Research article

Detection of differentially expressed circulating micro-RNA among Indians with Type 2 Diabetes using a global expression profile and systematic analysis

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ABSTRACT

Introduction and Aim: In recent years research on molecular mechanisms leading to Type 2 Diabetes Mellitus (T2DM) has discovered the promising role of microRNA (miRNA) as an early biomarker for the diagnosis of the clinical state. However, there is a lack of data from the Indian population exploring the global expression of circulating miRNA using precise and sensitive methods. The aim was to identify and quantify the circulating miRNAs from biological samples of T2DM and healthy controls using the Nanostring nCounter Platform.

Material and Methods: The miRNA analysis was performed using the NanoString nCounter platform. The T2DM patients within a duration of 5 to 15 years without any complications were recruited. The healthy controls were identified without any chronic diseases. The NanoString nCounter platform probes were used to detect the newly discovered miRNAs in the chosen panel of nearly 800 miRNAs.

Results: Our analysis has identified many unique miRNAs that are not reported earlier such as miR -25-3p, miR451a, miR365a, miR-129-2, miR-1271, miR572, and others.

Conclusion: Identification of differential patterns of circulating miRNAs could offer optimistic chances for managing T2DM diagnostically and prognostically. The specific expansion of the miRNAs to know the intercommunication among miRNAs and other genes involved in the development of T2DM must be further explored to uncover the potential role in clinical practice.

Keywords: MicroRNA; type 2 diabetes; circulating; global; expression.

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a major health problem across the world (1,2). A major proportion of the population across the world suffers from T2DM and its consequences. Growing rates of detection among the younger population indicate that the disease is likely to be driven by an inactive lifestyle, lack of physical activity, excess calorie consumption, and obesity among others (2,3). The role of genetic components in the pathogenesis of T2DM needs further exploration (4). The rapidly increasing percentage of T2DM calls for the need to develop efficient tools for the early detection of the disease and its progression.

MicroRNA's (miRNAs) are small noncoding endogenous RNAs that are 18-25 nucleotides long and involved in differentiation, proliferation, apoptosis, and development in health and disease (2). Their main function is to regulate various aspects of cellular function epigenetically by suppressing gene expression of target genes thereby controlling many biological processes within the cell (4). Their expression is tissue-specific and shows considerable redundancy among the targets. Though expressed in low levels in normal physiological conditions, their levels considerably raise during pathological stress and are often secreted in extracellular space including blood and body fluids. Due to their biochemical stability, they are considered potential biomarkers in T2DM and its complications (5).

Available studies among T2DM have explored the expression pattern of miRNA with no consistent pattern of miRNAs observed due to heterogeneity of the methods, different sampling techniques, and selection of most differentially regulated miRNAs. Therefore, we approached a systematic method after global profiling of miRNAs between the T2DM in comparison to normal controls to identify the set of miRNAs differentially regulated in T2DM.

MATERIALS AND METHODS

Participants

As part of the ongoing larger initiative in the department, T2DM patients are regularly screened including healthy controls. T2DM within a duration of 5 to 15 years without any complications were recruited. The objective of the present study including its risks was explained to the participants and written informed consent was obtained. The American Diabetes Association criteria for the diagnosis of T2DM were used (6). Any associated chronic disorders such as tuberculosis, cancer was excluded.

As part of the current study for the microRNA analysis using the NanoString nCounter platform n=6 samples (n=3, T2DM and n=3 age and gender-matched controls) were used. NanoString N Counter is a

multiplex nucleic acid hybridization technology used for the assessment of hundreds of genes in a single assay (7).

Sample processing

Serum samples from T2DM and control participants were collected. miRNA was isolated from these samples using a microRNA extraction kit (Qiagen) and quantified and purity of these samples was checked by calculating the 260/280 ratio for contamination with protein and 260/280 ratio for contamination with organic compounds. All six samples showed both ratios above 1.9 and were considered for the miRNA expression analysis using the Nanostring N counter Platform (NanoString Technologies, Seattle, WA, USA).

miRNA assay protocol

This assay provides highly multiplexed direct digital detection of miRNA in a single reaction without amplifying it. The amount of total miRNA used for each sample was 100 ng. Samples were prepared using miRNA sample preparation kit which included reagents for annealing, ligation, and purification steps. These purified samples were mixed with reporter probes, capture probes, and hybridization buffer and hybridized overnight as per the manufacturer's protocol. The N counter Human v2 miRNA platform contains 798 unique hsa-miRNA probes with barcodes. After hybridization, excess probes were washed using a two-step magnetic bead-based purification. The purified target/probe complexes were eluted off the beads and immobilized on the cartridge for data collection.

Data normalization and analysis

The expression level analysis of miRNA was conducted using nSolver analysis software (version 4.0). To standardize and reduce noise in the data, normalization was performed by using the mean expression of the top 100 highly expressed miRNAs, as recommended by Nanostring for miRNA analysis. Statistically significant differentially expressed miRNAs between the control and T2DM samples were screened using an absolute fold change ≥ 1.5 and a p-value < 0.05.

Validation in various miRNA-related databases

Identification of targets of miRNA**:** The experimentally validated microRNA target interactions (MTI) were retrieved from miRTarBase (8). The targets of the miRNAs were further filtered based on the support or evidence that verifies the interaction between them.

Literature and Database Survey**:** The miRNAs with at least 1 target were queried in PubMed to identify the association of miRNAs to T2DM. The search was designed in a such way that the "Title" or "Abstract" of the research article contains specified miRNA and the term type 2 diabetes mellitus (Type 2 diabetes [Title/Abstract]) AND (miR-34a-5p[Title/Abstract]). The number of articles matching the search criteria was collected. We explored miR2Diabetes (9), a manually curated microRNA database for diabetic microvascular complications, to see the overlapping miRNA in our filtered set. In addition, miRNAs were queried to the Human microRNA Disease Database (HMDD) (10) to find the overlap of curated miRNA disease association.

Functional enrichment analysis: Analysis of functional enrichment of miRNAs was performed using miRNA enrichment analysis and annotation (miEAA) (11) with Benjamini-Hochberg adjustment p value < 0.05 to select the relevant pathways associated with the shortlisted miRNAs.

RESULTS

Table 1 represents the descriptive data of the two study groups.

Table 1: Descriptive statistics of the study groups

Variables	Type 2 DM study group (n=3)	Control study group (n=3)
Age (year)	40±5	43±7
Gender (M:F)	2:1	2:1
Weight (kg)	71.4±5.7	65.5±6.4
Height (m)	1.5±0.1	1.6±0.05
BMI (kg/m^2)	30.0±3.0	23.6±2.5
FBS (mg/dl)	113.3±33.4	85.7±4.0
HbA1C (%)	9.7±3.9	5.4±0.50
HOMA %B	87.2±63.9	107.7±48.1
HOMA IR	1.3±0.20	1.0±0.53

BMI, Body mass index; FBS, Fasting blood sugar; HOMA, Homeostatic model assessment

Differentially expressed miRNAs (DEmiRNAs) were identified by setting the absolute fold change to 1.5 and a list of 51 miRNAs was obtained. Out of which 43 miRNAs were downregulated (Table 2).

Of the 8 upregulated miRNAs, hsa-miR-451a had the highest expression of 16 folds. Further to filter out the list of top DEmiRNAs, analysis was performed with absolute fold change set at ≥ 2 with p-value ≤ 0.05. With this analysis, a list of top 15 DE miRNA was obtained which have been represented in Table 3.

Table 2: List of differentially expressed miRNA with Fold change set at ≥1.5

Sr No.	miRNA	Fold change	p-value	Sr No.	miRNA	Fold change	p-value
1	hsa-miR-1178-3p	-1.62	0.03	26	hsa-miR-3180-5p	-2.1	0.04
2	hsa-miR-1224-5p	-1.61	0.03	27	hsa-miR-3185	-1.97	0.04
3	hsa-miR-1269b	-2.18	0.04	28	hsa-miR-323b-3p	-1.59	0.02
4	hsa-miR-1271-5p	-1.72	0.00	29	hsa-miR-34a-5p	-1.6	0.04
5	hsa-miR-1278	-1.98	0.04	30	hsa-miR-3605-3p	-1.63	0.01
6	hsa-miR-1281	-1.8	0.01	31	hsa-miR-365a-p+ hsa-miR-365b-3p	-1.79	0.01
7	hsa-miR-1287-5p	-1.66	0.01	32	hsa-miR-378b	-3.05	0.00
8	hsa-miR-1289	-1.86	0.04	33	hsa-miR-3918	-1.74	0.04
9	hsa-miR-129-2-3p	-1.81	0.03	34	hsa-miR-433-5p	2.13	0.04
10	hsa-miR-150-5p	3.68	0.01	35	hsa-miR-450a-2-3p	-1.79	0.01
11	hsa-miR-15a-5p	-1.61	0.03	36	hsa-miR-451a	16.22	0.03
12	hsa-miR-15b-5p	1.91	0.02	37	hsa-miR-486-3p	1.58	0.01
13	hsa-miR-16-5p	3.3	0.00	38	hsa-miR-503-3p	-1.64	0.02
14	hsa-miR-181b-5p+ hsa-miR-181d-5p	-3.08	0.01	39	hsa-miR-510-3p	-3.11	0.03
15	hsa-miR-182-5p	-1.65	0.04	40	hsa-miR-510-5p	1.83	0.04
16	hsa-miR-1908-5p	-2.48	0.00	41	hsa-miR-548v	-1.57	0.04
17	hsa-miR-1915-3p	-2.01	0.00	42	hsa-miR-548z+ hsa-miR-548h-3p	-1.51	0.03
18	hsa-miR-199b-5p	-1.53	0.00	43	hsa-miR-572	-1.9	0.03
19	hsa-miR-19b-3p	-1.53	0.02	44	hsa-miR-573	-1.64	0.02
20	hsa-miR-2053	-1.61	0.04	45	hsa-miR-578	-1.51	0.02
21	hsa-miR-208b-5p	-1.86	0.03	46	hsa-miR-615-5p	-1.65	0.00
22	hsa-miR-212-3p	-1.54	0.01	47	hsa-miR-637	-1.68	0.01
23	hsa-miR-223-3p	2.4	0.00	48	hsa-miR-6721-5p	-2.36	0.04
24	hsa-miR-25-3p	3.47	0.01	49	hsa-miR-924	-2.38	0.02
25	hsa-miR-26a-5p	-1.99	0.03	50	hsa-miR-941	-1.9	0.04
				51	hsa-miR-942-5p	-1.91	0.04

Table 3: List of top 15 DE miRNA with absolute fold change set at ≥ 2

Sr No.	**miRNA**	**Fold change**	**P-value**
1	hsa-miR-1269b	-2.18	0.04
2	hsa-miR-150-5p	3.68	0.01
3	hsa-miR-16-5p	3.3	0.00
4	hsa-miR-181b-5p +hsa-miR-181d-5p	-3.08	0.01
5	hsa-miR-1908-5p	-2.48	0.00
6	hsa-miR-1915-3p	-2.01	0.00
7	hsa-miR-223-3p	2.4	0.00
8	hsa-miR-25-3p	3.47	0.01
9	hsa-miR-3180-5p	-2.1	0.04
10	hsa-miR-378b	-3.05	0.00
11	hsa-miR-433-5p	2.13	0.04
12	hsa-miR-451a	16.22	0.03
13	hsa-miR-510-3p	-3.11	0.03
14	hsa-miR-6721-5p	-2.36	0.04
15	hsa-miR-924	-2.38	0.02

The set of 51 DEmiRNAs mentioned in Table 2 (FC ≥1.5 and p-value <0.05) was queried in mirTarbase to get experimentally validated miRNA target interactions (MTI). The functional interactions of these miRNAs were validated by various experiments such as Luciferase reporter assay, ELISA, Western blot, qRT-PCR, Immunofluorescence, GFP reporter assay, Immunohistochemistry, In situ hybridization, Northern blot, Immunoprecipitation, Immunoblot, Microarray, FACS, Chromatin immunoprecipitation, ChIP-seq, Proteomics, Flow cytometry, EMSA in published literature. Further, these 51 DEmiRNAs (Table 2) were filtered based on the support or evidence that verifies miRNA target interaction (MTI). These interactions

can be of 4 different types: Functional MTI, Functional MTI (Weak), Non-Functional MTI, and Non-Functional MTI (Weak). Among these miRNAs with Functional MTI were considered. We obtained 36 miRNAs with at least 1 target with functional MTI which are shown in Fig. 1.

Identification of miRNAs based on literature and database survey was performed. The 36 DEmiRNAs identified with at least 1 target were queried in PubMed to identify the association of miRNAs to T2DM. The number of articles matching the search criteria was collected. We queried the filtered miRNAs to HMDD (Human microRNA Disease Database) and miR2Diabetes (A Literature-Curated Database of microRNA Expression Patterns, in Diabetic Microvascular Complications) to find their association with the disease. Based on these search criteria, 19 DEmiRNAs were short-listed in Table 4.

Pathway analysis of the DEmiRs was performed to explore their association with T2DM. MiEAA is a web-based application that provides several commonly used statistical tests, including over-representation analysis and miRNA set enrichment analysis. The enrichment of shortlisted DEmiRNAs has shown their involvement in various pathways closely related to the pathogenesis of T2DM. The majority of the DEmiRNAs were involved in pathways like mTOR signalling pathway (19 DEmiRNAs, $p < 0.008$), Glucagon signalling pathway (17 DEmiRNAs, $p < 0.006$), Insulin signalling pathway (17 DEmiRNAs, $p < 0.03$), B cell receptor signalling pathway (16 DEmiRNAs, $p < 0.01$) and Insulin resistance (16 DEmiRNAs, $p < 0.03$). The word cloud plot for the major pathways enriched is represented in Fig. 2a. The patterns of regulation of these pathways (up or down) in which the miRNAs are involved are represented in Fig. 2b.

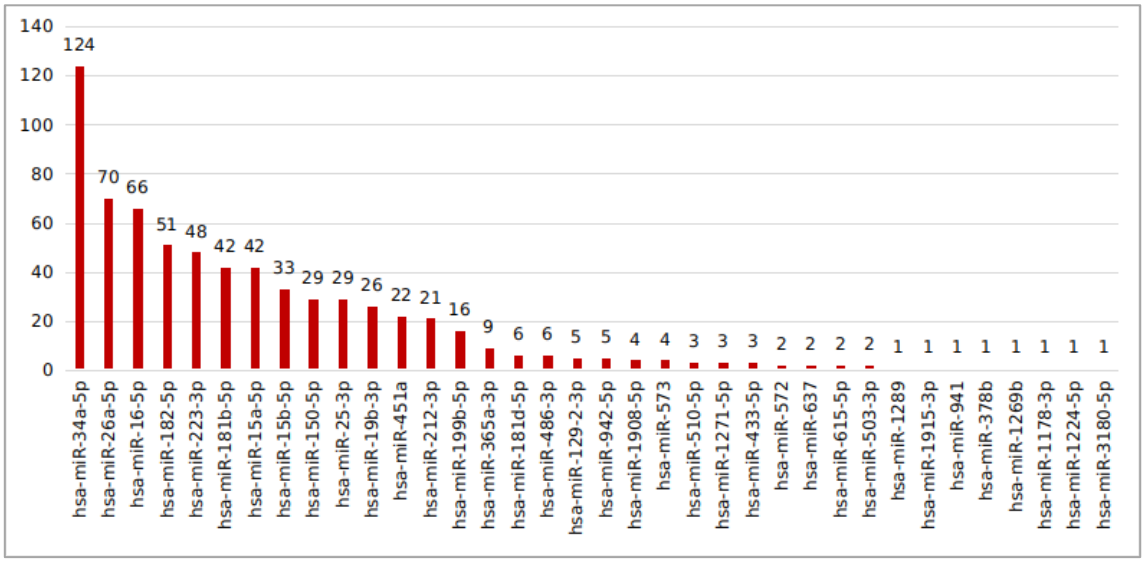

Fig. 1: Number of target genes for short-listed 36 differentially expressed miRNAs

Table 4: The list of 19 short-listed DEmiRNAs from the literature

miRNA	Number of targets	Fold Change	P-value	PubMed hits
hsa-miR-34a-5p	124	-1.6	0.04	7
hsa-miR-26a-5p	70	-1.99	0.03	2
hsa-miR-16-5p	66	3.3	0.00	7
hsa-miR-182-5p	51	-1.65	0.04	3
hsa-miR-223-3p	48	2.4	0.00	3
hsa-miR-181b-5p	42	-3.08	0.01	2
hsa-miR-15a-5p	42	-1.61	0.03	2
hsa-miR-15b-5p	33	1.91	0.02	4
hsa-miR-150-5p	29	3.68	0.01	3
hsa-miR-25-3p	29	3.47	0.01	2
hsa-miR-19b-3p	26	-1.53	0.02	2
hsa-miR-451a	22	16.22	0.03	6
hsa-miR-199b-5p	16	-1.53	0.00	1
hsa-miR-365a-3p	9	-1.79	0.01	1
hsa-miR-181d-5p	6	-3.08	0.01	1
hsa-miR-486-3p	6	1.58	0.01	1
hsa-miR-129-2-3p	5	-1.81	0.03	1
hsa-miR-1271-5p	3	-1.72	0.00	2
hsa-miR-572	2	-1.9	0.03	2

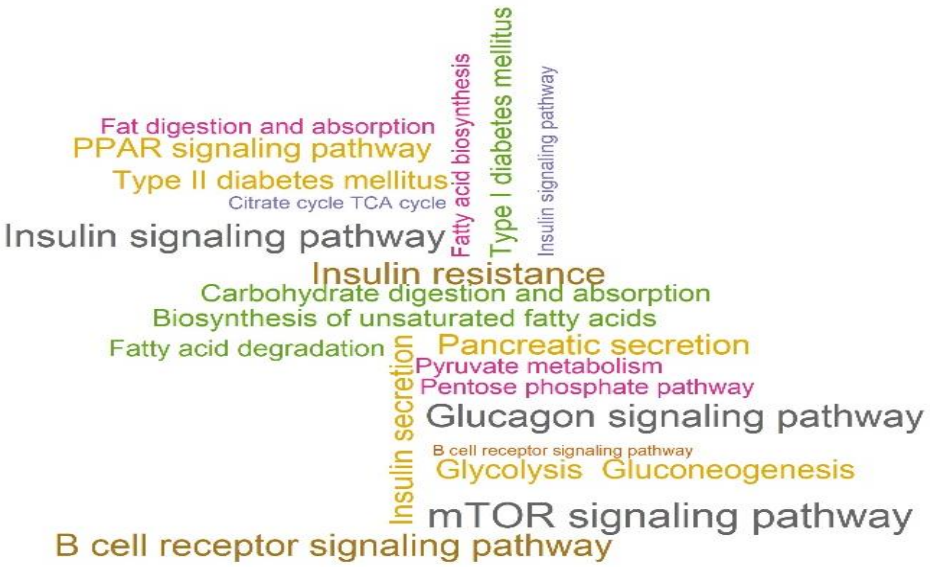

Fig. 2a: The word plot representing pathway enrichment analysis of shortlisted DEmiRNAs

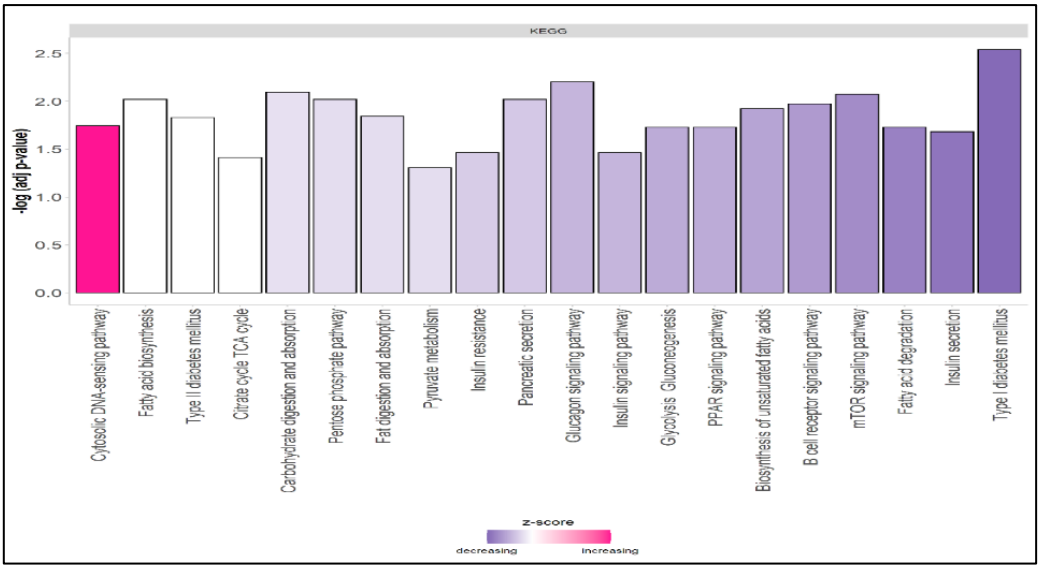

Fig. 2b: The word plot representing pathway enrichment analysis of shortlisted DEmiRNAs

DISCUSSION

The reported higher prevalence of T2DM and its complications is a growing problem worldwide, especially in India (12). The progression of T2DM till the development of complications raises the need to develop efficient methods for early identification and disease monitoring. The stability of small circulating molecules such as miRNAs with their tissue specificity makes them potential non-invasive biomarkers for T2DM. MicroRNAs exert their function through epigenetic regulatory networks where multiple miRNAs contribute to many target genes leading to significant redundancy. Hence, the estimation of a small set of miRNAs often presents differing results between various studies. We have used an approach to profile many miRNAs using a sensitive hybridization technology to identify patterns of circulating miRNAs in T2DM. The novelty of our study lies in using the nanostring platform that has availability of probes for newly discovered miRNAs in the chosen panel of nearly 800 miRNAs which has allowed the detection of novel miRNAs. Multiple methods have been used earlier for the detection of circulating miRNAs such as northern blot, quantitative PCR, and microarrays. All these technologies have limitations in that the northern blot is cumbersome and has low detection efficiency though very specific, q-PCR increases proficiency but uses an amplification strategy increasing false discoveries. Microarrays have the advantage of detecting several miRNAs at the same time while RT-PCR can detect only one miRNA at a time. Our approach using a multiplexed digital technology allowed the identification of circulating miRNAs from small amounts of biological samples from T2DM in comparison to normal controls. While newer methods like droplet digital PCR have the advantages of being very sensitive and next-generation sequencing can do global profiling, their use is limited by high cost, the requirement of expensive facilities, and trained personnel.

Earlier studies have reported several miRNAs such as in miR-9, miR-29a, miR-30d, miR-34a, miR-124a,

and miR146a, to name a few as differentially regulated in T2DM compared to controls (13-15). Karolina et al observed altered expression of miR-150, miR-192, miR-27a, miR-320a, and miR-375 in metabolic syndrome and T2DM suggesting their role in various biological processes involved in the pathogenesis of diabetes (16). In line with this another study evaluated miR-138, mir-376a, and mir-15b as potential serum biomarkers to distinguish obese patients from T2DM (17). Various meta-analysis studies have reported circulating miRNAs that are altered in T2DM which include miR-103, miR-107, miR-132, miR-144, miR-29a, miR-34a, and miR-375 (18).

Our analysis has identified many unique miRNAs along with the miRNAs that are previously reported by other studies on diabetes, such as miR-34a, miR-15a, miR-15b, miR-150, miR-19b, miR-223, and miR-486. Though other commonly reported miRNAs by many studies such as miR-375, miR-144, and miR 126 did not show up in top regulated miRNAs in our analysis. These differences in expression may be due to the sensitivity of the different methods used for the detection of miRNAs.

A recently published study including our study has identified miR34a and miR30d as novel regulators of dysfunction of pancreatic endocrine β cells and suggest its utility as a biomarker for diagnosis of T2DM (19, 20). The role of miR-26a, miR-16, miR15a, and miR-15b for maintaining β cell function is known. miR-182 is involved in glucose metabolism, miR-181 in insulin resistance, whereas miR-223 is involved in the maintenance of β cells and insulin secretion. Alongside these already reported miRNAs, we identified other miRNAs, that are not reported earlier for their expression and role in T2DM such as miR -25-3p, miR451a, miR365a, miR-129-2, miR-1271, miR572, and others. There is a need to validate the new list of miRNAs in well-curated large cohorts.

Pathway analysis showed that major pathways regulated by short-listed miRNAs were related to various diabetes-related processes such as insulin signalling and resistance, carbohydrate, and fat metabolism suggesting their role in the progression and development of diabetes. Along similar lines, previously a study by Kaur et al. has discussed the role of different miRNAs involved in various pathogenic mechanisms involving β cells (21). The authors further explained the role of PI3K, AKT/GSK, and mTOR pathways underlying insulin resistance supporting our findings (21).

CONCLUSION

Identification of differential patterns of circulating miRNAs could offer optimistic and promising results for diagnosis, monitoring, and prognosis for the treatment of T2DM. The specific miRNAs should be further explored to understand the complex intercommunications between miRNAs and the other relevant genes involved in the mechanisms leading to the development of T2DM.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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