

Original Article

Down-regulation of microRNA-28 in peripheral blood mononuclear cell plays a role in pathogenesis of type 1 diabetes

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Abstract: Objective: Differential network analysis was performed with mRNA expression data and microRNA (miRNA) expression data to identify critical miRNAs in peripheral blood mononuclear cell from type 1 diabetes patients. Methods: Gene expression profiles (dataset GSE55100) were downloaded from Gene Expression Omnibus, including 12 peripheral blood mononuclear cell samples from type 1 diabetes (T1D) and 10 from normal controls. Differentially expressed microRNAs (DEMs) were identified using package *limma* of R. Target genes of DEMs were predicted using miRanda, MirTarget2, PicTar, PITA and TargetScan. Differential analysis was performed for the target genes using *t* test. Functional enrichment analysis was done using DAVID. MiRNA functional synergistic network, pathway synergistic network and co-regulation network were constructed, from which core miRNAs and mRNAs were disclosed. Results: A total of 20 DEMs were revealed in T1D compared with normal control and 3752 target genes were predicted. A total of 695 differentially expressed miRNA-mRNA interactions were unveiled from the 7 DEMs and 651 mRNAs. Topological analysis of the three networks revealed two critical miRNAs: hsa-miR-28-5p and hsa-miR-146b-5p. Conclusion: miR-146 has been implicated in T1D. We speculated that down-regulated miR-28 played a critical role in pathogenesis of T1D. Further studies are needed to disclose the function of miR-28 in the T1D.

Keywords: Type 1 diabetes, peripheral blood mononuclear cell, microRNA-28, differentially expressed miRNAs, network analysis

Introduction

Type 1 diabetes (T1D) is featured by insufficient insulin. The exact cause of T1D remains unclear. However, it's believed to involve a combination of autoimmunity, genetics and environment. Type 1 diabetes makes up an estimated 5-10% of all diabetes cases. It's estimated that about 80,000 children develop the disease each year [1]. Immune system plays an important role in T1D [2, 3]. Increased immune cell infiltration of the exocrine pancreas might contribute to the pathogenesis of T1D [4]. Various aspects of immune system have been implicated in T1D, such as dendritic cells [5], B cells [6] and interleukin 2 [7]. Moreover, intestinal flora is considered an important risk factor for T1D [8].

MicroRNAs (miRNAs) participate various biological processes as well as pathogenesis of diseases via targeting a number of downstream

genes. Previous studies have indicated their roles in T1D via immune functions and other pathways. Hezova *et al.* report that miR-342, miR-191 and miR-510 are differentially expressed in T regulatory cells of T1D patients [9]. Up-regulation of miR-326 is found in T1D patients with ongoing islet autoimmunity [10]. The miR-21-PDCD4 axis prevents type 1 diabetes by blocking pancreatic beta cell death [11].

Identification of critical miRNAs could benefit treatment of T1D. However, current knowledge is far from enough to develop therapies. In present study, differential network analysis was performed to unveil key miRNAs in T1D. Differentially expressed miRNAs (DEMs) were uncovered and then target genes were predicted. MiRNA functional synergistic network, pathway synergistic network and co-regulation network were constructed, from which core miRNAs were revealed. They could advance the

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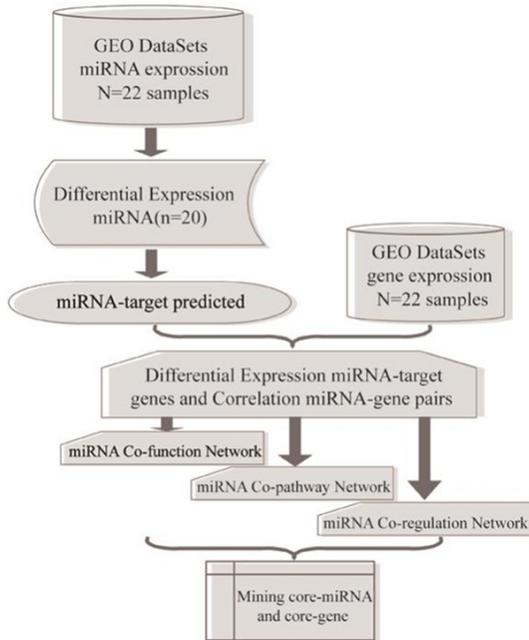


Figure 1. Data analysis process.

understanding about the disease and also provides potential therapeutic targets.

Materials and methods

Raw data and pre-treatment

Gene expression profiles (dataset GSE55100 [12]) and clinical information of T1D were downloaded from Gene Expression Omnibus (GEO). Affymetrix Human Genome U133 Plus 2.0 Array and Affymetrix miRNA Array were used to acquire mRNA expression profiles and miRNA expression profile, respectively. A total of 22 human peripheral blood mononuclear cell samples were included, 12 samples from T1D patients and 10 samples from normal controls.

Raw data were processed with package *affy* [13] of *R*. Normalization was performed with Robust Multichip Average (RMA) method. Probes corresponding to a same gene were averaged as the expression value of the gene. Finally, a total of 20534 mRNAs and 845 miRNAs were quantified. The data analysis process is shown in **Figure 1**.

Screening of DEMs

DEMs were identified using package *limma* [14] of *R*. Adjusted *P* value < 0.05 and $|\log(\text{fold change})| > 1$ were set as the cut-offs.

Prediction of target genes of DEMs

Target genes of DEMs were predicted using miRanda [15], MirTarget2 [16], PicTar [17], PITA [18] and TargetScan [19]. Genes predicted by at least 3 tools were regarded as the target genes of certain DEMs.

Screening of differentially expressed miRNA-mRNA interactions

Differential expression of target genes between T1D and normal controls was examined using *t* test. Target genes with *P* value < 0.01 were regarded as differentially expressed genes. Correlation between these genes and miRNAs was calculated. Correlation coefficient > 0.5 was set as the threshold to screen out differentially expressed miRNA-mRNA interactions.

Functional enrichment analysis

Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed for the target genes using DAVID (The Database for Annotation, Visualization and Integrated Discovery, <https://david.ncifcrf.gov/>) [20]. *P* value < 0.05 was set as the threshold.

Construction of miRNA functional synergistic network

MiRNAs targeting genes in the same biological process (BP) term were selected out and thus a miRNA functional synergistic network was constructed. Topological characteristics were measured for each node (miRNA) and those of high degree were screened out.

Construction of miRNA pathway synergistic network

MiRNAs targeting genes in the same KEGG pathway were selected out and thus a miRNA pathway synergistic network was constructed. Topological characteristics were calculated for each node (miRNA) and those of high degree were selected out.

Construction of miRNA co-regulation network

A miRNA co-regulation network was revealed using WGCNA [21]. Two miRNAs shared target genes were considered as co-regulation. The

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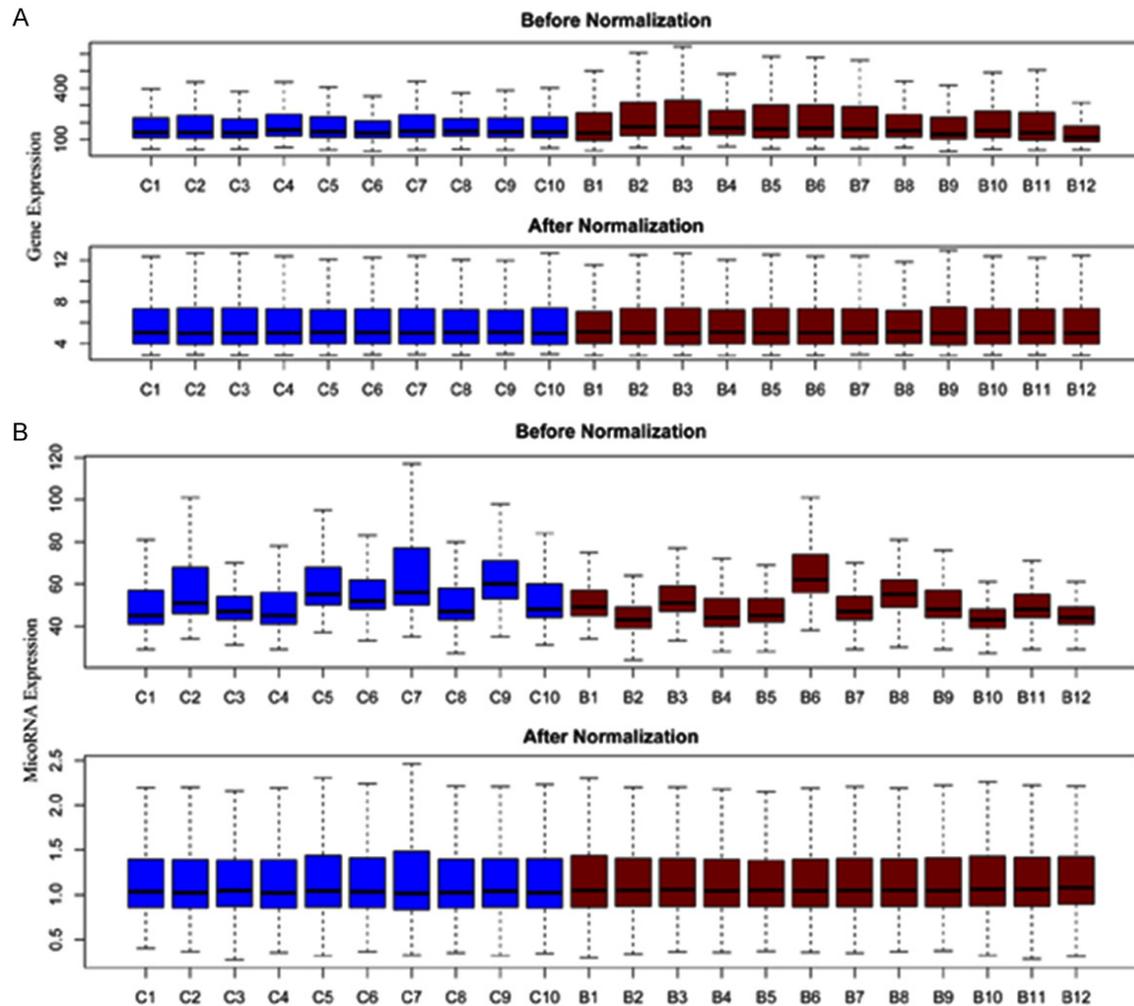


Figure 2. Box plots of gene expression data before and after normalization for mRNAs (A) and microRNAs (B).

number of common target genes correlates with the strength of the co-regulation. MiRNAs of high degree were then selected out.

Screening of core miRNAs

Core miRNAs were identified from the miRNA functional synergistic network, pathway synergistic network and miRNA co-regulation network. Co-regulated target genes of these core miRNAs were also selected out. Compared with all the 20 DEMs, co-regulated targets genes significantly associated with core miRNAs were revealed via Fisher's exact test.

KEGG pathway enrichment analysis

KEGG pathway enrichment analysis was performed for the genes using package *cluster*

Profiler [22] of *R* to identify disease-related biological pathways.

Results

Pre-treated gene expression data

Gene expression data before and after normalization are shown in **Figure 2**. A good performance of normalization using RMA method was achieved.

Differentially expressed miRNAs

A total of 20 DEMs (**Table 1**) were identified from the 845 miRNAs quantified in 22 samples. As shown in **Figure 3**, only 3 DEMs up-regulated in T1D compared with normal controls while others down-regulated. Cluster analysis using

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Table 1. 20 differentially expressed miRNAs

miRNA	Log (fold change)	Average expression	t	P value	Adjusted P value	B
hsa-miR-28-3p	-1.9283	2.7922	-6.1272	3.27E-06	0.0010	4.6183
hsa-miR-30a	-1.3646	1.9427	-6.0986	3.50E-06	0.0010	4.5545
hsa-miR-146b-5p	-2.6666	4.4375	-5.5516	1.29E-05	0.0027	3.3168
hsa-miR-146a	-1.3786	5.8602	-4.8923	6.43E-05	0.0059	1.7886
hsa-miR-181a-2	-1.8872	2.7055	-4.8633	6.90E-05	0.0059	1.7208
hsa-miR-28-5p	-1.3839	3.9232	-4.8628	6.91E-05	0.0059	1.7197
hsa-miR-1225-3p	1.0236	1.7381	4.7005	1.03E-04	0.0079	1.3395
hsa-miR-194	-1.3481	2.8294	-4.6676	1.12E-04	0.0079	1.2624
hsa-miR-15a	-1.7283	3.1916	-4.6038	1.31E-04	0.0085	1.1129
hsa-miR-374a	-1.8303	2.7578	-4.3915	2.21E-04	0.0117	0.6147
hsa-miR-25	-1.9026	4.7074	-4.2829	2.89E-04	0.0129	0.3601
hsa-miR-1249	1.0914	2.3621	3.9920	5.92E-04	0.0218	-0.3192
hsa-miR-29a	-1.2143	6.6021	-3.8759	7.88E-04	0.0275	-0.5890
hsa-miR-199a-5p	-1.2879	1.8501	-3.8636	8.12E-04	0.0275	-0.6174
hsa-miR-125b	-1.0752	1.9089	-3.7796	9.98E-04	0.0325	-0.8115
hsa-miR-126	-1.5444	3.0376	-3.7056	1.20E-03	0.0364	-0.9819
hsa-miR-625	1.1520	2.2399	3.6296	1.44E-03	0.0393	-1.1563
hsa-miR-19b	-1.1495	5.3557	-3.5950	1.57E-03	0.0415	-1.2353
hsa-let-7f	-1.6649	5.3560	-3.5674	1.68E-03	0.0426	-1.2981
hsa-miR-200c	-1.1715	3.6630	-3.5586	1.71E-03	0.0426	-1.3181

the 20 DEMs showed that they could separate T1D samples from normal controls (**Figure 4**).

Predicted target genes

A total of 3752 target genes of the DEMs were revealed by 5 prediction tools.

Differentially expressed miRNA-mRNA interactions

A total of 695 differentially expressed miRNA-mRNA interactions were unveiled among the 7 DEMs and 651 mRNAs.

Significantly enriched KEGG pathways

Ten KEGG pathways (**Table 2**) were significantly over-represented in the 651 genes, such as pathways in cancer, colorectal cancer, TGF-beta signaling pathway, Neurotrophin signaling pathway and cell cycle.

MiRNA functional synergetic network

A miRNA functional synergetic network was constructed with 7 DEMs. Five miRNAs exhibited high degrees: hsa-miR-374a, hsa-miR-146b-5p, hsa-miR-181a, hsa-miR-19b and hsa-miR-28-5p.

MiRNA pathway synergetic network

A miRNA pathway synergetic network was obtained (**Figure 5**). MiRNAs like hsa-miR-125b, hsa-miR-28-5p, hsa-miR-146b-5p, hsa-let-7f and hsa-miR-19b were of high degree, which suggested that they were involved in most of the KEGG pathways.

MiRNA co-regulation network

A miRNA co-regulation network was constructed (**Figure 6**). Four miRNAs co-regulated 39 genes. Compared with the 20 DEMs, the 4 miRNAs were significantly associated with 7 target genes (**Table 3**): ssemaphorin 4F (*SEMA4F*), neuroblastoma RAS viral oncogene homolog (*NRAS*), coiled-coil domain containing 71-like (*CCDC71L*), thyroid hormone receptor beta (*THRB*), IQ motif containing GTPase activating protein 1 (*IQGAP1*), nucleoporin 98 (*NUP98*) and syntaxin 3 (*STX3*).

Core miRNAs

Based upon information from miRNA functional synergetic network, miRNA pathway synergetic network and miRNA co-regulation network, two miRNAs (hsa-miR-28-5p and hsa-miR-146b-5p)

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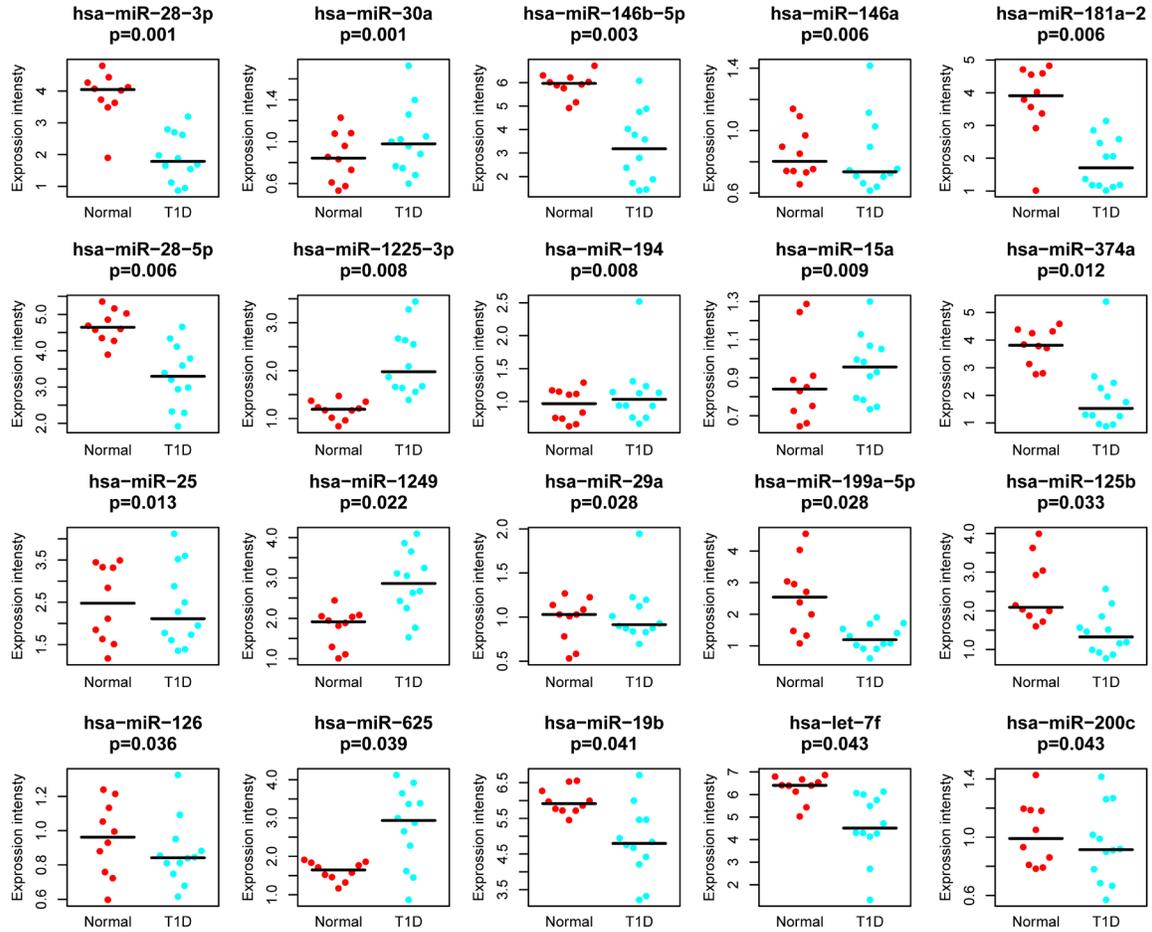


Figure 3. Expression levels of the 20 differentially expressed miRNAs in normal controls and type 1 diabetes (T1D). X-axis indicates samples while Y-axis indicates expression level.

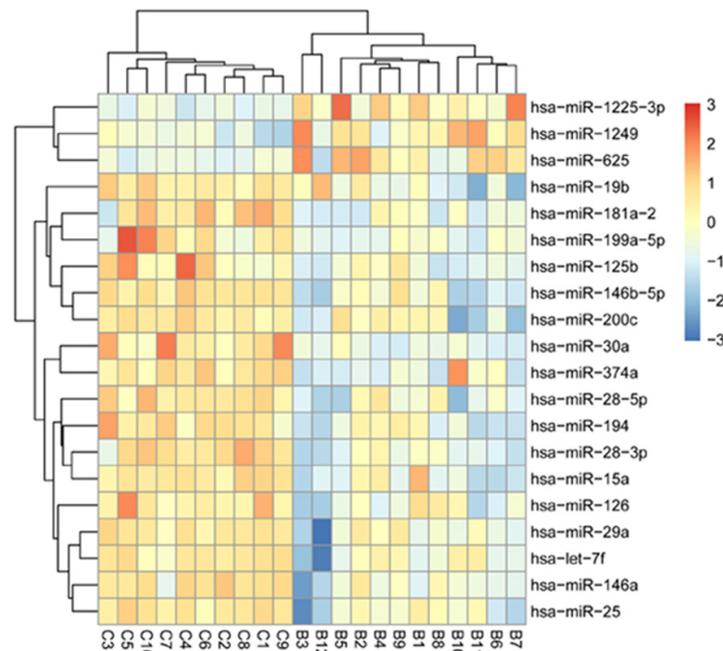


Figure 4. Cluster analysis result using the 20 differentially expressed miRNAs. Z-score standardization was applied to expression levels and Euclidean distance was adopted as metrics.

seemed to play critical roles. Previous study has illustrated that down-regulation of miR-146 in peripheral blood mononuclear cells is correlated with ongoing islet autoimmunity in T1D [12]. Out of the 7 genes, 6 were targeted by hsa-miR-28-5p. Thyroid hormone signaling pathway was significantly over-represented ($P = 0.0026$, **Figure 7**) in the 6 genes.

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Table 2. 10 significantly over-represented KEGG pathways

Term	Genes	P value
hsa04120: Ubiquitin mediated proteolysis	<i>UBE2Z, BTRC, UBE2G1, SOCS1, UBE2J1, UBE3C, PARK2, UBE2R2, MAP3K1, UBR5, FBXO4, ITCH, RCHY1, SMURF1</i>	0.0013
hsa05210: Colorectal cancer	<i>IGF1R, DVL3, TGFB1, PDGFRA, SMAD4, TP53, FZD1, FZD3, APPL1, FZD4</i>	0.0032
hsa05200: Pathways in cancer	<i>DVL3, E2F3, PTGS2, TGFB1, FGF11, FZD1, TP53, SMAD4, BRCA2, FOXO1, FZD3, BCL2L1, APPL1, FZD4, COL4A6, STAT3, DAPK1, CCDC6, NRAS, IGF1R, CDKN2A, PDGFRA, RARB</i>	0.0036
hsa04144: Endocytosis	<i>DNM3, PARD6B, ERBB4, ERBB3, TGFB1, PIP5K1B, EEA1, ADRB3, IGF1R, ADRB1, SH3GLB1, PDGFRA, GIT2, ITCH, SMURF1</i>	0.0067
hsa04350: TGF-beta signaling pathway	<i>PPP2R1B, E2F5, SMAD7, ZFYVE16, TGFB1, SMAD4, SMURF1, BMPR1B, CHRD</i>	0.0132
hsa04722: Neurotrophin signaling pathway	<i>IRAK1, NRAS, IRS2, YWHAH, MAP3K1, MAPK14, YWHAH, TP53, SORT1, SH2B1, MAP2K7</i>	0.0143
hsa04110: Cell cycle	<i>E2F3, MAD2L1, CDKN2A, YWHAH, CDC14A, E2F5, YWHAH, SMAD4, TP53, SMC1A, CDC25A</i>	0.0150
hsa05212: Pancreatic cancer	<i>E2F3, CDKN2A, TGFB1, SMAD4, TP53, BRCA2, BCL2L1, STAT3</i>	0.0151
hsa05218: Melanoma	<i>IGF1R, NRAS, E2F3, CDKN2A, PDGFRA, FGF11, TP53</i>	0.0434
hsa04114: Oocyte meiosis	<i>PPP2R1B, IGF1R, MAD2L1, YWHAH, BTRC, PPP2R5C, YWHAH, CPEB1, SMC1A</i>	0.0461

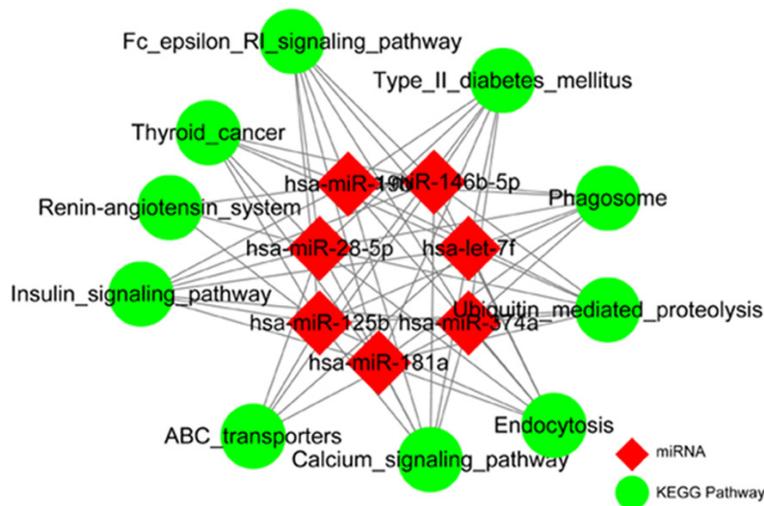


Figure 5. miRNA pathway synergetic network. Green circles represent KEGG pathways and red circles for miRNA.

Discussion

In present study, 20 DEMs were identified in T1D compared with normal controls. A total of 695 differentially expressed miRNA-mRNA interactions were unveiled from the 7 DEMs and 651 mRNAs. Ten KEGG pathways were sig-

nificantly over-represented in the 651 genes, such as TGF-beta signaling pathway, Neurotrophin signaling pathway and cell cycle. MiRNA functional synergistic network, miRNA pathway synergistic network and miRNA co-regulation network were constructed, from which miRNAs of high degree were selected out, such as hsa-miR-374a, hsa-miR-146b-5p, hsa-miR-181a, hsa-miR-19b and hsa-miR-28-5p.

Up-regulation of miR-29 expression is a key factor in the loss of pancreatic β cells and development of the first stage of type 1 diabetes mellitus (T1DM) [23]. MiR-29a is observed to be a positive regulator of insulin secretion in vivo, with dysregulation of the exocytotic machinery sensitizing β -cells to overt diabetes after unfolded protein stress [24]. Bacon et al. report up-regulation of miR-29a induced by ER

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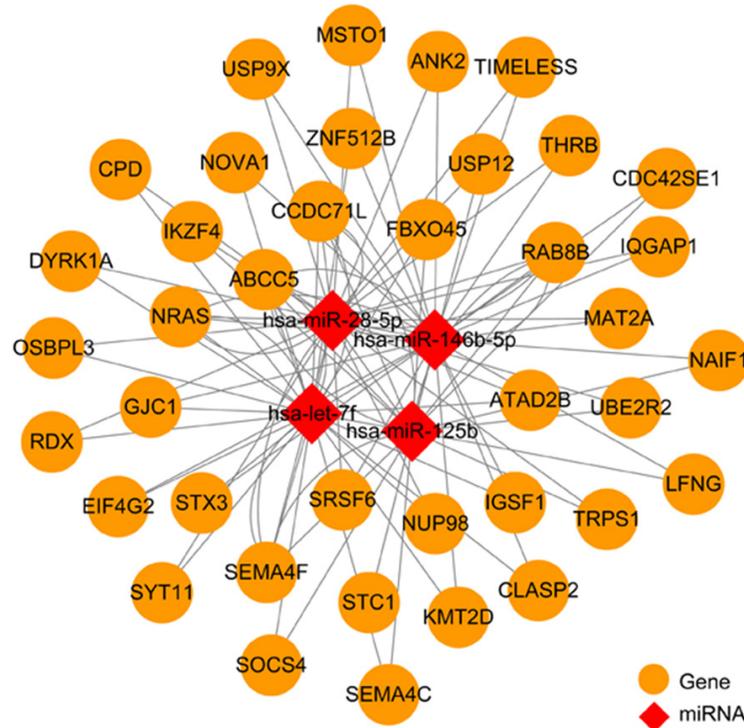


Figure 6. miRNA co-regulation network. Yellow circles represent target genes and red circles represent miRNAs. Edges indicate miRNAs co-regulation and miRNA-target gene interactions.

Table 3. Target genes of the four co-regulating miRNAs

Genes	P value	miRNAs
<i>SEMA4F</i>	0.0041	hsa-let-7f; hsa-miR-125b; hsa-miR-28-5p
<i>NRAS</i>	0.0157	hsa-miR-146b-5p; hsa-let-7f; hsa-miR-28-5p
<i>CCDC71L</i>	0.0157	hsa-let-7f; hsa-miR-125b; hsa-miR-28-5p
<i>THR8</i>	0.0351	hsa-miR-146b-5p; hsa-miR-28-5p
<i>IQGAP1</i>	0.0351	hsa-miR-146b-5p; hsa-miR-28-5p
<i>NUP98</i>	0.0351	hsa-let-7f; hsa-miR-28-5p
<i>STX3</i>	0.0351	hsa-miR-146b-5p; hsa-let-7f

stress in T1D patients [25]. It's reported that up-regulation of miR-199b-5p enhances proliferation of β -cells at least in part through down-regulation of mixed lineage kinase-3 (*MLK3*) [26], which might serve as a therapeutic approach for T1D. A significant deregulation of miR-126 is found in plasma and urinary samples from T1D patients [27, 28]. MiR-126 may be mediated by endothelial injury and participate in the development and progression of diabetic vascular complications [29]. It's reported that miR-200b and miR-200c are involved in diabetes via down-regulating FOG family member 2 (*FOG2*) which leads to Akt activation [30].

Murray et al. find that miR-200b down-regulates oxidation resistance 1 (*Oxr1*) expression in the retina of type 1 diabetes model [31]. We speculated that miR-200c might play a similar role. Bhatt et al. propose miR200-regulated DNA damage checkpoint pathway as a potential therapeutic target for treating complications of diabetes [32].

Two core miRNAs were further revealed via network analysis: hsa-miR-28-5p and hsa-miR-146b-5p. Decreased miR-146 expression in peripheral blood mononuclear cells is correlated with ongoing islet autoimmunity in T1D [12]. The target genes of miR-28 was involved in thyroid hormone signaling pathway. Previous study has indicated that autoimmune thyroid diseases (*AITDs*) is associated with T1D [33]. Cross-sectional studies have reported that the risk of thyroid dysfunction in patients with T1D is two- to threefold higher than in the general population [34]. Therefore, we speculated that miR-28 could play a critical role in pathogenesis of T1D. More studies are needed to unveil exact molecular mechanisms.

Overall, a number of DEMs were identified in T1D compared with normal controls. Most of them have been implicated in T1D. Network analysis revealed two critical miRNAs, one of them have been involved in T1D and the other was likely to take a part in the development of T1D. These findings could help to unveil the pathogenesis of T1D and provide potential therapeutic targets for treatment.

Disclosure of conflict of interest

None.

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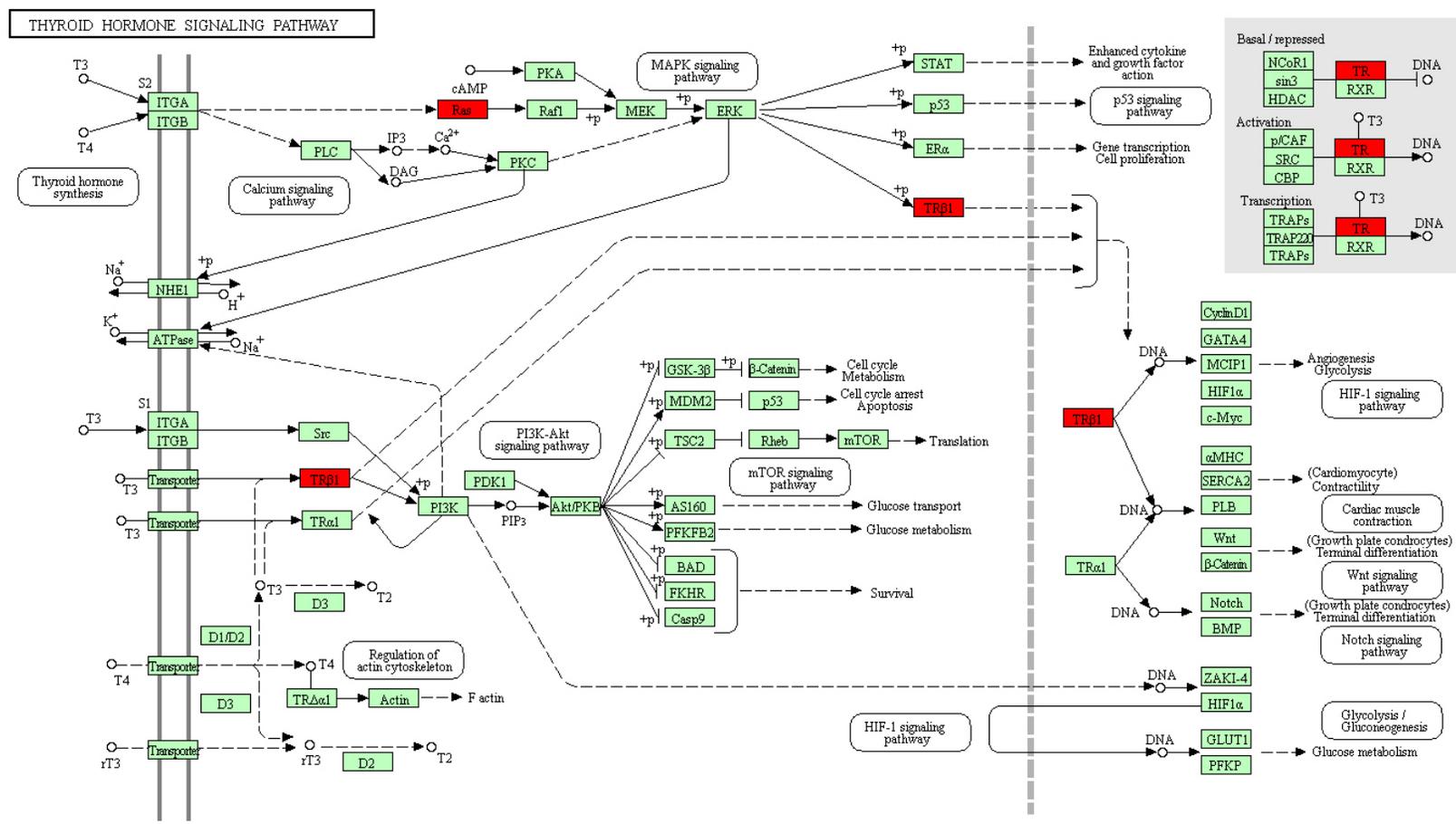


Figure 7. Thyroid hormone signaling pathway over-represented in the target genes of hsa-miR-28-5p. Up-regulated genes are shown in red squares.

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