# Analysis of Micro-RNAs and Gene Expression Profiles in Gestational Diabetes Mellitus: A Consensus Approach

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Genet. Mol. Res. 20 (1): gmr16039993
Received: December 22, 2020
Accepted: December 29, 2020
Published: January 05, 2021



## ABSTRACT.

Gestational Diabetes Mellitus (GDM) is a metabolic disorder characterized by insulin resistance. Lack of complete mechanisms involved in its pathophysiology makes its early diagnosis and treatment a difficult task. Recently, micro-RNAs are associated with many diseases including GDM. Its high stability in biological fluids and the ability to modulate genes at large scale makes it potent bio-markers. Here, we analyzed the transcriptomic datasets (GSE98043 and GSE19649) to gain a deeper understanding of the role of miRNAs in GDM. We processed and analyzed the microarray datasets to find differentially expressed miRNAs. Then we used a consensus approach to find the predicted as well as validated GDM related target genes. We then constructed the miRNA-mRNA gene regulatory module to have a better understanding of its regulation. These target genes were further enriched for their functions and pathways. We identified a total of 128 DE miRNAs, of which the top 20 were selected for downstream processing, and 49 validated

GDM related target genes among predicted ones that may contribute to the regulatory alterations behind GDM. The micro-RNAs were linked to carbohydrate metabolism, insulin signaling, and cell proliferation and apoptosis. We then focused on miRNAs which were regulating most of the genes related to GDM, this lead to the identification of four potential GDM miRNAs biomarkers, miR-3065-3p, miR-4650-3p, miR-29b-2-5p and miR-3915 that were significantly altered in GDM. The pathways enrichment analysis shows that they are involved in insulin signaling and pathways related to cancer. We demonstrated the most regulatory and novel miRNAs, miRNA-mRNA interactions, and their related pathways in GDM using Bioinformatics methods. Accordingly, our defined miRNAs and genes could be used for future molecular studies and can be useful in early diagnosis and treatment of GDM.

**Keywords:** Gestational Diabetes Mellitus; Micro-RNAs; Bio-Markers; Genes; Gene Ontology

## INTRODUCTION

Gestational Diabetes Mellitus (GDM) is defined as any degree of carbohydrate intolerance, with onset or first recognition during the second or third trimester of pregnancy. GDM complicates around 7% of all pregnancies while it comprises 90-95% of all cases of diabetes in pregnancy. It is a major cause of perinatal morbidity and mortality, as well as maternal long term morbidity. Major risk factors of GDM are the history of macrosomia, familiarity for type 2 diabetes (T2D), elevated maternal age, and pre-Pregnancy obesity. The pathophysiology of GDM is still not fully characterized. Gestational diabetes mellitus is a metabolic disorder characterized by insulin resistance accompanied by low/absent beta-cell compensatory adaptation to the increased insulin demands. During normal pregnancy, the mother develops insulin resistance during the second or third trimester to properly nourish the fetus. This insulin resistance is compensated by increasing insulin demands associated with hypertrophy and/or hyperplasia of β-cells. The inability of beta-cells to meet this increased demand for insulin leads to glucose intolerance and hyperglycemia that characterizes GDM. It is supposed that a cascade of molecules and several pathways are involved in these adaptive changes, thus contributing to gene expression changes necessary to beta-cells to fulfill the compensatory request. However, the application of molecular mechanisms in the diagnosis of GDM is not clear; therefore deciphering molecular mechanisms and biomarkers related to it should have a high impact on the diagnosis and treatment of GDM (Landon MB, Ashwal E, Sebastiani G, Bowes SB, Schiavone M, Guarino E, Lewis BP, et al.).

MicroRNAs (miRNAs) are endogenous ~19-24 nt small non-coding RNAs that modulate gene expression by inducing the translational arrest and degradation of messenger RNAs. Micro-RNA is highly versatile as a single miRNA can potentially modulate multiple genes, whereas a single gene can be regulated by several miRNAs. Such complex nature of miRNAs justifies its role in virtually every cellular process, as well as in development or differentiation, regulation of cell cycle, and immune system homeostasis. Recently, several studies have reported the role of miRNAs in multiple sides of beta-cell function and differentiation, both in normal and diabetic conditions, as well as in beta-cell compensatory processes during pregnancy. Besides their classical role in negative regulation of gene expression, they have also been shown to stimulate gene expression, and also act like hormones. Furthermore, miRNAs have been reported to be present in biological fluids and are highly stable there which can be easily detected and measured. Several studies have reported the expression of microRNAs in the plasma/serum of diabetic patient11; their association with the regulation of β cell mass and function and with the immune system homeostasis and certainly represent master players in the progression of this group of chronic metabolic disorders. Therefore a deep understanding of microRNA functions and genes and pathways related to it could improve the knowledge on the etiology and pathophysiology of GDM and its complications. Furthermore, due to their high stability in body fluids and their accessibility from maternal blood throughout gestation, they could serve as biomarkers for the early diagnosis and treatment of GDM (Sebastiani G, Faruq O, Iljas JD, Guay C, Barrett T, Davis S, et al).

This study aims to find altered miRNAs in GDM and their potential validated target genes, the determination of the most important miRNAs, and their related genes and pathways in Gestational Diabetes Mellitus. Here we investigated and identified GDM related differentially expressed miRNAs (DEmiRs), validated GDM related target genes, miRNA-mRNA interactions, and signaling pathways. Our results showed 128 DEmiRNAs of which the top 20 was considered for further analysis. Target genes were predicted and a consensus-based approach leads to the identification of validated GDM related target genes. Of the 128miRNAs, miR-3065-3p, miR-4650-3p, miR-29b-2-5p, and miR-3915 are the most novel promising biomarker. Besides, Functional and pathways enrichment showed that these miRNA and their target genes have important roles in GDM and insulin metabolism.

## MATERIALS AND METHODS

We have identified miRNA and their target genes in GDM as well as the Gene ontology and their signaling pathways. First, the top 20 differentially expressed miRNAs (DEmiRNAs) were selected based on their log fold change expression (highest log FC for up-miRNA and lowest logFC for down-miRNA). Target genes of the DEmiRNAs were predicted and GDM specific target genes were selected from gene cards and transcriptomic datasets GSE19649 for GDM blood. Then using the Cytoscape 3.2.1 software miRNA-mRNA Regulatory Module (MMRM) was constructed for up and down miRNAs separately. Furthermore, Gene Ontology and pathway enrichment analysis were performed using DAVID 7.6, significant functions and pathways involved in GDM were identified.

### Microarray data

Two microarray data sets of human GDM have been selected that are available in the public repository: NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) 14: GSE98043 and GSE19649, in which GSE98043 was used for its miRNA expression profile while GSE19649 was used for its mRNA expression profile. The dataset GSE98043, submitted by wang et al., contains 4 chips derived from a study using GPL21575 Agilent-070156 Human_miRNA_V21.0_Microarray 046064. The samples used for this study were plasma from 4 pregnant women (2 from normal controls and 2 from GDM patients). Another dataset GSE19649, submitted by Zhao et al., contains a total of 5 chips three for blood and two for the placenta, derived from a study using GPL7350. Aalborg University Illumina human-6 v2.0 expression bead chip. Only blood samples namely GSM490133 (blood tissue from GDM woman), GSM490134 (pooled blood tissues from four GDM women), and GSM490135 (pooled blood tissues from four healthy women) were used in this study. The Subjects in both data sets were of Asian (Chinese) ethnicity. Both microarray studies were earlier approved by local Chinese ethics committee and participants; here we are just accessing the data from NCBI and analyzing them.

### Data pre-processing and Differentially Expressed miRNA in GDM

The Series matrix file of both datasets was downloaded and processed. The probe-level symbols were converted into gene-level symbols by using GEO2R. Analyzing GDM has been done in two groups NGT (Normal Glucose Tolerant) control and GDM patients using the GEO2R tool, to detect the differentially expressed miRNAs. Top 20 differentially expressed miRNAs have been selected, of which 10 were highly up-regulated while 10 were highly down-regulated. The selection of miRNA was based on fold change value. All miRNA selected have P-values less than 0.05.

### Identification of miRNA-target gene

To identify the target genes of DEmiRNAs, two databases namely Target scan16 and miRDB were searched and results were downloaded. Results were tabulated and compared to find out the common target gene in both the data sets. Target score >90 were taken as criteria for gene selection. Only those target genes which were common in both databases and have a target score >90 were selected and uploaded in STRING18 for further analysis. Further selection and sorting of target genes were based on Combined Score (CS), here CS>0.9 were taken

as criteria for gene selection. All genes which have TS >90 and CS >0.9 were selected. Target prediction and gene sorting were performed separately for up-miRNA and down-miRNA.

**Construction of miRNAs and mRNAs Regulatory Module (MMRM)**

Target genes with a combined score >0.9 were then uploaded in Cytoscape 3.2.1.19. Protein-protein interaction network and the miRNA-mRNA interaction network were then merged to get microRNA-mRNA Regulatory Module (MMRM). MMRM were constructed separately for up-miRNA and down-miRNA each. The network was analyzed and edge betweenness was taken as criteria for network construction. After that, an organic layout was applied to the network. Furthermore, node size and color have been done on the miRNA-target genes network to identify miRNA, mRNA, and GDM specific genes.

**Identification of potential active miRNA-target gene in GDM**

To determine the validated GDM related miRNA-target genes as compared to predicted ones that were studied in this analysis; we searched a database Gene Cards and a transcriptomic data sets GSE19649. Those target genes which were common in both were identified as GDM specific miRNA-target genes.

**Enriched Gene Ontology and pathway analysis**

To identify the biological processes, molecular function, cellular component, and their related pathways in GDM related miRNA-target gene, DAVID 7.6 was used. Based on hypergeometric distribution, DAVID takes the genes with similar or related functions as a whole set. Significant functions were plotted against –log10 of the p-value for up and down miRNA-target genes separately. In this analysis p-value <0.05 was set as the criterion.

**GDM specific target Gene expression profile with RNA-seq**

We were then interested to know the mRNA expression of all GDM related miRNA-target genes of up-miRNA and down-miRNA both. To achieve this, transcriptomic data sets GSE19649 of GDM-blood was accessed and expression value was plotted for all genes. All the genes were plotted against average gene expression and log fold change (log FC) values. Graph pad prism 7.0 was used to plot the graph.

**Functional analysis of potential GDM specific target genes**

The biological process network was created for GDM specific target genes of up-miRNA and down-miRNA separately. All the significant biological processes being regulated by these target genes were selected and uploaded in Cytoscape 3.2.1 for network construction.

## RESULTS

**Differentially expressed miRNAs in GDM**

Both the data sets (one for miRNA and mRNA each) were analyzed according to the workflow (Figure 1). After pre-processing and data normalization, expression profiles for both data sets were created. Based on fold change value, exclusive differentially expressed miRNAs were divided into Up-or Down-regulated miRNAs. The p-value <0.005 and fold change ≥ 1.5 were set as the cut off values of DEmiRs. Our results showed a total of 128 differentially expressed miRNAs, 63 Up-regulated, and 65 Down-regulated miRNAs in GDM. The heat map was constructed for the DEmiRNAs. Out of these 128, we selected the top 20 differentially expressed miRNAs (10 from Up-miRs and 10 from Down-miRs). For visualizing the Differentially Expressed miRNAs, we sorted them and categorized the top 10 Up-and Down-regulated DEmiRNAs in GDM (Table 1). Further, for better visualization of differential expression, a heat map was constructed for these DEmiRNAs based on their average gene expression (Figure 2A) and bar graph were plotted against its log fold change value (Figure 2B).

**Table 1.** List of 20 differentially expressed miRNAs (DEmiRNAs) with their average expression value.

| Sl. NO. | miR ID | Log Fc | Adj. p Values | p Values | GDM1 | GDM2 | HC1 | HC2 | |
|---|---|---|---|---|---|---|---|---|---|
| **1** | hsa-miR-7703 | 9.7474035 | 0.688 | 0.00474 | 9.16E+00 | 3.95E+00 | -4.24E+00 | -2.73E+00 | |
| **2** | hsa-miR-3065-3p | 9.1267026 | 0.688 | 0.005803 | 2.64E+00 | 2.33E+00 | -1.32E+00 | -2.21E-01 | |
| **3** | hsa-miR-4752 | 10.6873786 | 0.688 | 0.003563 | 1.07E+00 | 4.44E-01 | -2.61E+00 | -9.18E-01 | |
| **4** | hsa-miR-146a-5p | 10.8218352 | 0.688 | 0.00042 | 7.67E+00 | 3.54E+00 | 1.73E+00 | 1.08E+00 | |
| **5** | hsa-miR-4722-3p | 10.873676 | 0.688 | 0.003376 | 3.31E-01 | 4.68E+01 | -2.64E+00 | -3.49E+00 | **Significantly UP-regulated miRNAs** |
| **6** | hsa-miR-4650-3p | 11.1341598 | 0.688 | 0.003136 | 8.82E-01 | 9.25E-01 | -4.30E-01 | -1.41E+00 | |
| **7** | hsa-miR-1272 | 11.5237329 | 0.688 | 0.002816 | 5.12E+00 | 2.34E-01 | -2.37E+00 | -7.23E-01 | |
| **8** | hsa-miR-6731-3p | 11.7662513 | 0.688 | 0.002638 | 7.52E-01 | 5.66E-01 | -1.90E+00 | -3.84E+00 | |
| **9** | hsa-miR-567 | 12.1642951 | 0.688 | 0.002376 | 8.18E-01 | 1.66E-01 | -7.91E-01 | -9.54E-01 | |
| **10** | hsa-miR-2110 | 13.2913349 | 0.688 | 0.001797 | 1.44E+00 | 2.11E-01 | -5.48E+00 | -3.10E-01 | |
| **Sl. NO.** | **miR ID** | **Log Fc** | **Adj. p Values** | **p Values** | **GDM1** | **GDM2** | **HC1** | **HC2** | |
| **1** | hsa-miR-4756-5p | -14.9916289 | 0.688 | 0.001227 | -1.05E-01 | -1.27E+00 | 1.43E+00 | 1.51E+01 | |
| **2** | hsa-miR-146a-3p | -12.7578448 | 0.688 | 0.000204 | -6.30E-01 | -6.10E-01 | 4.78E-01 | 1.74E+00 | |
| **3** | hsa-miR-2467-3p | -12.7154383 | 0.688 | 0.002067 | 1.37E+00 | -1.17E+00 | 2.82E+00 | 2.05E+00 | |
| **4** | hsa-miR-3915 | -11.4513811 | 0.688 | 0.002872 | -1.13E-01 | -1.55E+00 | 2.92E+00 | 4.41E+00 | |
| **5** | hsa-miR-99b-5p | -11.1771781 | 0.688 | 0.001339 | -8.47E-01 | -8.41E-01 | -1.95E+00 | 2.32E+00 | **Significantly Down regulated miRNAs** |
| **6** | hsa-miR-29b-2-5p | -11.1600859 | 0.688 | 0.003113 | -1.52E+00 | -1.85E+00 | 1.00E+00 | 1.56E+00 | |
| **7** | hsa-miR-873-3p | -11.1212139 | 0.688 | 0.003147 | -3.30E+00 | -3.84E+00 | 3.19E-01 | 2.62E-01 | |
| **8** | hsa-miR-4330 | -10.9450941 | 0.688 | 0.003308 | -4.16E+00 | -5.32E-02 | 3.84E+00 | 1.52E+01 | |
| **9** | hsa-miR-2682-3p | -10.4099057 | 0.688 | 0.003867 | -1.74E+00 | -5.64E+00 | 2.02E+00 | 1.36E+00 | |
| **10** | hsa-miR-6739-3p | -10.4081275 | 0.688 | 0.003869 | -1.28E+00 | -5.14E-01 | 2.27E+00 | 8.83E-01 | |

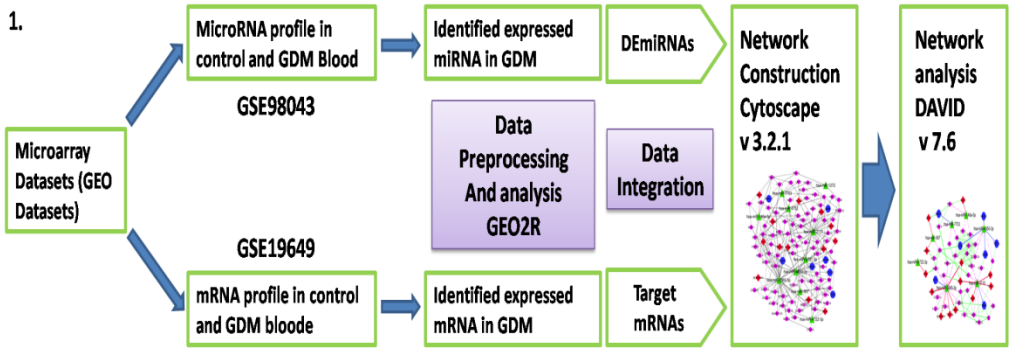


**Figure 1.** Workflow and analysis process. Bioinformatics workflow, illustrating the databases and tools employed to reveal the molecules and interactions in the gestational diabetes mellitus (GDM)-associated gene- and microRNA (miRNA)-based regulatory networks.

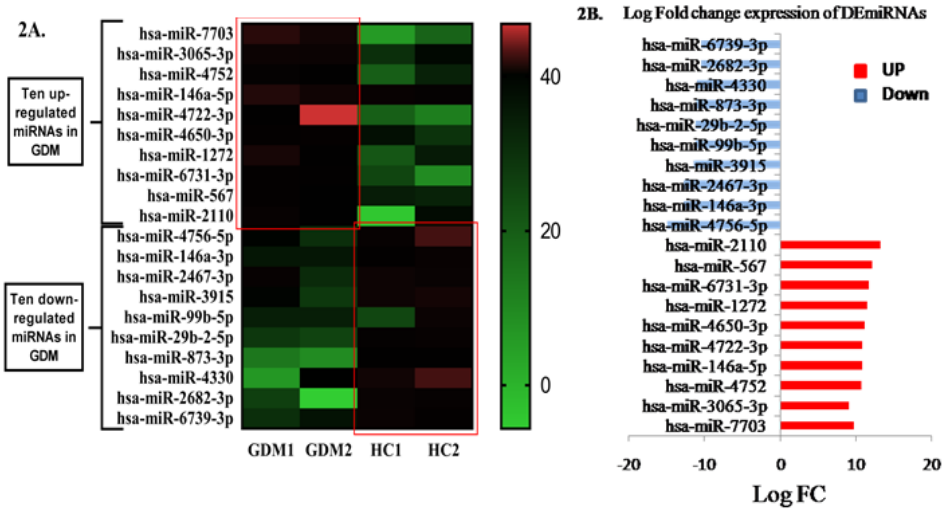


**Figure 2**. Heat map and Log fold change expression of the twenties differentially express miRNA sets. A. Heat map showing the average gene expression of differentially expressed miRNAs (DEmiRNAs) among gestational diabetes mellitus (GDM) and healthy control (HC). The green to red gradation represents the gene expression values change from small to large. Graph pad prism 7 tools were used to draw a heat map. B. Bar graph showing log fold change expression of the twenties differentially expresses miRNA sets. The red and blue bar represents the up and down-regulated miRNAs respectively.

## Target genes of DEmiRNAs

Scanning of target genes from two databases Target scan and miRDB resulted in a total of 41,685 target genes for Up- and 50,831 genes for Down-regulated miRs. Target genes for each miRNA were categorized for Up- and Down-regulated miRNAs (Table 2 and Figure 3). These target genes were further selected and sorted down based on the target score and combined score. Target score >90 and combined score >0.9 were set as the criteria for selection. Based on the target score we get a total of 795 target genes, 370 for Up-miRNAs, and 425 for Down-regulated miRNAs (Figure 4A). Further selection based on combined score led to the identification of a total of 162 target genes, 72 for Up-miRNAs, and 90 for Down-regulated miRNAs (Figure 4A). The next level of sorting was done to identify GDM specific target genes (discussed in other sub-section). Different steps involved in the selection of target genes are depicted diagrammatically in Figure 3 and number of genes sorted at each steps are depicted in Table 3.

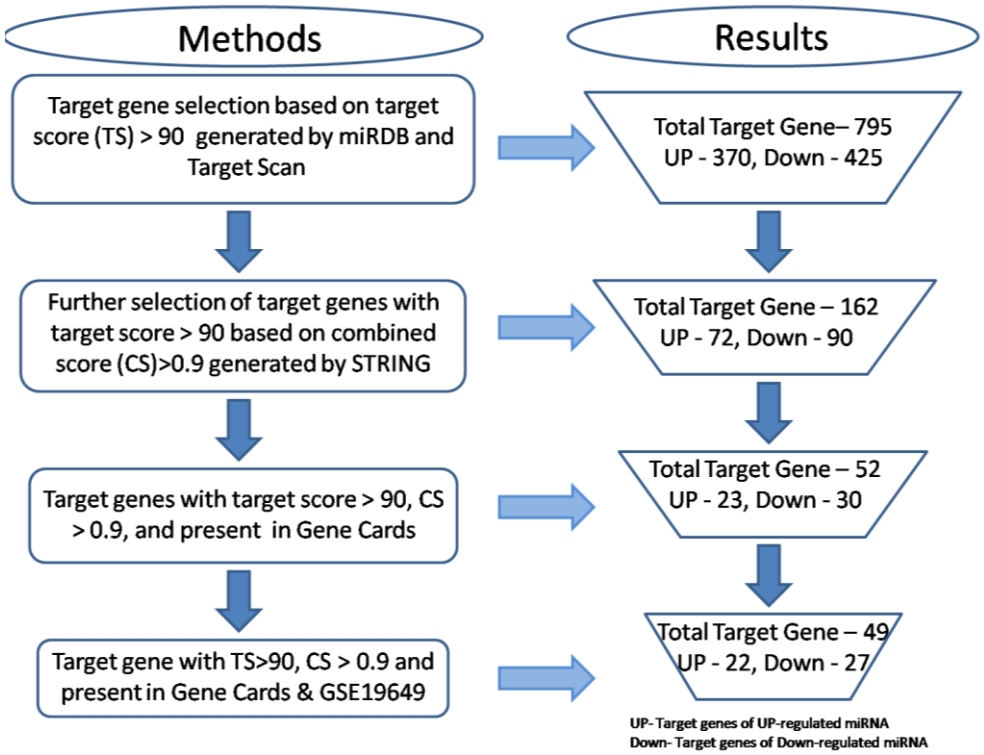


**Figure 3.** Flow chart. Flow chart showing the different steps and criteria being involved in the selection and sorting of GDM specific target genes of DEmiRNAs.

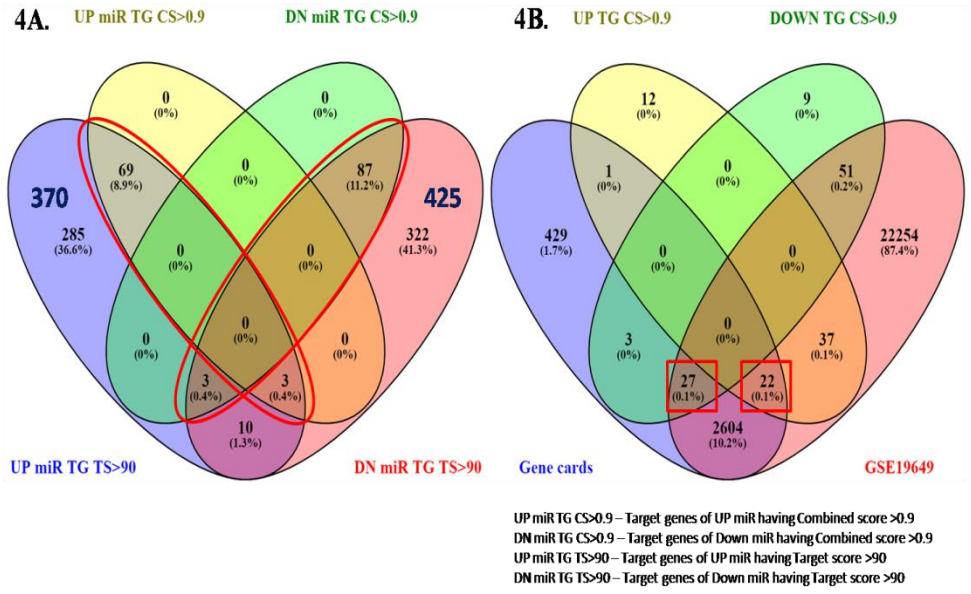


**Figure 4.** Venn diagram showing the total number of target genes being regulated by DEmiRNAs at each level of selection and sorting. A. Selection of target genes based on target score (TS>90) and combined score (CS) >0.9 B. A further selection of GDM specific target genes based on Gene Cards and GSE19649. The red rectangle and ellipse highlight the total number of target genes UP and DOWN regulated miRNAs. Venny tool v 2.1.0 was used to draw the vein diagram.

**Table 2.** List of DEmiRNAs and their respective common target genes with target score>90 searched from different database.

| | **Target Genes searched** |
|---|---|

| miR ID | Expression | Total target genes searched by Target Scan | Total genes searched by miRDB | Genes common in both with target score>90 |
|---|---|---|---|---|
| hsa-miR-7703 | UP-MIR | 4617 | 460 | 36 |
| hsa-miR-3065-3p | UP-MIR | 4255 | 718 | 102 |
| hsa-miR-4752 | UP-MIR | 3298 | 378 | 26 |
| hsa-miR-146a-5p | UP-MIR | 275 | 488 | 21 |
| hsa-miR-4722-3p | UP-MIR | 5859 | 560 | 17 |
| hsa-miR-4650-3p | UP-MIR | 3120 | 504 | 47 |
| hsa-miR-1272 | UP-MIR | 3388 | 432 | 22 |
| hsa-miR-6731-3p | UP-MIR | 2791 | 353 | 23 |
| hsa-miR-567 | UP-MIR | 3395 | 545 | 30 |
| hsa-miR-2110 | UP-MIR | 5362 | 887 | 50 |
| **Total** | | **36, 360** | **5, 325** | **374** |
| **Total target Genes of UP-regulated miRNAs=374 [Unique value=370]** | | | | |
| hsa-miR-4756-5p | Down-MIR | 5507 | 875 | 44 |
| hsa-miR-146a-3p | Down-MIR | 5150 | 881 | 68 |
| hsa-miR-2467-3p | Down-MIR | 6074 | 945 | 53 |
| hsa-miR-3915 | Down-MIR | 4841 | 873 | 57 |
| hsa-miR-99b-5p | Down-MIR | 59 | 47 | 8 |
| hsa-miR-29b-2-5p | Down-MIR | 5077 | 859 | 97 |
| hsa-miR-873-3p | Down-MIR | 4295 | 358 | 12 |
| hsa-miR-4330 | Down-MIR | 4533 | 506 | 22 |
| hsa-miR-2682-3p | Down-MIR | 4274 | 416 | 17 |
| hsa-miR-6739-3p | Down-MIR | 4408 | 853 | 62 |
| **Total** | | **44, 218** | **6, 613** | **440** |
| **Total target Genes of Down-regulated miRNAs=440 [Unique value=425]** | | | | |

**Table 3.** Sorted target genes with their respective DEmiRNAs and expression value from GSE19649.

| Sl.No. | miRID | Expression | Gene Symbol | adj.P.Val | P.Value | logFC | Expression |
|---|---|---|---|---|---|---|---|
| 1 | hsa-miR-146a-5p | UP-MIR | IRAK1 | 0.97 | 0.881307 | 0.0751153 | UP |
| 2 | hsa-miR-2110 | UP-MIR | IL6ST | 0.661 | 0.063488 | 2.707043 | UP |
| 3 | hsa-miR-2110 | UP-MIR | FGFR1 | 0.667 | 0.079481 | 2.3631764 | UP |
| 4 | hsa-miR-2110 | UP-MIR | PYGB | 0.768 | 0.262578 | 0.6297874 | UP |
| 5 | hsa-miR-2110 | UP-MIR | ELK1 | 0.932 | 0.767384 | 0.1534358 | UP |
| 6 | hsa-miR-2110 | UP-MIR | BRAF | 0.96 | 0.849252 | -0.096696 | DOWN |
| 7 | hsa-miR-2110 | UP-MIR | SEMA5A | NA | NA | NA | |
| 8 | hsa-miR-3065-3p | UP-MIR | SERPINH1 | 0.661 | 0.004637 | 5.1647439 | UP |
| 9 | hsa-miR-3065-3p | UP-MIR | HBEGF | 0.861 | 0.547787 | 0.3590278 | UP |
| 10 | hsa-miR-3065-3p | UP-MIR | VHL | 0.884 | 0.623891 | 0.2491753 | UP |
| 11 | hsa-miR-3065-3p | UP-MIR | STX1A | 0.921 | 0.73254 | -0.1732861 | DOWN |
| 12 | hsa-miR-3065-3p | UP-MIR | CCND2 | 0.926 | 0.751809 | 0.2108157 | UP |
| 13 | hsa-miR-3065-3p | UP-MIR | TBC1D4 | 0.929 | 0.759318 | 0.3543628 | UP |
| 14 | hsa-miR-3065-3p | UP-MIR | MED12L | NA | NA | NA | |
| 15 | hsa-miR-4650-3p | UP-MIR | HMGA2 | 0.666 | 0.075853 | -3.1973274 | DOWN |
| 16 | hsa-miR-4650-3p | UP-MIR | TCF7L2 | 0.722 | 0.159903 | -0.869586 | DOWN |
| 17 | hsa-miR-4650-3p | UP-MIR | CD36 | 0.831 | 0.459778 | -0.4184764 | DOWN |
| 18 | hsa-miR-4650-3p | UP-MIR | IL10RB | 0.853 | 0.522855 | -0.3637919 | DOWN |
| 19 | hsa-miR-4722-3p | UP-MIR | PBX1 | 0.988 | 0.94737 | 0.0439 | UP |
| 20 | hsa-miR-567 | UP-MIR | ARID1A | 0.661 | 0.03859 | 3.6287296 | UP |
| 21 | hsa-miR-567 | UP-MIR | ATG5 | 0.837 | 0.480002 | 0.368994 | UP |
| 22 | hsa-miR-7703 | UP-MIR | RNF135 | 0.876 | 0.59697 | -0.2870281 | DOWN |
| 1 | hsa-miR-146a-3p | Down-MIR | TXNIP | 0.947 | 0.806965 | 0.12428 | UP |
| 2 | hsa-miR-146a-3p | Down-MIR | FZD4 | NA | NA | NA | |
| 3 | hsa-miR-146a-3p | Down-MIR | SORT1 | 0.776 | 0.279027 | -0.60279 | DOWN |
| 4 | hsa-miR-2467-3p | Down-MIR | FKBP1A | 0.746 | 0.216253 | -0.72693 | DOWN |
| 5 | hsa-miR-2467-3p | Down-MIR | IL1RN | 0.798 | 0.335011 | 0.844496 | UP |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 6 | hsa-miR-2467-3p | Down-MIR | AR | 0.754 | 0.233983 | -0.86218 | DOWN |
| 7 | hsa-miR-2467-3p | Down-MIR | KCNB1 | NA | NA | NA | |
| 8 | hsa-miR-2467-3p | Down-MIR | SULT2A1 | NA | NA | NA | |
| 9 | hsa-miR-2682-3p | Down-MIR | SPRY2 | 0.661 | 0.024107 | -2.10976 | DOWN |
| 10 | hsa-miR-29b-2-5p | Down-MIR | FBN1 | 0.756 | 0.242347 | -1.11274 | DOWN |
| 11 | hsa-miR-29b-2-5p | Down-MIR | PLXNA1 | 0.801 | 0.349435 | -0.92566 | DOWN |
| 12 | hsa-miR-29b-2-5p | Down-MIR | CACNA2D3 | 0.811 | 0.379452 | 0.515053 | UP |
| 13 | hsa-miR-29b-2-5p | Down-MIR | ACSL1 | 0.823 | 0.425851 | | |
| 14 | hsa-miR-29b-2-5p | Down-MIR | POLR3A | 0.983 | 0.930515 | 0.043018 | UP |
| 15 | hsa-miR-29b-2-5p | Down-MIR | PRKG1 | 0.986 | 0.943176 | 0.039746 | UP |
| 16 | hsa-miR-29b-2-5p | Down-MIR | TBL1X | 0.993 | 0.972287 | 0.01833 | UP |
| 17 | hsa-miR-29b-2-5p | Down-MIR | CCL11 | NA | NA | NA | |
| 18 | hsa-miR-3915 | Down-MIR | IL1RAP | 0.661 | 0.036744 | -2.25565 | DOWN |
| 19 | hsa-miR-3915 | Down-MIR | FKBP5 | 0.681 | 0.101399 | -1.20476 | DOWN |
| 20 | hsa-miR-3915 | Down-MIR | CACNA2D1 | 0.733 | 0.185701 | 1.181032 | UP |
| 21 | hsa-miR-3915 | Down-MIR | RPS6KB1 | 0.971 | 0.883808 | -0.0722 | DOWN |
| 22 | hsa-miR-3915 | Down-MIR | GRIA3 | NA | NA | NA | |
| 23 | hsa-miR-3915 | Down-MIR | AVPR1A | NA | NA | NA | |
| 24 | hsa-miR-4330 | Down-MIR | NRXN1 | 0.681 | 0.099354 | 1.601328 | UP |
| 25 | hsa-miR-4756-5p | Down-MIR | FGF23 | 0.783 | 0.301097 | -0.68339 | DOWN |
| 26 | hsa-miR-6739-3p | Down-MIR | PRLR | 0.801 | 0.345088 | -3.2489 | DOWN |
| 27 | hsa-miR-6739-3p | Down-MIR | EIF2S1 | 0.834 | 0.471175 | 0.552658 | UP |

**Enrichment of GDM specific target genes**

In this study, we tried to select those target genes which are validated potential players in GDM. For this, we compared our computationally predicted target gene list with genes obtained from the Gene Cards database, containing experimentally validated GDM related gene and was further validated from transcriptomic data set GSE19649. Venn diagram by VENNY 2.1 tool has been drawn for common genes between Gene Cards, GSE19649, and our gene list of Up-and Down-miRs. Thus the result showed a total of 49 target genes, 22 for Up-miRNAs and 27 for Down-miRNAs as potential GDM specific target genes which are experimentally validated (Figure 4).

**miRNA-mRNA regulatory module (MMRM) in GDM**

All target genes with combined score >0.9 along with their respective miRNAs were transferred into the Cytoscape 3.2.1, and the network was constructed separately for Up-miRs and Down-miRs. The regulatory miRNA-target genes network (MMRM) for Up-miRs included a total of 380 genes 10 Up-regulated miRNAs and 528 edges, while MMRM for Down-miRs contain total 435 genes, 10 Down-regulated miRNAs and 621 edges. We filtered the unique target genes for each miRNA and GDM specific target genes were given different shapes and colors based on expression value. The node shape (diamond-ellipse) and color (red-blue) represented the expression value of Down- and Up-regulated respectively. The final MMRM for Up-miRs included a total of 114 genes (20 GDM specific target genes), 10 Up-miRs, and 257 edges (Figure 5A) while MMRM for Down-miRs included total 166 genes (21 GDM specific target genes), 10 Down-miRs and 350 edges (Figure 5B). Out of 20 GDM specific target genes in Up-MMRM, 13 were up-regulated while 7 were down-regulated. Similarly, in Down-MMRM, out of 21, 9 were Up-regulated while 12 were down-regulated.

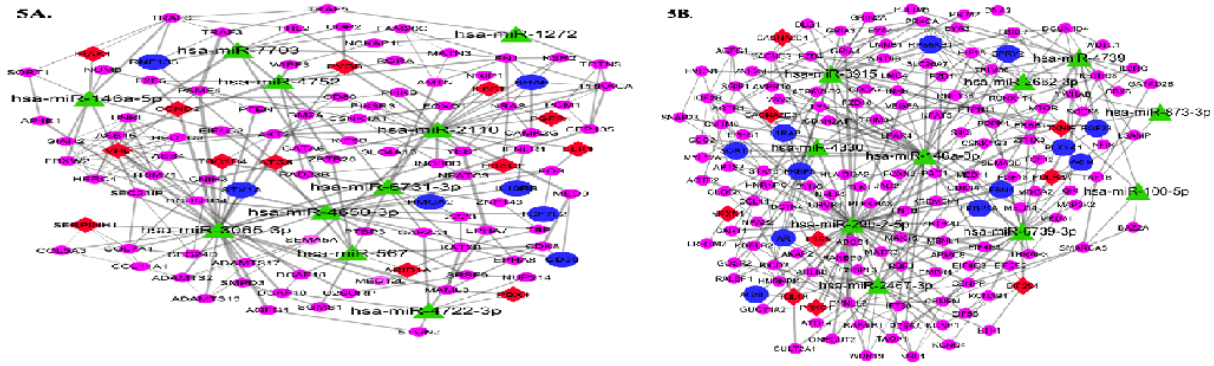


**Figure 5.** A: MicroRNA-mRNA regulatory Module (MMRM) of up-regulated miRNA and their target genes. Red diamond GDM specific up-regulated genes, Blue ellipse GDM specific down-regulated genes, Purple ellipse other target genes, Green triangle up-regulated miRNAs. Lines the correlation between genes Thickness of lines (edges) is proportional to the combined score. Cytoscape v 3.2.1 was used to construct the network. 5B: MicroRNA-mRNA regulatory Module (MMRM) of Down-regulated miRNA and their target genes. Red diamond GDM specific up-regulated genes, Blue ellipse GDM specific down-regulated genes, Purple ellipse other target genes, Green triangle up-regulated miRNAs. Lines the correlation between genes Thickness of lines (edges) is proportional to the combined score. Cytoscape v 3.2.1 was used to construct the network.

## Functional enrichment analysis

Gene ontology enrichment analysis for target genes involved in the MMRM network was performed and significantly enriched functions, processes, and cellular components (p-value <0.05) were listed in tables 4 and 5 (for target genes of up-regulated miRs) and table 6 (for target genes of down-miRs). Response to insulin stimulus, lipid storage, regulation of apoptosis, and cell proliferation are major significant processes being regulated by the Up-regulated miRNAs through regulation of expression of target genes involved in it (Figure 6A). Major significant processes regulated by Down-miRNAs are regulation of fatty acid metabolism, regulation of immune response, tyrosine kinase signaling, Wnt receptor signaling, and response to carbohydrate stimulus (Figure 6B).

**Table 4.** Criteria applied for gene sorting and number of genes selected at each step.

| Criteria of Gene selection being employed | Target Genes of UP miRNAs | Target Genes of Down miRNAs |
|---|---|---|
| Target genes with target score (TS) >90 | 370 | 425 |
| Target genes with target score (TS) >90 and combined score (CS)>0.9 | 72 | 90 |
| Target genes with target score (TS) >90, combined score (CS)>0.9 and present in Gene cards | 23 | 30 |
| Target genes with target score (TS) >90, combined score (CS)>0.9, present in Gene cards and GSE19649 | 22 | 27 |

**Table 5.** Gene ontology and pathway enrichment analysis for target genes of up-mirs.

| Category | Term | P-Value |
|---|---|---|
| GOTERM_BP_FAT | GO:0010628~positive regulation of gene expression | 0.001239229 |
| GOTERM_BP_FAT | GO:0010604~positive regulation of macromolecule metabolic process | 0.00160204 |
| GOTERM_BP_FAT | GO:0045935~positive regulation of nucleotide and nucleic acid metabolic process | 0.002028601 |
| GOTERM_BP_FAT | GO:0051173~positive regulation of nitrogen compound metabolic process | 0.002512839 |
| GOTERM_BP_FAT | GO:0019915~lipid storage | 0.00301611 |
| GOTERM_BP_FAT | GO:0031328~positive regulation of the cellular biosynthetic process | 0.003793497 |

| | | |
|---|---|---|
| GOTERM_BP_FAT | GO:0007167~enzyme linked receptor protein signaling pathway | 0.00467085 |
| GOTERM_BP_FAT | GO:0043066~negative regulation of apoptosis | 0.00551467 |
| GOTERM_BP_FAT | GO:0051254~positive regulation of RNA metabolic process | 0.006246808 |
| GOTERM_BP_FAT | GO:0045944~positive regulation of transcription from RNA polymerase II promoter | 0.006895316 |
| GOTERM_BP_FAT | GO:0032868~response to insulin stimulus | 0.010815146 |
| GOTERM_BP_FAT | GO:0006897~endocytosis | 0.018212366 |
| GOTERM_BP_FAT | GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway | 0.019321416 |
| GOTERM_BP_FAT | GO:0006886~intracellular protein transport | 0.028128857 |
| GOTERM_BP_FAT | GO:0016310~phosphorylation | 0.028892007 |
| GOTERM_BP_FAT | GO:0007548~sex differentiation | 0.031943201 |
| GOTERM_BP_FAT | GO:0003006~reproductive developmental process | 0.031958933 |
| GOTERM_BP_FAT | GO:0043434~response to peptide hormone stimulus | 0.033575483 |
| GOTERM_BP_FAT | GO:0034613~cellular protein localization | 0.0397918 |
| GOTERM_BP_FAT | GO:0008284~positive regulation of cell proliferation | 0.040849818 |
| GOTERM_BP_FAT | GO:0006665~sphingolipid metabolic process | 0.046317716 |
| GOTERM_BP_FAT | GO:0016192~vesicle-mediated transport | 0.047719234 |
| GOTERM_BP_FAT | GO:0006643~membrane lipid metabolic process | 0.053149906 |
| GOTERM_MF_FAT | GO:0030528~transcription regulator activity | 0.002110548 |
| GOTERM_MF_FAT | GO:0019899~enzyme binding | 0.008397377 |
| GOTERM_MF_FAT | GO:0019838~growth factor binding | 0.01149475 |
| GOTERM_MF_FAT | GO:0008134~transcription factor binding | 0.026362292 |
| GOTERM_MF_FAT | GO:0019901~protein kinase binding | 0.027898848 |
| GOTERM_MF_FAT | GO:0016563~transcription activator activity | 0.035783067 |
| GOTERM_MF_FAT | GO:0019900~kinase binding | 0.045787287 |
| GOTERM_MF_FAT | GO:0004672~protein kinase activity | 0.052488424 |
| GOTERM_CC_FAT | GO:0044451~nucleoplasm part | 0.006173003 |
| GOTERM_CC_FAT | GO:0005654~nucleoplasm | 0.02251137 |
| GOTERM_CC_FAT | GO:0016585~chromatin remodeling complex | 0.032561638 |
| GOTERM_CC_FAT | GO:0012505~endomembrane system | 0.034566827 |
| GOTERM_CC_FAT | GO:0005887~integral to the plasma membrane | 0.043489378 |
| GOTERM_CC_FAT | GO:0031226~intrinsic to the plasma membrane | 0.049052532 |
| GOTERM_CC_FAT | GO:0031981~nuclear lumen | 0.057813502 |

**Table 6.** Gene ontology and pathway enrichment analysis for target genes of down-miRs.

| Category | Term | P-Value |
|---|---|---|
| GOTERM_BP_FAT | GO:0016055~Wnt receptor signaling pathway | 1.08E-04 |
| GOTERM_BP_FAT | GO:0032868~response to insulin stimulus | 0.002517288 |
| GOTERM_BP_FAT | GO:0009628~response to abiotic stimulus | 0.004890961 |
| GOTERM_BP_FAT | GO:0032774~RNA biosynthetic process | 0.006742312 |
| GOTERM_BP_FAT | GO:0007166~cell surface receptor linked signal transduction | 0.006932356 |
| GOTERM_BP_FAT | GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway | 0.008956942 |
| GOTERM_BP_FAT | GO:0043434~response to peptide hormone stimulus | 0.011494766 |
| GOTERM_BP_FAT | GO:0007167~enzyme linked receptor protein signaling pathway | 0.013165622 |
| GOTERM_BP_FAT | GO:0045621~positive regulation of lymphocyte differentiation | 0.016852376 |
| GOTERM_BP_FAT | GO:0019216~regulation of lipid metabolic process | 0.025958885 |
| GOTERM_BP_FAT | GO:0032844~regulation of the homeostatic process | 0.027168996 |
| GOTERM_BP_FAT | GO:0050863~regulation of T cell activation | 0.029040041 |
| GOTERM_BP_FAT | GO:0014070~response to organic cyclic substance | 0.031638785 |
| GOTERM_BP_FAT | GO:0048193~Golgi vesicle transport | 0.038651759 |
| GOTERM_BP_FAT | GO:0060429~epithelium development | 0.040404439 |

| | | |
|---|---|---|
| GOTERM_BP_FAT | GO:0010565~regulation of cellular ketone metabolic process | 0.041728975 |
| GOTERM_BP_FAT | GO:0006955~immune response | 0.041975455 |
| GOTERM_BP_FAT | GO:0009743~response to carbohydrate stimulus | 0.045779062 |
| GOTERM_BP_FAT | GO:0002684~positive regulation of immune system process | 0.046685551 |
| GOTERM_BP_FAT | GO:0006796~phosphate metabolic process | 0.048512996 |
| GOTERM_BP_FAT | GO:0045449~regulation of transcription | 0.053658147 |
| GOTERM_BP_FAT | GO:0010604~positive regulation of macromolecule metabolic process | 0.053765873 |
| GOTERM_BP_FAT | GO:0051924~regulation of calcium ion transport | 0.058738904 |
| GOTERM_MF_FAT | GO:0022838~substrate specific channel activity | 0.001566895 |
| GOTERM_MF_FAT | GO:0022803~passive transmembrane transporter activity | 0.00197717 |
| GOTERM_MF_FAT | GO:0046873~metal ion transmembrane transporter activity | 0.002223261 |
| GOTERM_MF_FAT | GO:0005262~calcium channel activity | 0.009570277 |
| GOTERM_MF_FAT | GO:0005245~voltage-gated calcium channel activity | 0.010444832 |
| GOTERM_MF_FAT | GO:0042393~histone binding | 0.024695146 |
| GOTERM_MF_FAT | GO:0042562~hormone binding | 0.030161026 |
| GOTERM_MF_FAT | GO:0004896~cytokine receptor activity | 0.037287959 |
| GOTERM_MF_FAT | GO:0042813~Wnt receptor activity | 0.048833168 |
| GOTERM_MF_FAT | GO:0005528~FK506 binding | 0.048833168 |
| GOTERM_MF_FAT | GO:0005527~macrolide binding | 0.048833168 |
| GOTERM_MF_FAT | GO:0005161~platelet-derived growth factor receptor binding | 0.059361395 |
| GOTERM_CC_FAT | GO:0005624~membrane fraction | 0.016195535 |
| GOTERM_CC_FAT | GO:0005626~insoluble fraction | 0.020370953 |
| GOTERM_CC_FAT | GO:0005654~nucleoplasm | 0.027677113 |
| GOTERM_CC_FAT | GO:0031974~membrane-enclosed lumen | 0.030116453 |
| GOTERM_CC_FAT | GO:0031981~nuclear lumen | 0.033309162 |
| GOTERM_CC_FAT | GO:0044451~nucleoplasm part | 0.038658183 |
| GOTERM_CC_FAT | GO:0034703~cation channel complex | 0.039679963 |

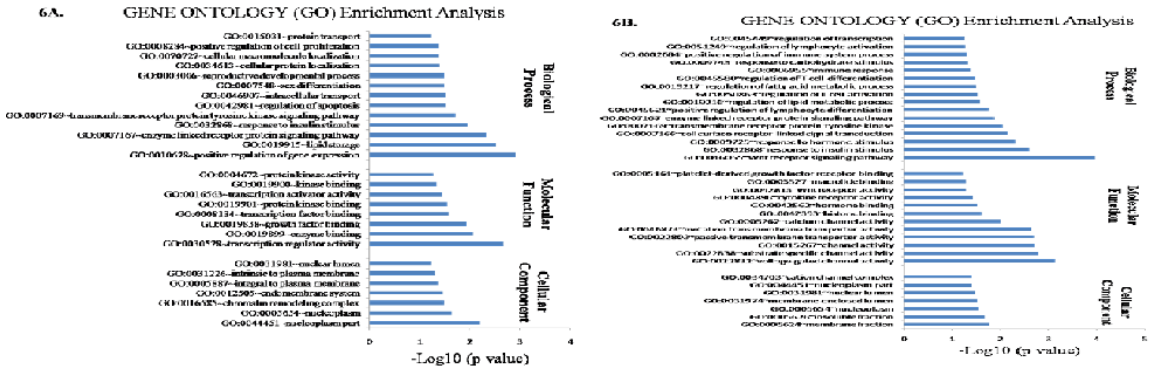


**Figure 6.** A: Gene Ontology (GO) analysis for target Genes of up-miR in PPI network. Bar graph showing significant processes, function, and cellular component enriched in diabetic mothers for target genes of up-miR. DAVID v 6.7 was used for annotation. 6B: Gene Ontology (GO) analysis for target Genes of down-miR in PPI network. Bar graph showing significant processes, function, and cellular component enriched in diabetic mothers for target genes of down-miR. DAVID v 6.7 was used for annotation.

## KEGG Pathway enrichment analysis

According to the KEGG pathway analysis of DAVID 7.6 software, we demonstrated the significant pathways for Up-miRs and Down-miRs. Significant pathways enriched for target genes of Down-miRs were long term depression and pathways in cancer while JAK-STAT signaling, Wnt signaling, Insulin signaling, and ErbB signaling are some of the major significant pathways being regulated by target genes of Up-miRs (Figure 6C). It

should be noted that GDM specific target genes of Up-miRs and Down-miRs were enriched in GO functions and/or KEGG pathways together with their other related genes (Figures 7-9).

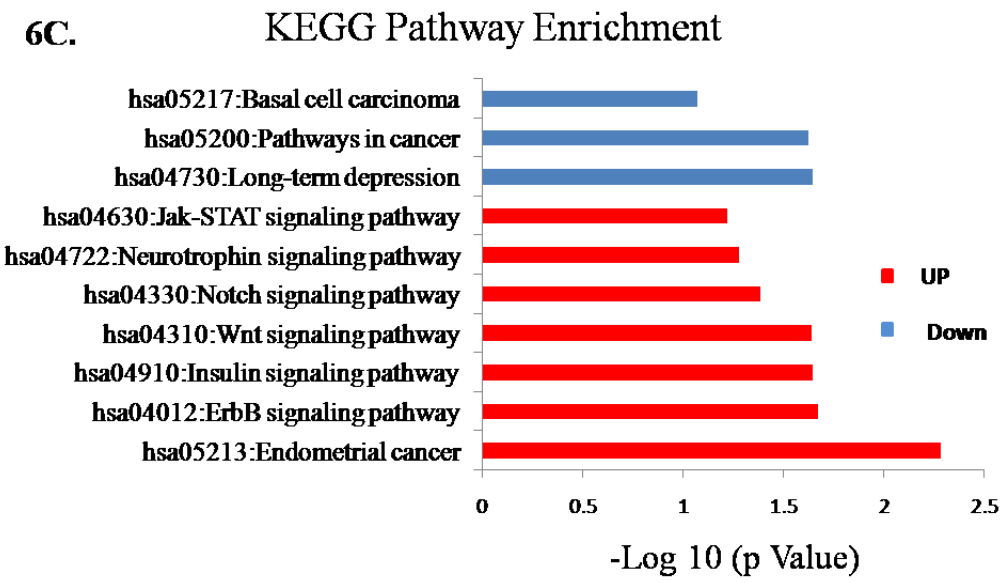


**Figure 6C.** KEGG Pathway analysis for target genes of DEmiRNAs in diabetic mothers. Pathway enrichment for DEmiR leads to the identification of 3 significant pathways for up-miR while 7 significant pathways for down-miR. DAVID v 6.7 was used for annotation. Red and blue bar represents pathways of up-miR and down-miR respectively.

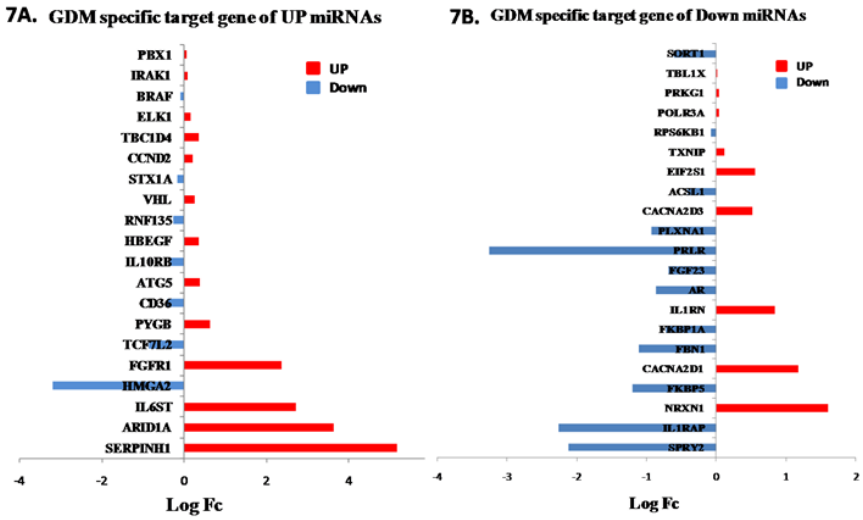


**Figure 7.** Log fold change gene expression of GDM specific target genes of up and down miRNAs. A. Expression of GDM specific target genes of up-miRNAs. B. Expression of GDM specific target genes of down-miRNAs.

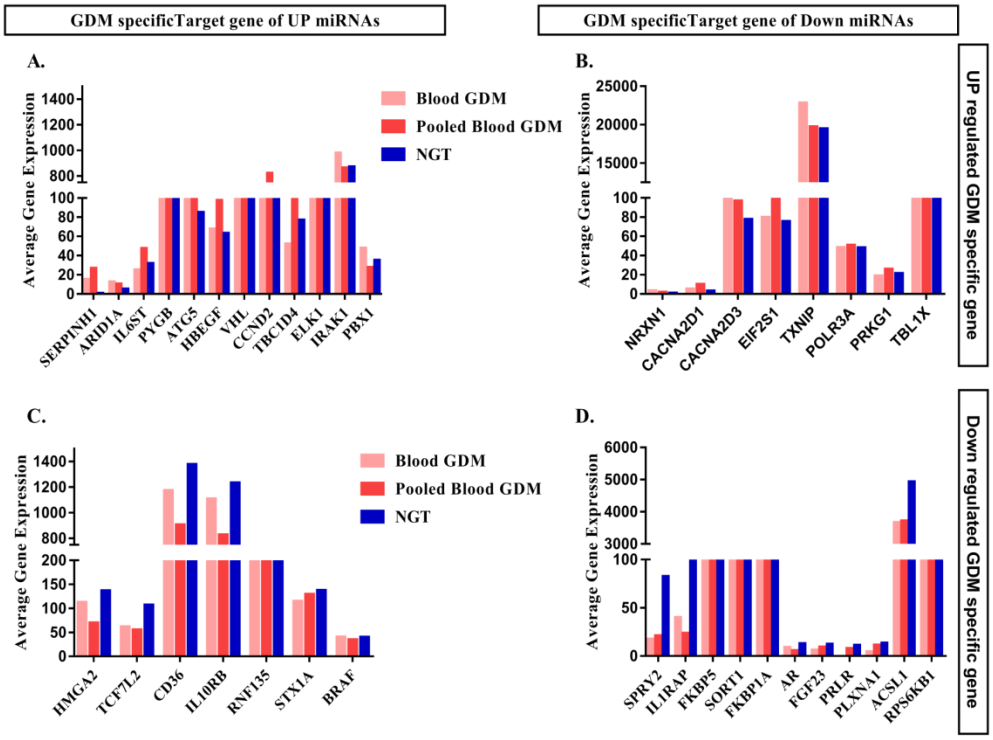


**Figure 8.** Average gene expression of GDM specific target genes of up and down miRNAs. Expression of A. GDM specific up-regulated target genes B. GDM specific down-regulated target genes of up-miRNAs. C. GDM specific up-regulated target genes D. GDM specific down-regulated target genes of down-miRNAs.

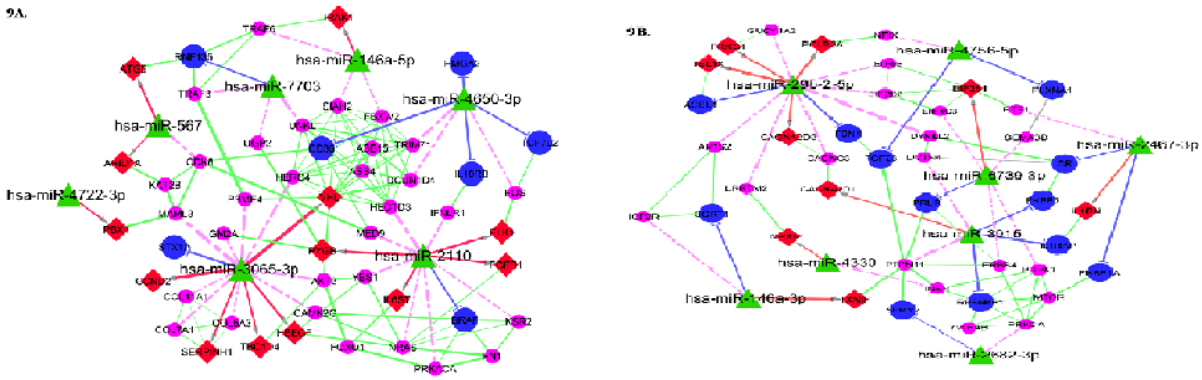


**Figure 9.** Micro-RNA-gene regulatory network of DEmiRNAs. Regulatory network of A. Up-miRNAs and B. Down-miRs. Red diamond GDM specific up-regulated genes, Blue ellipse GDM specific down-regulated genes, Purple ellipse related target genes. Red edges stimulatory effect, Blue edges inhibitory effect, Purple edges unknown

## Study of significant processes being regulated by GDM specific target Genes

The validated target genes for DEmiRNAs were enriched for their biological processes. Biological processes specific to validate GDM related genes of DEmiRNAs were extracted and transferred to Cytoscape 3.2.1, and the network was constructed. Target genes of Up-miRNAs were involved in several significant processes including response to insulin stimulus, regulation of cell-proliferation and cell-death, Tyrosine kinase signaling, oxidative phosphorylation, lipid storage, regulation of gene expression and glycoprotein biosynthesis (Figure 10A). Similarly, target genes of Down-miRNAs were regulating some of the major processes like regulation of transcription, Wnt signaling pathway, response to carbohydrate stimulus, regulation of lipid metabolism, immune

response and metal ion transport (Figure 10B). On the basis of our findings, it may suggest that among others, miR-3065-3p, miR-4650-3p, miR-29b-2-5p, and miR-3915 might be the most promising blood-derived miRNA biomarkers in GDM (Figure 11).

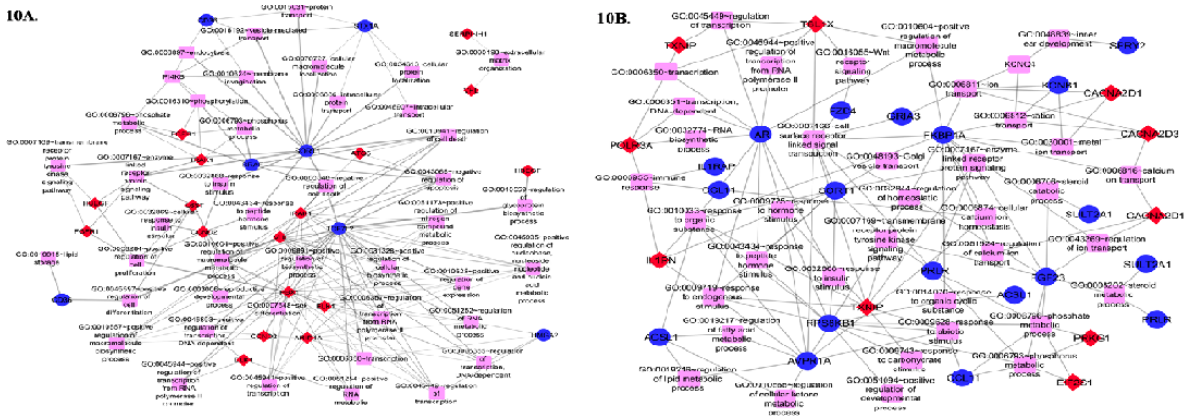


**Figure 10.** A: Functional analysis of GDM specific target genes of up miRNAs. The functional analysis uncovers many significant processes being regulated by them. Red diamond GDM specific up-regulated genes, Blue ellipse GDM specific down-regulated genes, Purple rectangle biological processes. 10B: Functional analysis of GDM specific target genes of down miRNAs. The functional analysis uncovers many significant processes being regulated by them. Red diamond GDM specific up-regulated genes, Blue ellipse GDM specific down-regulated genes, Purple rectangle biological processes.

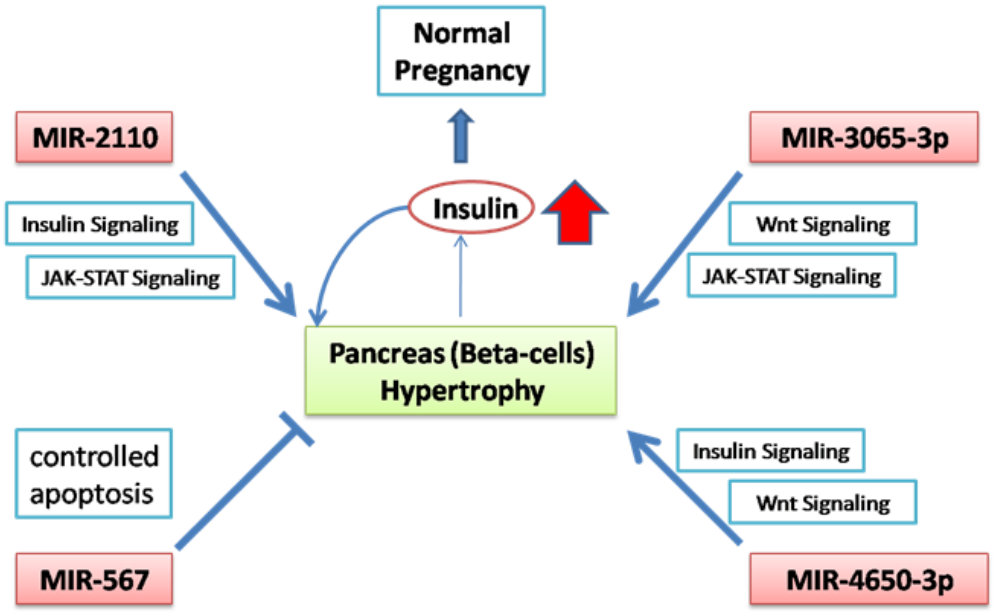


**Figure 11.** Hypothetical view of regulation of pancreas hypertrophy by up-miRNAs. MIR-2110 and 3065-3p were increasing the cell proliferation of the pancreas via tyrosine kinase and insulin pathway, while miR-567 may involve in controlled apoptosis to prevent over proliferation. A delicate balance between proliferation and apoptosis may maintain by these Up-miRNAs. Controlled pancreas hypertrophy may be able to produce enough insulin to meet the demands, while any alteration can lead to GDM.

## DISCUSSION

Gestational Diabetes Mellitus is a general metabolic disorder of glucose metabolism and its pathophysiological process mostly begins before clinical diagnosis. Currently, screening and diagnosis of GDM are accomplished at 24-28 weeks of gestation, this is the period by which diabetes has already been established and presents a high risk of fetal morbidity and mortality (Garcia, Liu W, Franceschini A, Kohl M, Huang DW, Jang w,

Sweeting A, Guay C, Ventriglia G). Further, its increasing incidences demand additional biomarkers to predict the onset and to accurately monitor the status of gestational diabetes so that early screening in the first or second trimester of pregnancy could be feasible to promptly set up an adequate therapy which normalizes blood glucose levels22, thereby reducing GDM incidences and its associated adverse pregnancy outcomes. Therefore, the molecular study of GDM could have an important role in detecting biomarkers involved in the prognosis of GDM. Recently several studies have reported the diverse role of miRNA in many diseases. Due to its endocrine nature and ability to modulate gene expression, it can be considered as optimal biomarkers and sensors in GDM. However, due to the limited number of public miRNA microarray expression profiles for GDM, candidate miRNAs for GDM has not been identified yet. In the present study, we have tried to identify the blood-derived miRNAs and mRNA biomarkers as well as molecular interactions that clarify biochemical mechanisms involved in GDM. Further, we created a miRNA-mRNA based network based on which we were able to identify some miRNAs biomarkers and pathways potentially involved in GDM.

The two microarray datasets, GSE8043-miRNA, and GSE19649-mRNA were statistically analyzed in this study. We have identified a total of 128 differentially expressed miRNAs, 63 Up-regulated, and 65 Down-regulated miRNAs in GDM. For further analysis, only the top 20 differentially expressed miRNAs (10 Up and 10 Down) were selected and plotted for their fold change values and a heat map was generated (Figures 2A and 2B). Target gene prediction led to the identification of a total of 41,685 target genes for Up- and 50,831 genes for Down-regulated miRs. These genes were further sorted based on target score and combined score leading to the identification of 49 potential validated GDM related genes (22 for up- and 27 for down-miRs) were identified (Figure 4). Then, the miRNA-mRNA regulatory module was constructed for Up-miRNAs and Down-miRNAs (Figure 5A and 5B). Validated GDM related target genes along with other neighbor-genes were enriched for their functions and pathways. Major significant functions and pathways regulated by target genes of Up-miRs were a response to insulin stimulus, lipid storage, regulation of apoptosis, cell proliferation, response to carbohydrate stimulus, Notch signaling, Wnt signaling, insulin signaling, and ErbB signaling pathways. Down-miRs were found to be involved in the regulation of fatty acid metabolism, regulation of immune response, pathways in cancer and long term depression, tyrosine kinase signaling (Figures 6A-6C). Expression analysis of all 49 GDM specific-target genes reveals 13 up- and 7 down-regulated target genes of Up-miRs while 9 up- and 12 down-regulated target genes of Down-miRs (Figure 7 and 8). Analysis of gene regulatory network led to the identification of 7 Up-miRs and 8 Down-miRs regulating maximum GDM related target genes (Figure 9A and 9B).

We identified four potential GDM miRNAs biomarkers, miR-3065-3p, miR-4650-3p, miR-29b-2-5p, and miR-3915 that were significantly altered in GDM. Among these miRNAs, miR-3065-3p and miR-4650-3p were Up-regulated while miR-29b-2-5p and miR-3915 were Down-regulated. When these miRNA was searched for an article on Pub Med, it returned no results for miR-4650-3p and miR-3915, only one result for miR-3065-3p while 11 for miR-29b-2-5p. No, any study reports the role of these miRNAs in GDM. Very less/no study on these four miRNAs makes them novel biomarkers for GDM. However, further experimental studies are required to validate these findings.

MIR-29b-2-5p has been shown to activate p53 expression and induce the p53-mediated apoptosis in Hela cells. In another study, mir-29b-2-5p inhibited cell proliferation, induced cell cycle arrest, and promoted apoptosis27. Furthermore, in a variety of tumors including chronic lymphocytic leukemia, lung cancer, prostate cancer, and breast cancer, miR-29b-2-5p was down-regulated. Matthaei et al. have demonstrated that decreased endothelial expression of miR-29 family members may be associated with increased sub-endothelial extracellular matrix accumulation in corneal dystrophy. We exposed the down-regulation of miR-29b-2-5p and also identified its role in apoptosis and cell proliferation which is consistent with the other findings. Although, our result is in accordance with the other findings, however, further experiments are required to investigate the role of miR-29b-2-5p in context of GDM (Park, Li C, Merkel O, Avasarala S, Matthaei M, Palsgaard J, Chodick G, Abiola M, Cabrae R, et al.).

In our Up-miRNA-based network, miR-3065-3p, miR-2110, miR-4650-3p, and miR-567 have 6, 5, 4, and 2 GDM specific target genes respectively. Similarly, in the Down-miR network, miR-29b-2-5p, miR-3915, and miR-2467-3p have 6, 4, and 3 GDM related target genes respectively. These four up-miRs and three Down-miRs regulate more GDM related target genes than other thirteen miRNAs in the network in combination. This indicates

that these miRNAs may have a more important role in GDM. Even though microarray experiments we have selected are performed on different subjects, our predicted target genes show overlap with genes found in GSE19649. Although, these genes were not significant which may be due to very little sample size, however, these genes were a potential player in GDM as they were confirmed from Gene cards. At last, we cannot deny from the fact that inter-individual differences may potentially mask the largely fine-tuning regulatory effects of miRNAs since the expression datasets of mRNA and miRNA are unpaired and truly independent samples. The availability of paired miRNA and mRNA expression datasets for large patient cohorts should provide additional insights. Ultimately, validating predicted regulatory mechanisms requires experiments with miRNA mimics/inhibitors.

Pathway enrichment analysis of Up-regulated miRs revealed many important pathways like insulin signaling, Notch signaling, Wnt-receptor signaling, JAK-STAT signaling, and ErbB signaling which are altered in GDM. Insulin signaling is a major pathway that is altered in any kind of diabetes including GDM. Disturbed Wnt and Insulin signaling have been implicated in many diseases including type 2 diabetes. Furthermore, Insulin has known mitogenic activity in cells, and activation of the Insulin pathway is the main factor regulating cell proliferation. From network analysis, we revealed that mir-3065-3p, which has increased expression in GDM and enriched in insulin metabolism, has a role in the regulation of insulin signaling pathways. Besides its role in insulin signaling, it has also been found to regulate Wnt and JAK-STAT pathways. This implies that Insulin signaling is somehow related to Wnt and JAK-STAT. In accordance with our study, Abiola et al. showed that activation of Wnt/b-catenin signaling in skeletal muscle cells improved insulin sensitivity. Another report states that Insulin activates hepatic Wnt/β-catenin signaling. Furthermore, altered Wnt activity can serve as modifiers of insulin action and insulin resistance in the pathophysiology of diabetes and metabolic syndrome. JAK/STAT signaling has also been shown to be associated with metabolic abnormalities including insulin resistance and obesity. Interestingly all these pathways are directly involved in cell proliferation, differentiation, and apoptosis. Wnt signaling is both necessary and sufficient for islet β cell proliferation while Insulin and JAK-STAT is a potent inducer of cell proliferation in normal development. Altogether, all these three pathways converge and regulate pancreas development and insulin metabolism, which is being supported by the findings of other groups.

Besides miR-3065-3p, we unveiled two other miRs highly connected to the insulin signaling pathway along with Wnt and JAK-STAT pathway. Our analysis revealed that miR-567 was associated with GO terms concerning the regulation of transcription, cell death, and apoptosis. Another Up- miRNA, MIR-2110 was enriched for processes like cell proliferation and differentiation, regulation of transcription, and tyrosine kinase signaling pathway. Receptor tyrosine kinase has been shown to be altered in cancer and causes deregulated cell proliferation. In the context of GDM, tyrosine kinase-mediated cell proliferation and apoptosis are relevant, as the pancreas (particularly β-cells) undergo hyper-proliferation as a compensatory mechanism to fulfill the increasing insulin demands. Hence these Up-miRNAs are somehow involved in the regulation of molecular mechanisms involved in these compensatory adaptive changes of the pancreas. Based on these findings we can postulate a hypothesis that a very delicate and fine-tuned balance among the tyrosine kinase-mediated cell proliferation by MIR-2110, the mitogenic activity of insulin-mediated by MIR-3065-3p and controlled apoptosis regulated by MIR-567 will be interplaying to maintain the hyper-proliferation in a controlled way. This hyper-proliferating pancreas wills secret enough insulin to meet the demands in normal pregnancy whiles their deregulation may cause GDM (Figure 11). However, no study reports the similar role of these Up-miRNAs in GDM and hence requires further experimental validation. Hence, hypertrophy or hyperplasia of the pancreas which is a compensatory mechanism to fulfill the increased demand for insulin is somehow regulated by these Up-miRNAs via Insulin-Wnt -JAK/STAT pathways ().

Pathway enrichment analysis revealed that all Down-regulated miRNAs were involved in pathways of cancer. Few studies have shown the correlation of diabetes with cancer. Type 2 diabetes has an association with cancer of many specific organs like pancreas, liver, colorectal, bladder, endometrial, non-Hodgkin's lymphoma, and breast32. Around 16% of breast cancer has T2D. Here, we exposed that miR-146a-3p, which was decreased in expression in GDM patients compared to HCs, is associated with several cancers. Xiang et al. report the down-regulation of miR-146a-3p in bladder cancer and its overexpression inhibited migration, invasion, metastasis and growth, and induced senescence of bladder cancer cells. Another study reports that miR-146a polymorphism correlates with lung cancer risk in Chinese nonsmoking females. Besides the miR-146a-3p, other Down-regulated miRs: miR-2467-3p was also found to be associated with cancer. In accordance with our study, miR-2467-3p was previously found to be down-regulated in diabetic nephropathy patient and STZ induced type 1 diabetic mouse.

Down-regulation of miR-2467-3p was also reported in cervical cancer. However, no study reports the association of mir-3915 with any cancer. Besides their association with cancer, these down-miRNAs were enriched for insulin metabolism and immune response (Dodington DW, Rulifson IC, Straßburger K, Rawlings JS, Paul MK, Wolf I, Xiang W, Yin Z, Xu Y, Valinezhad Orang A, et al.)

Generally, micro-RNA is known to mediate posttranscriptional down-regulation of expression; translational repression, and deadenylation-dependent decay of messages through partially complementary microRNA target sites in mRNA Untranslated Regions (UTRs). Interestingly we find that some of the target genes of these miRNAs were down-regulated while some up-regulated irrespective of the expression of the miRNAs. When we went back to literature, we found some of the articles which were reporting the role of miRNA also as a stimulator of gene expression and thus supporting our findings. This makes the miRNA all-rounder in nature, further complicating the mechanism of its action in GDM. The consensus approach led to the identification of GDM specific target genes of DEmiRNAs in GSE19649 datasets. Although these genes were not significant, however, they were found to be validated potential role players in GDM as confirmed from the results of Gene Cards search. These genes were TCF7L2, BRAF, IL10RB, CD36, and IL6ST regulated by Up-miRs namely miR-3065-3p, miR-2110, miR-4650-3p, and miR-567 while ACSL1, AR, IL1RN, PRKG1 are genes regulated by Down-miRs namely miR-146a-3p, miR-2467-3p and mir-3915. The functional enrichment of these GDM related target genes showed major significant processes related to GDM including carbohydrate and lipid metabolism and insulin signaling (Figures 10A and 10B). An altered miRNA may have a greater impact on the progression of GDM compared to a deregulated protein-coding gene since a miRNA may regulate hundreds of mRNA targets. Genetic variants such as single nucleotide polymorphisms can lead to the aberrant expression of miRNAs and increase the risk of developing certain diseases (Lewis BP, Hecker M, Xu Q, et al.).

## CONCLUSION

In conclusion, we presented a consensus-based method to analyze and integrate gene and miRNA expression data. Our finding suggests that the different expressions of genes and miRNAs are one of the most important variables in GDM. Hence, for a better understanding of the gene regulatory network, molecular mechanisms of GDM, developing new therapeutic approaches, future studying of miRNA function and regulation, and their potential as diagnostic biomarkers for GDM, bioinformatic analysis is required. We argued that, among others, miR-3065-3p, miR-4650-3p, miR-29b-2-5p, and miR-3915 might be the most promising blood-derived miRNA biomarkers in GDM. Although, our analysis is based on the high throughput data and is not derived in our laboratory, a large number of experimental studies confirm that the pathways and genes which were involved in GDM are supported.

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