

ORIGINAL ARTICLE

Exosomal miR-23a and miR-192, Potential Diagnostic Biomarkers for Type 2 Diabetes

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SUMMARY

Background: Type 2 diabetes mellitus (T2D) is a common chronic disease, which lacks a specific diagnostic method. A substantial body of literature has demonstrated that the molecular constituents of extracellular vesicles (EVs) are promising as a novel biomarker for clinical diagnosis, especially exosomal miRNAs in biological fluids.

Methods: To find a diagnostic biomarker for T2D, we isolated exosomes from plasma and then quantitative PCR (qPCR) was used to detect the miRNA expression in plasma and exosomes from control subjects and T2D subjects. The ROC (receiver operating characteristic) curve and AUC (area under curve) were used to evaluate the diagnostic value of exosomal miRNAs.

Results: We found the exosomal levels of miR-23a and miR-192 were both significantly higher in T2D subjects. The AUC of miR-23a and miR-192 were 0.828 and 0.717, respectively. Further bioinformatics analysis was performed to speculate possible mechanisms.

Conclusions: In conclusion, exosomal miR-23a and miR-192 have good potential as diagnostic biomarkers for type 2 diabetes.

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KEY WORDS

exosome, biomarker, type 2 diabetes, microRNA

LIST OF ABBREVIATIONS

EV - extracellular vesicle
T2D - type 2 diabetes
GLU - glucose
HbA1c - hemoglobin A1c
ROC - receiver operating characteristic
AUC - the area under the ROC
KEGG - Kyoto Encyclopedia of Genes and Genomes
GO - Gene Ontology annotation

INTRODUCTION

Diabetes mellitus is a common chronic disease with growing incidence rate. According to the IDF (International Diabetes Federation), China has the largest num-

ber of adults with diabetes (116.4 million) [1]. In 2019, diabetes and its complications caused about 4.2 million deaths and occurred in patients 20 to 79 years old. Type 2 diabetes mellitus (T2D) accounts for most adult diabetes with increasing morbidity [2]. The golden standard for T2D diagnosis is the oral glucose tolerance test (OGTT). However, OGTT is limited by inconvenient operation, low reproducibility, and financial cost [3]. Glycated hemoglobin A1C (HbA1C) represents long term glucose level and is recommended for diabetes diagnosis these years. However, HbA1c has 60 genetic variants, some are related with the function, structure, and lifespan of red blood cells, some are related with blood glucose control. Too many variants of HbA1c may result in its high false negative, especially in different populations [4,5]. Thus, other types of biomarkers for T2D are necessary.

MicroRNAs (miRNAs) are a kind of single-stranded, noncoding RNA that consists of approximately 20-mer nucleotides [6]. The miRNAs can regulate gene expression by binding with the 3'UTR (3'-untranslated regions) of target genes [7,8]. Therefore, miRNAs can affect a range of physiological and biochemical functions, and they have good potential as biomarkers of diseases. It has been reported by some researchers that circulating miRNAs in plasma or serum could be potential biomarkers and therapeutic targets for T2D. For example, Wan et al. found that increased serum miR-7 was a promising biomarker for T2D [9]. Yan et al. also showed that differentially expressed miRNAs, such as miR-320b and miR-572, existed in the plasma of T2D and prediabetes patients [10].

Exosomes are small vesicles derived from the multivesicular body (MVB) and shed to the extracellular spaces. During the production of exosomes, protein, DNA, miRNAs and other molecules are packaged and secreted. The contents in exosomes could be delivered to near or distant recipient cells and modify their physiological state [11]. Hence, the miRNAs in exosomes could also be the biomarkers of diseases. Exosomal miRNAs such as miR-15a and miR-144 could participate in the diagnosis and treatment of T2D [12,13]. In addition, compared with the circulating miRNAs from serum or plasma, exosomal miRNAs have a more accurate earlier diagnostic potential and an earlier and broader diagnostic time window [14].

Based on the reported data from GSE26167, we found miR-144, miR-23a, and miR-192 changed significantly in exosomes from both impaired fasting glucose and T2D patients [13]. Besides, Ying et al. reported that miR-155 has an important regulatory effect on insulin sensitivity [15]. Then, we conducted screening and verification on our samples of the four miRNAs. MiR-23a and miR-192 were selected for further research. The diagnostic potential of miR-23a and miR-192 was evaluated by ROC curve analysis. We further used bioinformatics to predicate the potential mechanism of the two miRNAs in T2D.

MATERIALS AND METHODS

Subjects

A total of 44 type 2 diabetics (T2D) and 36 control subjects were recruited from the Huadong Sanatorium. Possible gestational diabetes mellitus subjects were not included. The Ethics Review Board of the Huadong Sanatorium approved the study. The subjects were treated according to the Declaration of Helsinki. The clinical parameters including gender, age and others were collected by reviewing the medical records. Glucose (GLU) is the blood glucose level (normal range is 3.6 - 6.1 mmol/L). Hemoglobin A1c (HbA1c) refers to glycated hemoglobin, which identifies average plasma glucose concentration with a normal range of 4.8 - 5.9%. Urine GLU test measures the amount of GLU in urine samples and is semiquantitative. The results of “-” are defined as “negative” and the results of “+”, “++” or multi “+” are defined as “positive”. Informed consent was obtained from all individual participants in the study.

Sample collection and plasma processing

Blood samples were drawn from both controls and type 2 diabetics and stored in EDTA blood tubes. The tubes were transferred to the lab and centrifuged at 1,600 g for 10 minutes within 12 hours. Judged by hemolysis color charts, the samples with hemolysis were excluded [16]. Plasma was stored at -80°C. Before used, plasma was centrifuged at 3,000 g for 10 minutes and 10,000 g for 25 minutes at 4°C to deplete cell debris and big vesicles.

Exosome isolation and identification

A total of 500 µL 15% PEG4000 (1 M NaCl) was added to 500 µL plasma in a 2 mL centrifuge tube and mixed by inversion. After 3 hours incubation at 4°C, the mixture was centrifuged at 3,000 g for 15 minutes at 4°C. The total number of pellets was used for RNA detection.

For the identification of exosomes using the isolation protocol, six control subjects and six T2D subjects were randomly selected. Exosomes were lysed by RIPA buffer (Biochem, China), and the concentration was assayed by BCA protein assay kit (Thermo, USA). The signature proteins including TSG101 (dilution ratio 1:500, Abcam, Cambridge, MA, USA) and CD9 (dilution ratio 1:500, Abcam, Cambridge, MA, USA) were detected by western blot with Actin (dilution ratio 1:1,000, Abcam, Cambridge, MA, USA) as positive control and Calnexin (dilution ratio 1:500, Abcam, Cambridge, MA, USA) as negative control. The concentration and size distribution of exosomes from all samples were analyzed by the Nano platform (iZON Science). First, standard particles were used to calibrate the NP100 nanopores of the system. PBS, which had been repeatedly filtered by a 0.22 µm filter, was used to dilute the exosome pellets to the proper concentration. The concentration and size distribution were detected by the particle analyzer and the

supporting software.

RNA isolation and qPCR

For all samples, RNAiso Plus (Takara, China) was used to isolate total RNA from exosomes contained in 500 μ L plasma or 200 μ L plasma according to the instructions. In detail, 1 mL RNAiso Plus was added to the pellets or plasma and mixed by homogenizer. Next, 200 μ L chloroform was added and mixed by vortex oscillator. The mixture was placed at room temperature for 10 minutes and centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was transferred to a new 1.5 mL centrifuge tube and mixed with an equal volume of isopropyl alcohol. After an overnight incubation at -20°C, the solution was centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was discarded and 75% ethanol was prepared with DEPC water to wash the RNA. After centrifuging at 12,000 g for 5 minutes at 4°C, the remaining liquid was removed using blotting paper and was maintained in an inverted position on a new piece of blotting paper for 15 to 30 minutes. The RNA was dissolved in 10 μ L DEPC water.

Four Hairpin-it miRNAs qPCR Quantitation Kits (Genepharma, China) containing different synthetic miRNAs were used to detect the target miRNA levels. The reverse transcription PCR (RT-PCR) system was 20 μ L as the Kits provided, and the standard process was as follows: 25°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes, and then stored at 4°C. We diluted synthetic miRNAs to 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8} nM and established four standard curves for absolute quantification. The qPCR system was 20 μ L and the standard process was 95°C for 3 minutes; 95°C for 12 seconds, 62°C for 40 seconds, 40 cycles; the signal was collected at 62°C.

Bioinformatics analysis

The bioinformatics analysis was performed by the web applications of TarBase v.8, TargetScan, microT-CDS, and MirPath v.3 in DIANA tools [17-19]. The KEGG analysis and GO analysis were performed by mirPath v.3 based on the databases of Tarbase, TargetScan, and microT-CDS.

Statistical analysis

Data were analyzed by SPSS v.19 and GraphPad v5.0. Student's *t*-test was used to compare the miRNA expression between the groups, and $p < 0.05$ was considered statistically significant. ROC curve and the area under the ROC (AUC) were used to assess the biomarker potential of miR-23a and miR-192.

RESULTS

Subject description

The subject characteristics are shown in Table 1. A total of 80 subjects were enrolled in this study. Among them, there were 44 T2D subjects and 36 control subjects. The

GLU, HbA1c, and urine GLU level in the T2D group were significantly higher than the level in the control group ($p < 0.001$, $p < 0.001$, and $p < 0.05$).

Exosome identification

To identify whether the isolated pellets were exosomes, we used iZon to analyze the size distribution and concentration of all samples. The peak of particle size distribution of control subjects and T2D subjects was 120 nm and 130 nm, respectively (Figure 1A and 1B). There was no significant difference in concentration between the two groups (Figure 1C). We also used western blot to detect the exosomal signature proteins of TSG101 and CD9 (Figure 1D), and both of the two groups showed them.

Detection of miRNA expression

First, we tested some common indices of T2D, including GLU, HbA1c and urine GLU. The T2D subjects had higher levels of these three indices than control subjects (Figure S1 A, B, and C). According to the previous reports, the four miRNAs, miR-146a, miR-155, miR-23a, and miR-192, might be the potential biomarkers for T2D [15]. We first built the standard curves of the four miRNAs (Figure S2). The exosomal expression of miR-23a and miR-192 was significantly higher in T2D subjects (Figure 2A and 2B), and the exosomal expression of miR-146a and miR-155 showed no significant difference (Figure 2C and 2D). We also detected the expression of miR-23a and miR-192 in the plasma. The result showed that the concentration of miR-23a and miR-192 in plasma was higher than in exosomes, but there was no significant difference between control subjects and T2D subjects (Figure 2E and 2F).

In addition, we compared the relationship between various clinical-pathological features and exosomal miR-23a or miR-192 level, including gender, age, GLU, HbA1c, and urine GLU (Table 2). Neither miR-23a nor miR-192 level differed significantly in age and gender. The exosomal miR-23a level was significantly related to GLU and HbA1c level, while the exosomal miR-192 level was significantly related to GLU, HbA1c, and urine GLU level.

ROC curve analysis

To investigate the diagnostic value of exosomal miR-23a and miR-192, ROC curve and AUC analysis were performed. The ROC curve of exosomal miR-23a is shown in Figure 3A, and the ROC analysis yields an AUC of 0.828 (95% CI, 0.735 to 0.920, $p < 0.001$) in the differentiation of control subjects from T2D subjects. The ROC curve of exosomal miR-192 is shown in Figure 3B, and the ROC analysis yields an AUC of 0.717 (95% CI, 0.607 to 0.828, $p = 0.001$). Youden's index was used to find the cutoff value. Youden's index reaches max with exosomal miR-23a of 5.61×10^{-6} nM and miR-192 of 8.05×10^{-7} nM.

Table 1. The characteristics of control subjects and T2D subjects.

		Total	Control	T2D
No.		80	36	44
Gender	male	42	19	23
	female	38	17	21
Age (years)	mean	54.4	54.5	54.4
	range	32 - 72	34 - 72	32 - 72
Glu (mmol/L)		7.18 ± 2.63	5.02 ± 0.41	8.96 ± 2.34
HbA1c (%)		6.48 ± 1.50	5.35 ± 0.23	7.64 ± 1.28
Urine Glu (mmol/L)		0.85 ± 1.42	0.10 ± 0.32	1.13 ± 1.57

Table 2. The Relation between exosomal miR-23a or miR-192 level and various clinicopathological features.

	No.	Mean ± SD (miR-23a, 10 ⁻⁵ nM)	p-value	Mean ± SD (miR-192, 10 ⁻⁷ nM)	p-value
Gender					
Male	42	2.320 ± 5.680	0.3278	6.630 ± 7.986	0.4135
Female	38	0.695 ± 0.709		5.036 ± 2.753	
Age (years)					
≥ 60	27	1.990 ± 4.512	0.9175	6.977 ± 7.871	0.5481
< 60	53	2.120 ± 5.658		6.092 ± 5.170	
GLU					
Normal	27	0.623 ± 1.173	0.0486	4.340 ± 3.388	0.0105
Abnormal	26	3.675 ± 7.751		7.912 ± 6.075	
HbA1c					
Normal	23	0.431 ± 0.471	0.0467	4.216 ± 3.162	0.0385
Abnormal	20	4.189 ± 8.791		7.197 ± 5.772	
Urine GLU					
Normal	24	3.481 ± 8.161	0.6307	5.028 ± 4.673	0.0070
Abnormal	10	2.207 ± 1.671		1.085 ± 6.830	

Bioinformatics analysis

To further study the potential role of miR-23a and miR-192 in T2D, we used the online software DIANA tools for bioinformatics analysis [17]. DIANA TOOLS is a website established by Paraskevopoulou and other co-workers. This website provides low-threshold and easy-to-use miRNA biochemical analysis methods. In this study, we mainly used the MirPath v.3, a miRNA pathway analysis web server [20]. The web server could collect data from multiple databases, including TargetScan, TarBase and microT-CDS. Targetscan is a widely used website for miRNA target prediction [21]. TarBase is a database of experimentally supported miRNA-gene interactions [22]. In addition, microT-CDS searches for the target of annotated miRNAs based on the popular microT-CDS algorithm [23]. For miR-23a, there were

1,400 target genes from Tarbase, 3,477 from Targetscan, and 903 from microT-CDS. Among all the genes, 27 genes were found in the three databases (Figure 4A). For miR-192, there were 1,451 target genes from Tarbase, 2,058 target genes from Targetscan, and 109 target genes from microT-CDS. Among all the genes, there was no common target gene in the three databases (Figure 4B). For KEGG analysis, we found 5 intersection pathways for miR-23a (Figure 4C) and 2 intersection pathways for miR-192 (Figure 4D). For GO analysis, we found 64 intersection categories for miR-23a (Figure 4E) and 16 intersection categories for miR-192 (Figure 4F). Among them, FoxO signaling pathway was reported to participate in glucose metabolism [24,25]. Prolactin pathway was reported to have protective effects on T2D with dopamine [26,27]. The KEGG path-

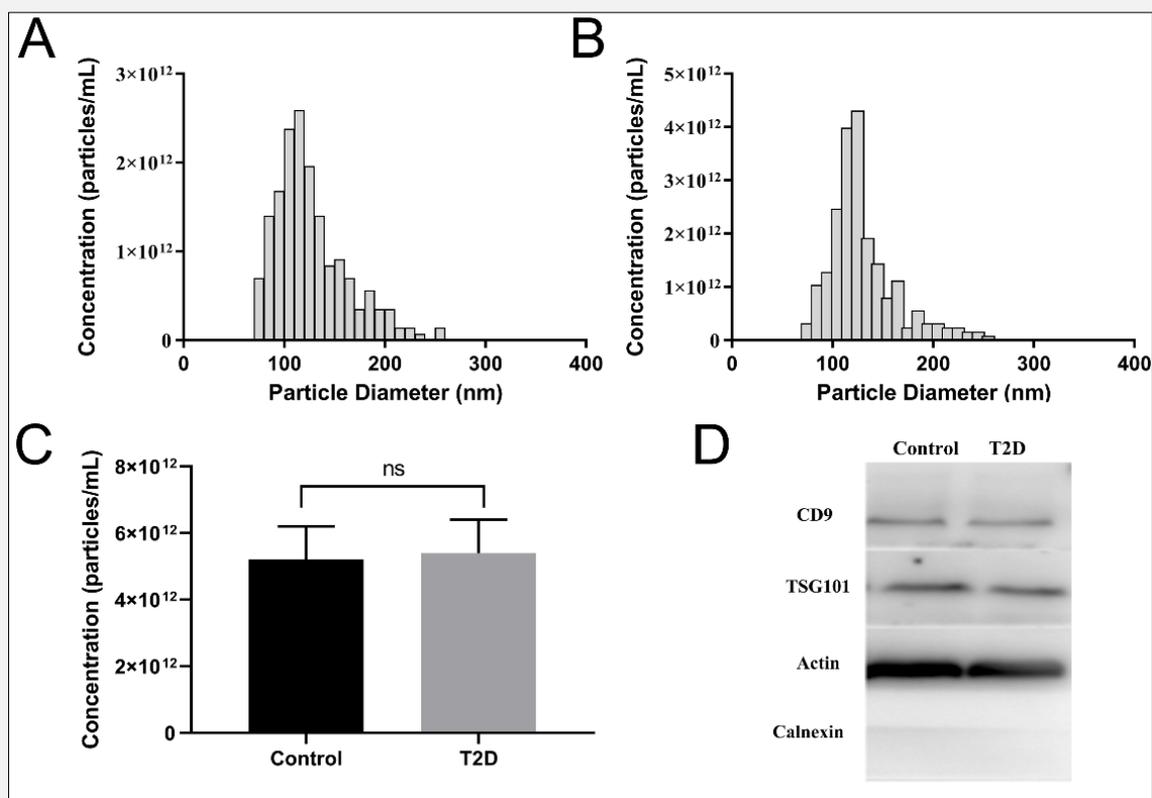


Figure 1. Exosome isolation and identification.

A - The size distribution of exosomes from control subjects. **B** - The size distribution of exosomes from T2D subjects. **C** - The concentration of exosomes from control and T2D subjects. **D** - Western blot of exosomes from control and T2D subjects in the same gel. ns - not significant.

way analysis of miR-192 showed two pathways, and apoptosis of pancreatic beta cells or other cells would influence T2D [28,29]. These bioinformatics analyses guided our future research direction for the potential molecular mechanism. However, the regulation mechanisms of T2D by miR-23a and miR-192 are still unclear and need to be studied in the future.

DISCUSSION

The diagnostic criteria for diabetes include fasting plasma glucose, two-hour plasma glucose, HbA1c, and random plasma glucose. Among them, plasma glucose indicates glucose level at one time point. HbA1c indicates glucose level for long term and is widely used. However, some studies found that HbA1c exosomes were first discovered in the 1970s as platelet microparticles from tumor cells and blood [30]. In 1987, these released vesicles were formally named “exosomes” [31]. Ultracentri-

fugation is the gold standard for exosome isolation [32]. However, ultracentrifugation is not suitable for clinical studies with large numbers of samples, which is limited by the ultracentrifuge. miRNAs were first discovered by Ambros et al. in 1993 and introduced as a new mechanism of gene regulation [33]. Recent studies have shown that miRNAs can participate in a series of physiological and pathological processes [34]. In 2007, Valadi et al. found that exosomes contained mRNA and miRNA [35]. From then on, exosomal miRNAs have gradually become a research hotspot. As a worldwide disease, T2D also has drawn attention of many researchers, some of whom study the relationship of exosomal miRNAs and T2D.

For exosome isolation, there have been several methods, including ultracentrifugation, size-based techniques, immunoaffinity capture-based techniques, microfluidics-based isolation techniques, and precipitation [36]. Among them, ultracentrifugation is considered to be the gold standard for exosome isolation, requiring an

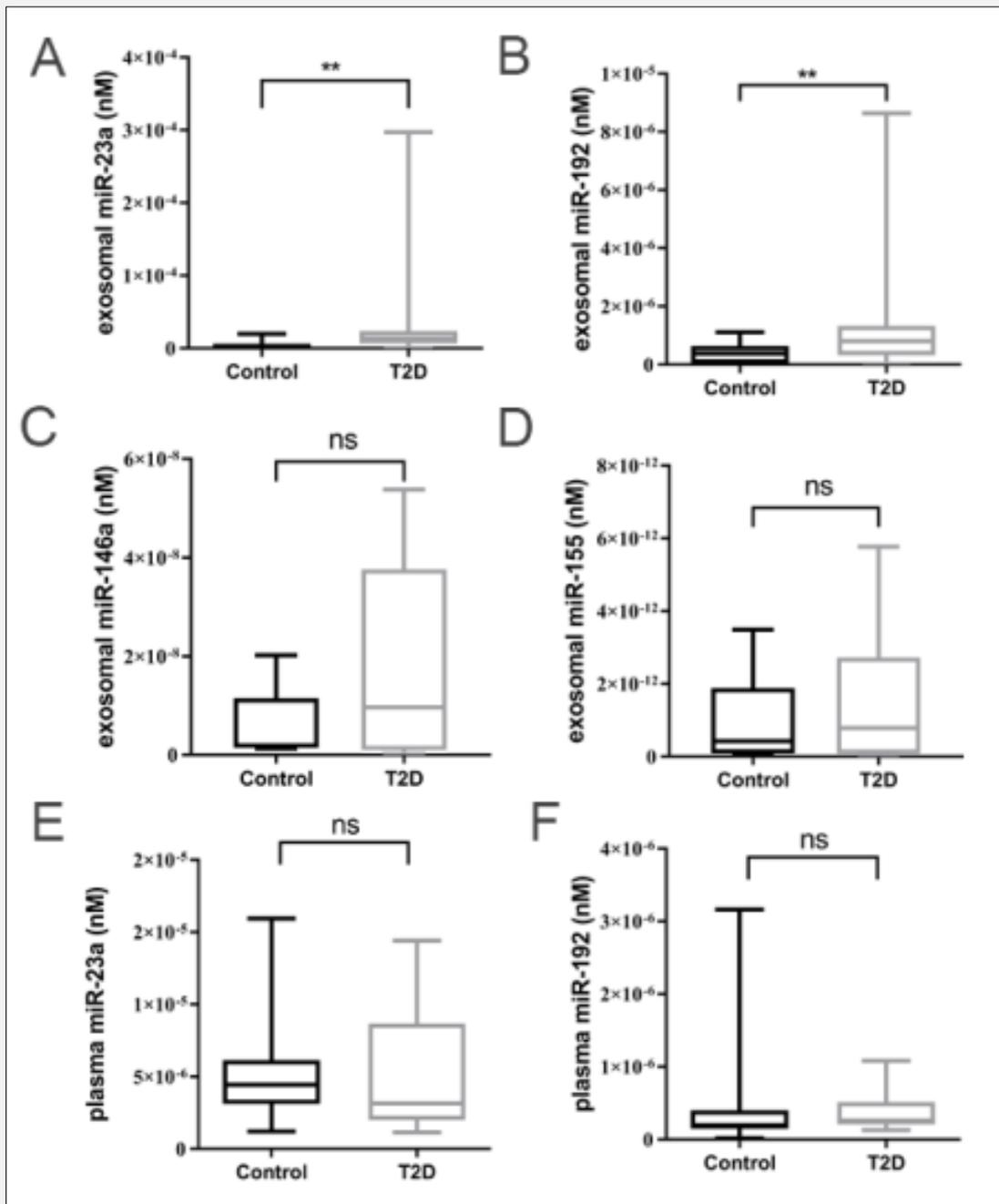


Figure 2. The level of different miRNAs.

A - The level of exosomal miR-23a. B - The level of exosomal miR-192. C - The level of exosomal miR-146a. D - The level of exosomal miR-155. E - The level of plasma miR-23a. F - The level of plasma miR-192. ** - $p < 0.01$, * - $p < 0.05$; ns, not significant.

ultracentrifuge which is available in only a few professional labs. The size-based techniques, immunoaffinity capture-based techniques, and microfluidics-based isolation techniques also require specific devices or mate-

rials. Precipitation might be a better choice for most common labs because it only requires an ordinary cryogenic high-speed centrifuge. In this study, we used PEG4000 to isolate exosomes from plasma [37]. To

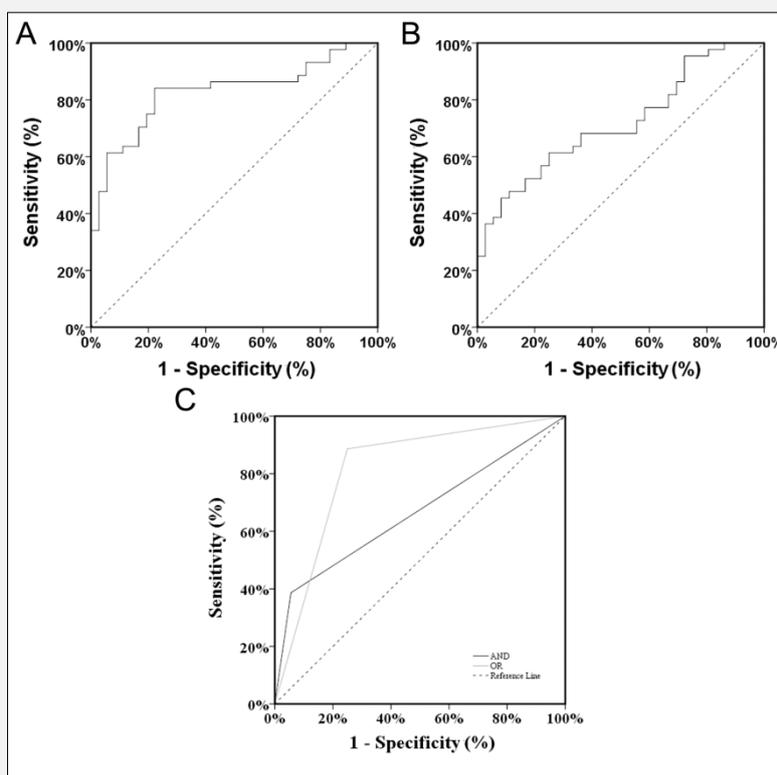


Figure 3. The ROC curve of exosomal miR-23a and miR-192 by SPSS v19.0.

A - The ROC curve of exosomal miR-23a. **B** - The ROC curve of exosomal miR-192. **C** - The ROC curve of exosomal miR-23a and/or miR-192. AND, those samples with both exosomal miR-23a and miR-192 positive can be defined as positive, the others are defined as negative. OR, those samples with both exosomal miR-23a and miR-192 negative can be defined as negative, the others are defined as positive.

confirm the reliability of this method, we used iZON to detect the concentration and size distribution and western blot to detect the signature protein expression of exosomes. The peaks of the size distribution of exosomes from controls and T2D subjects were both approximately 120 nm, which was consistent with the previous reports [38].

Karolina et al. [13] used miRNA microarray and qPCR to compare the exosomal miRNA expression in healthy controls, impaired fasting glucose, and T2D male patients. Based on their microarray results, we picked miR-23a, miR-146a, and miR-192, which were differently expressed and had been reported to be related to T2D [39-41]. We also chose miR-155 for our research according to the report of Ying et al. [15]. For the four miRNAs, we first detected their expression of exosomes in control subjects and T2D subjects. miR-23a and miR-192 of T2D subjects showed significantly higher expression than control subjects, while miR-146a and miR-155 did not. Hence, we further analyzed the diagnostic value of miR-23a and miR-192 by ROC analysis. The AUC of miR-23a and miR-192 were both more

than 0.7, which mean that exosomal miR-23a and miR-192 had a medium diagnostic value.

Some studies have reported the possible relationship of miR-23a and miR-192 with T2D. Serum miR-23a and miR-192 might be potential biomarkers for diagnosis of type 2 diabetes [42,43]. Pancreatic β -cell plays an important role in both type 1 and 2 diabetes [44]. miR-23a regulates the apoptosis of pancreatic β -cells by targeting PUMA and DP5, while miR-192 regulates the development of pancreatic β -cells by targeting GLP1 [45,46]. Besides, miR-23a and miR-192 are makers of diabetes derived diseases, including diabetic kidney disease, diabetic foot, and diabetic retinopathy [47-49]. In addition, miR-23a and miR-192 are related to other functions. For example, they function as oncogenes or tumor suppressors in colorectal cancer and lung cancer [50-53]. They are also related with immune system regulation. MiR-23a enforces intracellular reactive oxygen species equilibrium to curb necrosis during early T cell activation [54]. MiR-192 affects the expression of innate and adaptive immunity proteins in celiac disease [55]. Therefore, we will evaluate the relative risk of the com-

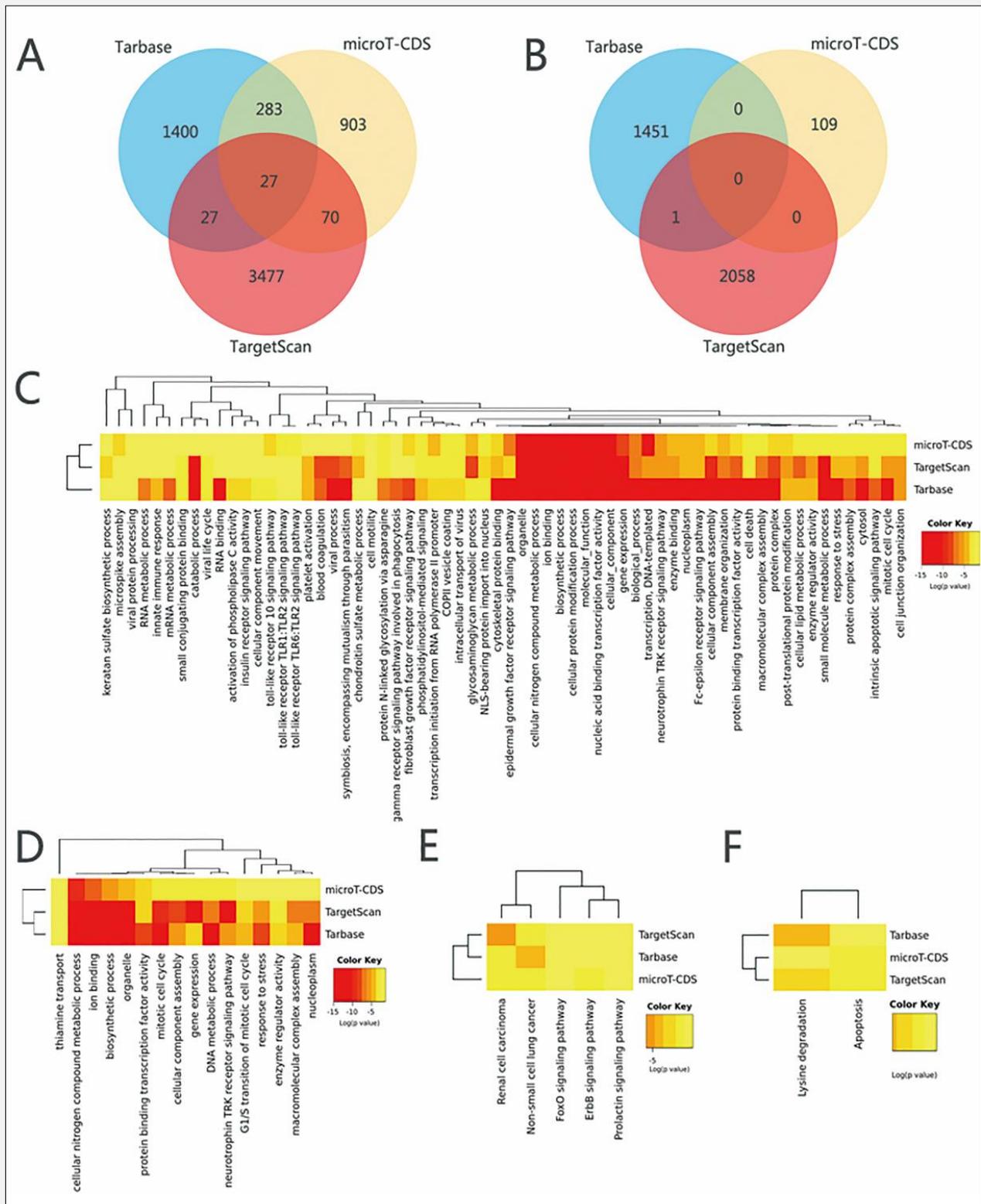


Figure 4. The bioinformatics analysis of miR-23a and miR-192 by DIANA Web Server V5.0.

A - Target gene prediction of miR-23a. B - Target gene prediction of miR-192. C - GO analysis of miR-23a. D - GO analysis of miR-192. E - KEGG pathway analysis of miR-23a. F - KEGG pathway analysis of miR-192.

complications of diabetes derived diseases in our future study.

However, there were some limitations in our study. The information of weight, BMI (body mass index), and menopausal state for subjects were not included in our study, which were proven to be related to the occurrence of diabetes [56,57]. In the follow-up study, we will include other essential indicators including weight, BMI and menopausal state.

CONCLUSION

In conclusion, our results suggested that exosomal miR-23a and miR-192 were differently expressed in control and T2D subjects. The miR-23a and miR-192 in exosomes from plasma may serve as potential biomarkers for the diagnosis of T2D patients.

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Declaration of Interest:

The authors have declared that no competing interests exist.

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