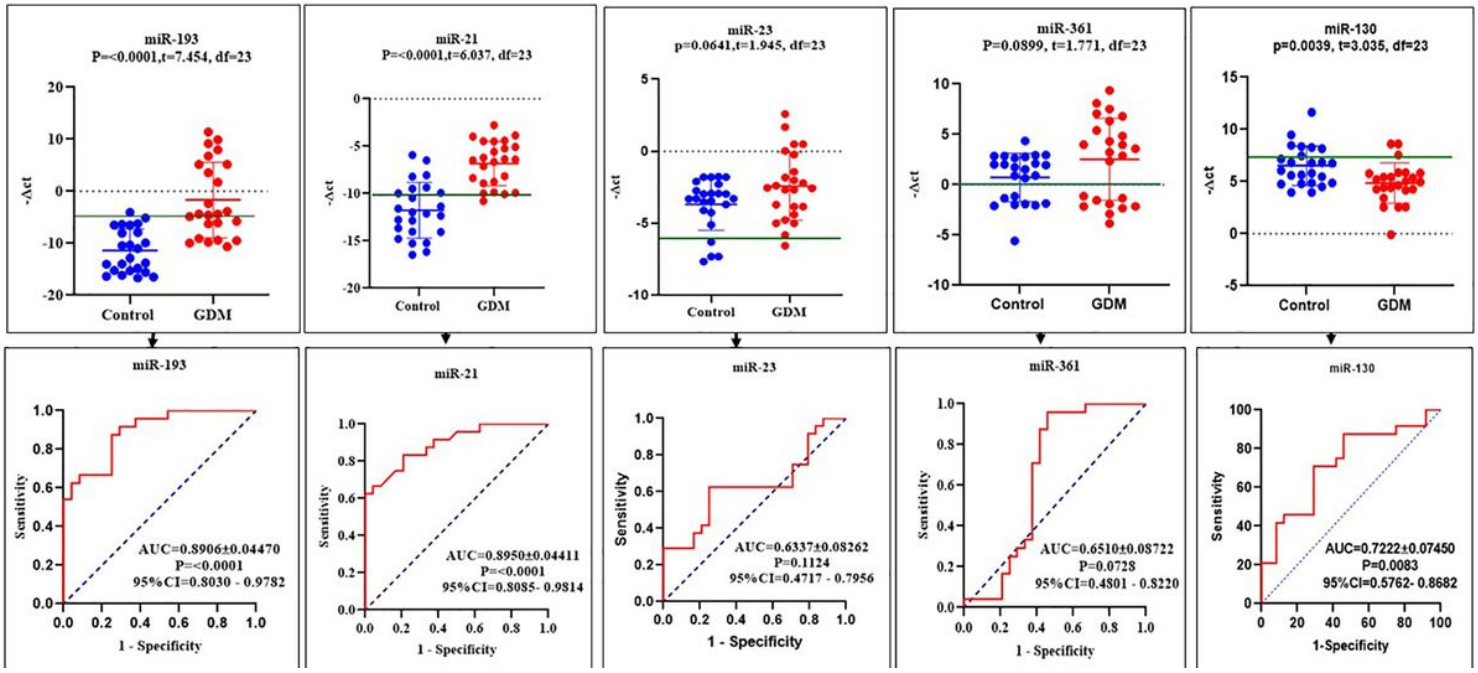

Figure 3

ROC curve analysis. The figure shows the comparison of five dysregulated microRNAs and their specific AUC based on their ΔCt distribution in the first trimester of pregnancy. The ROC curve is derived from Graf Pad Prism version 8. Normality distribution of samples was performed using Shapiro-Wilk test. P-value calculated using non-parametric analytical test.

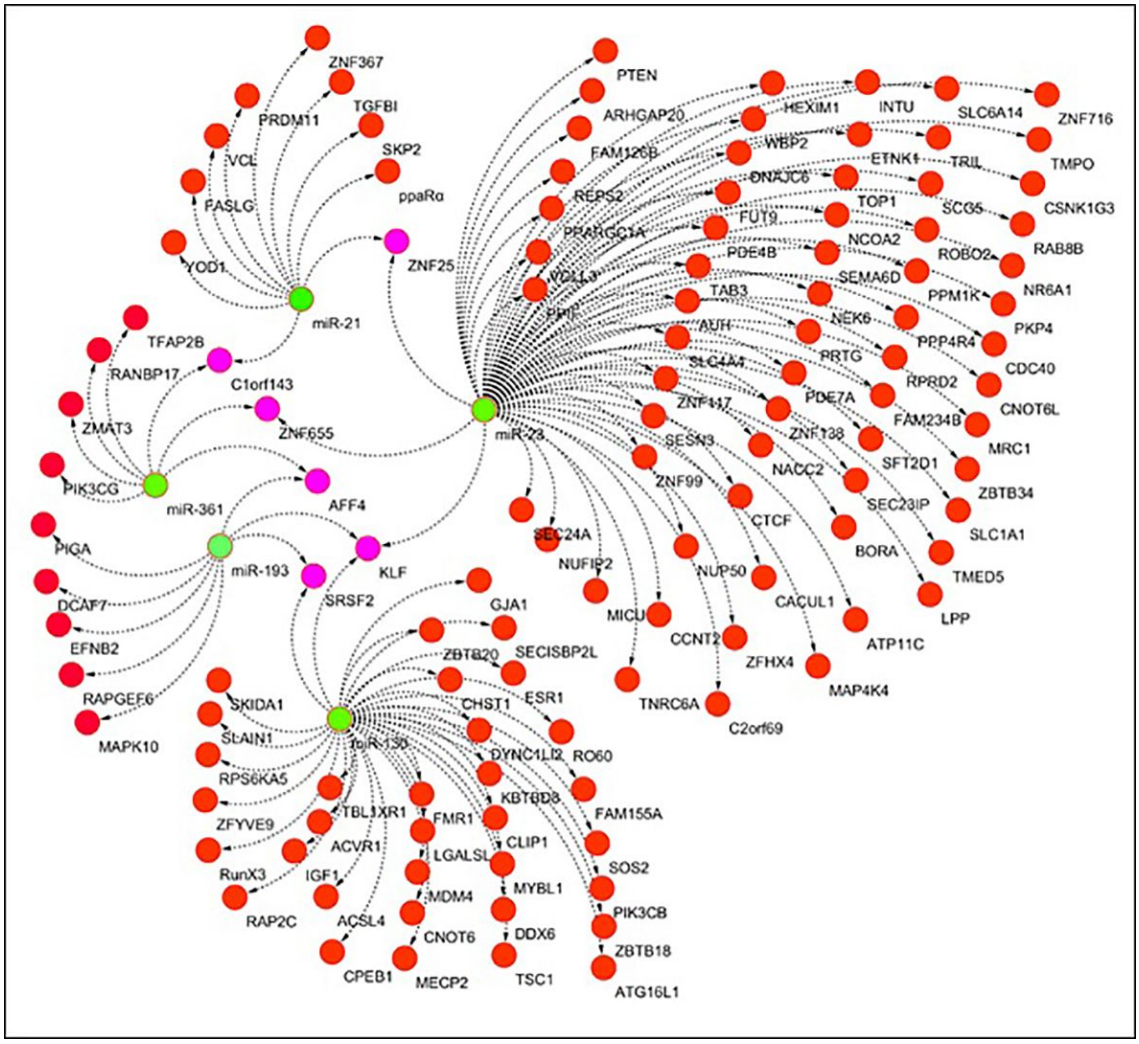

Figure 4

In-silico analysis of dysregulated microRNAs in the first trimester. Green and purple colors indicate dysregulated microRNAs and their common target genes, respectively. Interaction pathway provided by Cytoscape tool version 3.7.1.