Basing on microRNA-mRNA analysis identifies microRNA in exosomes associated with wound repair of diabetic ulcers

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Key words: Exosome, Diabetic ulcer, Angiogenesis, Biological analysis, miRNA

Abstract: The diabetic ulcer is one of the serious complications of diabetes. In this study, we aimed to establish an exosomal microRNA (miRNA)-targeted messenger RNA (mRNA) regulatory network for screening new biomarkers for diabetic ulcer treatment. For this purpose, exosomes were extracted from bone marrow stem cells (BMSCs) collected from diabetic ulcer patients and healthy adults. The miRNAs in exosomes was detected by high-throughput sequencing analysis. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the differential miRNAs were performed. The miRNA-mRNA regulatory network between candidate miRNAs and their target genes were constructed by Cytoscape software basing on mRNA expression profiles data of diabetic ulcer patients from Gene Expression Omnibus (GEO). GO and KEGG analyses of the core genes were performed. A total of 63 differential expressed miRNAs in BMSCs exosomes were identified between diabetic ulcer patients and healthy adults. The GO analysis of miRNAs showed that it was mainly related to signal transduction and intercellular transport, and KEGG analysis showed that it was related to the vascular endothelial growth factor (VEGF) signaling pathway. The core genes of the miRNA-mRNA network were thioredoxin interacting protein (TXNIP), cell division cycle 14A (CDC14A), cache domain containing 1 (CACHD1), interferon-induced protein 44 like (IFI44L), late cornified envelope 1AL (LCE1A), leucine-rich repeats and immunoglobulin-like domains 2 (LRIG2), palmdelphin (PALMD) and serine and arginine-rich splicing factor 11 (SRSF11). GO analysis of the core genes was related to platelet-derived growth factor receptor signaling pathway. The KEGG analysis of the core genes was related to the cell cycle and nucleotide-binding oligomerization domain (NOD)-like receptor signaling pathway. A potential miRNA-mRNA regulatory network provides a comprehensive understanding of the molecular mechanisms and promising new targets such as miR-130a-5p, SESN2, LRIG2, and CDC14A for the wound repair of diabetic ulcers.

Abbreviations

- miRNAs: microRNAs
- DM: Diabetes mellitus
- AGEs: advancedglycation end products
- BMSCs: bone marrow stromal cells
- CD63: lysosomal membrane-associated glycoprotein 3
- TXNIP: Thioredoxin interacting protein
- CDC14A: Cell division cycle 14A
- CACHD1: Cache domain containing 1
- IFI44L: interferon induced protein 44 like
- LCE1A: late cornified envelope 1A
- LRIG2: leucine rich repeats and immunoglobulin like domains 2
- PALMD: Palmdelphin
- SRSF11: serine and arginine rich splicing factor 11
- FIH: hypoxia inducible factor inhibitor
- ECM: extracellular matrix
- DDL4: Delta like ligand 4
- FDR: false detection rate
- 3’UTR: 3’ untranslated region
- VEGF: vascular endothelial growth factor
- ERK: extracellular signal-regulated kinase
- STAT3: signal transducer and activator of transcription 3

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Received: 06 July 2020; Accepted: 23 September 2020

Doi: 10.32604/biocell.2021.012601 www.techscience.com/journal/biocell

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HGF: hepatocyte growth factor
IGF: insulin-like growth factor
NGF: nerve growth factor
SDF-1: stromal cell-derived factor-1
AGES: advanced glycation end products
hESCs: human Embryonic Stem Cells
EMT: epithelial-to-mesenchymal transforming

Introduction

Diabetes mellitus (DM) is a metabolic disorder syndrome caused by various pathogenic factors such as genetic factors, obesity, immune dysfunction, microbial infection, toxins, free radicals, and mental factors (Schmidt, 2018). Nowadays, with the improvement of living standards, lifestyle changes, and social aging, the incidence of diabetes was increasing year by year (Schmidt, 2018). The diabetic ulcer was one of the serious complications of DM; the risk of foot ulcers in diabetic patients is up to 25% (Al-Rubeaan et al., 2015). A diabetic foot ulcer is the leading cause of local distal amputation (Millington and Ellenzweig, 2005). Delayed or nonunion of wound healing after skin injury is a pressing problem in clinical practice. Some studies have shown that the accumulation of advanced glycation end products (AGEs) and hyperglycemia will not only damage peripheral blood vessels and microvessels but also inhibit the expression of many kinds of neurotrophic factors and vascular factors, which may be the main factors that cause diabetic skin ulcers difficult to heal (Bukowiecki et al., 2017; Lalla et al., 2006; Negre-Salvayre et al., 2009; Sun et al., 2016).

A variety of physiological or pathophysiological events such as the decreased proliferation of fibroblasts, decreased growth factors, reduced keratinocytes, reduced angiogenesis, abnormal collagen deposition, a small number of macrophages, and impaired function, are related to poor wound healing in DM (Bukowiecki et al., 2017). Exosomes are extracellular vesicles with a diameter of 30–150 nm; they have a small bilayer lipid membrane, which can enter the extracellular matrix directly by budding and then release the internal components after being ingested by the target cells to complete cell communication and information exchange (Pegtel and Gould, 2019). Exosomes as a subcellular component secreted by cells, which were widely involved in cell communication and can play a dominant role in tissue repair and regeneration (Chen et al., 2017; Han et al., 2016). They can be identified by their expression of exosome-associated markers such as Tsg101 and CD63 (Wubbolts et al., 2003). In recent years, there are many reports on the application of mesenchymal stem cell-derived exosomes in the treatment of diabetic ulcers (Geiger et al., 2015; Zhu et al., 2018). It has been found that the bone marrow stromal cells (BMSCs) exosomes can promote the proliferation and migration of fibroblasts extracted from diabetic chronic ulcer wounds in a concentration-and dose-dependent manner (Shabbir et al., 2015). The exosomes derived from lysosomal membrane-associated glycoprotein 3 (CD63)+ BMSCs have a stronger ability to uptake exogenous Wnt family member 3A (Wnt3a) and promote the proliferation and migration of fibroblasts through the Wnt/β-catenin signal pathway (Hu et al., 2016). BMSCs exosomes can also activate other pathways that play an important role in skin wound healing, such as AKT serine/threonine kinase (AKT), extracellular signal-regulated kinase (ERK), and signal transducer and activator of transcription 3 (STAT3) (Ding et al., 2019), and promote hepatocyte growth factor (HGF), insulin-like growth factor (IGF), nerve growth factor (NGF) and stromal cell-derived factor-1 (SDF-1) and other growth factors (Cui et al., 2016; Dai et al., 2019; Umezu et al., 2017).

MicroRNA (miRNA) is a non-coding RNA with a length of 22-24 nucleotides (Bartel, 2004). It participates in the regulation of the post-transcriptional expression of genes. Mature miRNA can bind to the 3’ untranslated region (3’-UTR) of target gene messenger RNA (mRNA), and negatively regulate target genes by degrading mRNA and inhibit protein translation of target genes (Fang and Rajewsky, 2011). MiRNAs is an important participant in the healing of diabetic ulcers, miRNAs can promote the healing of diabetic ulcers by up-regulating or down-regulating the expression of some genes and activating specific signaling pathways (Jhamb et al., 2016). MiRNA is an important substance for exosomes to promote angiogenesis. It is found that miR-31 can promote the proliferation and migration of endothelial cells and endothelial progenitor cells and induce angiogenesis by inhibiting hypoxia-inducible factor inhibitor (FIH) (Liu et al., 2010), miR-125a can inhibit the expression of Delta-like ligand 4 (DLL4), and promotes endothelial cell formation (Liang et al., 2016). Exosomes of mesenchymal stem cells (MSCs) overexpressing miR126 can promote angiogenesis in the diabetic wound model (Zgheib et al., 2013).

In this study, we used high throughput sequencing of miRNA to analyze the difference in MSCs exosomal miRNAs expression between diabetic ulcer patients and healthy adults. We constructed a miRNA-miRNA network and analyzed the core genes in the network by bioinformatics analysis methods, which aimed to discover key targets related to wound healing.

Materials and Methods

Patients

The BMSCs were obtained from 5 male patients with diabetic foot ulcers and 5 male healthy adults in the General Hospital of Southern Theatre Command of the Chinese People’s Liberation Army (PLA) between 2018 JAN to 2019 JAN. The age of patients is 58 ± 10.5 years old, and the duration of DM is 8.5 ± 3.1 years. The age of healthy adults (57 ± 6.2 years old) was matched with diabetic ulcer patients. Informed consent was obtained from all subjects. Inclusion criteria: refer to the World Health Organization (WHO) diagnostic criteria for DM. Exclusion criteria: Severe purulent infections such as sepsis, severe heart, liver, and kidney diseases and malignant tumors, blood system diseases, connective tissue diseases, and mental diseases. All experimental procedures were approved by the Ethical Committee of General Hospital of Southern Theatre Command of PLA (No. 201712) and were performed in accordance with the Helsinki Declaration (2000).
Cell culture
In this study, human BMSCs (hBMSCs) were isolated and cultured by the whole bone marrow culture method [18]. Bone marrow was diluted with phosphate buffer saline (PBS) and then centrifuged for 10 min at 4°C at 1500×g discarded the supernatant, and then washed the cells with PBS twice. The cell precipitation were collected and cultured in the complete medium [20% fetal bovine serum (FBS, Hyclone, Erembodegem-Aalst, Belgium), 2 mmol/L sodium pyruvate and 1 mmol/L L-glutamine in alpha-minimum essential medium (αMEM, Corning, USA)] in 37°C and 5% CO₂ incubator, with adjusting the cell density to 1 × 10⁶ cells/mL. After 72 h, the medium was half-changed every 2 to 3 days according to the cell growth condition.

Extraction and identification of hBMSCs exosome
In this study, we extract exosomes using the Exosomes Isolation Kit (#EQ806TC-1, SBI, Mountain View, USA) according to the manufacturer’s protocol. The hBMSCs derived from patients and healthy adults were cultured with serum-free medium. After 3 days, the culture medium was collected and centrifuged at 2000×g for 30 min. Then, the cells and debris were removed, and the supernatant was absorbed. Exosomes were then isolated by polymer precipitation using the Exosomes Isolation Kit and resuspended in PBS.

Transmission electron microscopy
The exosome sample (10 μL) was dripped on the sample-carrying copper grids with diameter 2 mm for 10 min at room temperature, re-stained with 3% sodium phosphotungstate solution (pH 6.8) at room temperature for 5 min, washed gently with double distilled water and dried at room temperature for 2 min, and then observed and photographed under the transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

Nanoparticle Tracking Analysis (NTA)
The size and number of exosomes were measured by Nanosight-N5000 (Nanosight, Amesbury, UK). A total of 1 μL exosome sample was diluted in an equal volume of filtered PBS to obtain no more than 100 particles in the NTA software screen. The modal size and the number of particles were measured in triplicates, and data were expressed as mean ± standard deviation (SD).

Exosomes protein extraction and western blotting analysis
Exosomes precipitates were extracted with protein lysate (25 mmol/L Tris-HCl PH7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and 1 mmol/L PMSF) on ice for 30 min, and then centrifugation at 12000×g for 10 min at 4°C to collect the supernatant. Exosomes protein extraction and western blotting analysis were measured in triplicates, and data were expressed as mean ± standard deviation (SD).

The expression was quantified using RPKM (Reads Per Kilo bases per Million reads) as the unit.

Microarray data acquisition
Skin mRNA expression profiling data download from the Gene Expression Omnibus (GEO) database (GSE.ncbi.nlm.nih.gov/geo) GSE80178 datasets on the expression profiles of diabetic foot ulcers. Platforms: GPL 16686 [HsGene-2.0-st] Affymetrix Human Gene 2.0 ST Array (transcript [gene] version), contains >30000 coding transcripts mRNA. The microarray consisted of 6 diabetic foot ulcers and 3 healthy controls.

Bioinformatics analysis
Data processing and differential gene analysis
The expression data were analyzed on the R (version 3.5.3) statistics environment (http://www.r-project.org). Affy, Limma, Pheatmap, ggplot2, and other software packages are used for data processing, and the RMA algorithm is used for background correction, standardization, and expression value calculation. The processed data were screened by Fold change (FC) and T-test for differential genes and defined as effective genes|log2FC| ≥ 2 (p < 0.05 or FDR < 0.05).

Enrichment analysis of GO analysis and KEGG pathways
The Gene Ontology (Pegtel and Gould) database mainly describes the functions of genes synthetically, including biological process (BP), cell composition (cellular component, CC), and molecular function (Molecular Function, MF). KEGG (Kyoto Encyclopedia of Genes and Genomes) is a comprehensive database that combines genomic, chemical, and functional data. In this study, both

Total RNA extraction and high-throughput sequencing analysis
The total RNA of the exosome of BMSCs was extracted by ExoRN easy Serum/Plasma Kit (Qiagen, German). The total RNA was quantified by NanoDropND-2000 (Thermo Scientific), and the quality and purity of total Nanodrop were analyzed and detected by ND-1000 spectrophotometer (Nanodrop company, USA). Quality control qualified sample RNA integrity count >8.0, and the A260/A280 ratio between 1.8 to 2 can be carried out after the test. High-throughput sequencing analysis was performed by the ChiBiotech Company (http://www.chi-biotech.com). The fastx_toolkit software was used to remove the low-quality reads in the sequencing data and truncate the ends of the reads, and the reads with a length greater than 17 nt were retained. FANse3 ultra-high-precision sequence alignment algorithm was used to align the reads of each sample with the reference sequence (human mature miRNA). Gene expression was quantified using RPKM (Reads Per Kilo bases per Million reads) as the unit.

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The primary antibody anti-CD63 (1:100, Abcam, Cambridge, UK) and anti-TSG101 (1:500, Abcam, Cambridge, UK) overnight at 4°C. The membrane was washed with TBST 3 times and incubated with the second antibody conjugated with horseradish peroxidase (1:2000, Biotechnology, Shanghai, China) at room temperature for 1 h. The Enhanced chemiluminescence Kit (Biotechnology, Shanghai, China) was used for the detection, and the Quantity One program (BioRad, Hercules, CA) was used for photography and data analysis.

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the unit of counting is p/mL. N: Normal healthy; D: Diabetic ulcer. NTA

CD63 and TSG101 were detected. C. The modal size and yield of protein extracted from exosomes. The exosome-associated markers hBMSCs derived exosomes were observed by transmission electron microscopy. The scale bar = 100 nm. B. Western blot analysis of protein extracted from exosomes. The exosome-associated markers CD63 and TSG101 were detected. C. The modal size and yield of exosomes were analyzed by NTA. The unit of particle size is nm; the unit of counting is p/mL. N: Normal healthy; D: Diabetic ulcer.

GO and KEGG enrichment analyses of miRNAs were performed using FunRich (version 3.1.3). The standard setting with a statistically significant difference was \( p < 0.05 \). The key genes GO and KEGG enrichment analyses were performed by R software (version 3.5.3), applied cluster profiler, org.Hs.eg.db, enrichplot, and ggplot2 package for data processing and visualization.

MiRNA target gene prediction
In this study, we have applied miRDB (http://mirdb.org), miRTarBase (http://miRTarbase.mbc.nctu.edu.tw/php/index.php) and TargetScan (http://www.targetscan.org/vert_72/) miRNA target gene prediction tool to predict miRNA target genes. In this study, we screened out the interactions between miRNA and targets that coexisted in at least two databases and identified on the 3′ untranslated region (3′UTR) of all known human genes for further analysis.

MiRNA-mRNA regulatory network construction
We use the Venny tool (Oliveros, 2007) to obtain the intersection of differentially expressed miRNAs target genes and differentially expressed mRNA. Cytoscape3.8.0 (https://cytoscape.org/) was used to construct and visualize the miRNAs-mRNA regulatory network.

Results

Identification of exosome secreted by hBMSCs
The white precipitate collected was dissolved in PBS and observed by a transmission electron microscope that exosome was a vesicle structure with a diameter of 40–100 nm (Fig. 1A). The expression levels of CD63 and TSG101 were detected by Western Blot, and the results showed that the precipitates were strongly positive for CD63 and TSG101 (Fig. 1B). We also used NTA to detect the particle modal size and quantity. The results showed that the modal size of our extracted particles was 152.3 ± 11.2 nm in the diabetic ulcer group and 172.4 ± 8.5 nm in the healthy adult group. The particle size count was 1.36 ± 0.28 × 1010 p/mL in the diabetic ulcer group and 1.56 ± 0.33 × 1010 p/mL in the healthy adult group. There was no difference in the number and size of exosomes between the two groups (\( p > 0.05 \)).

Different exosome MiRNAs profiles between diabetic ulcer patients and health subjects
In order to identify the difference of miRNAs expression in the hBMSCs exosomes of diabetic ulcer patients and healthy adults, we analyzed the miRNA-Seq data, normalized the data of miRNA expression, calculated logFC, FDR, and \( P \) values of miRNA in the two groups, and screened out \( p < 0.05 \) and \(|\log FC| > 2\). The results showed that a total of 63 miRNAs. Among them, 60 miRNAs were up-regulation and 3 miRNAs (hsa-miR-2116-3p, hsa-miR-1-5p and hsa-miR-130a-5p) were down-regulation. In up-regulated expression of miRNAs, hsa-miR-665, hsa-miR-6731-5p, hsa-miR-2115-5p, hsa-miR-873-5p, hsa-miR-214-3p, hsa-miR-2115-5p were more obvious up-regulated. The up-regulation of miRNAs were more common than the down-regulated (Fig. 2). It should be noted that in our results, FDR values are more than 0.05, which suggests that miRNAs between patients and healthy groups are not significantly regulated.

Heatmaps demonstrate differential expressed miRNAs between diabetic ulcer patients (D, blue) and normal healthy adults (N, pink). The x-axis denotes differential expressed miRNAs, and the y-axis represents the samples. The expression values are shown in line with the color scale.

Functional annotation and pathway enrichment analysis of differential expressed miRNAs
In this study, we analyzed the function of differential expression of miRNAs in patients with diabetic ulcers and healthy adults. GO analysis showed that candidate target genes of differential expressed miRNAs were significantly enriched in CC terms including “Nucleus,” “Cytoplasm” and “Golgi apparatus” (Fig. 3A), MF terms including “Transcription factor activity” (Fig. 3B), and BP including “Signal transduction,” “Cell communication” and “Regulation of nucleic acid metabolism” (Fig. 3C). The GO results suggested that the differential expressed miRNA may play an important regulatory role in cell to cell communication.

KEGG pathway enrichment analysis was further conducted for candidate target genes of differential expressed miRNAs. The top 10 significantly enriched KEGG items were listed in Fig. 3D, including “TRAIL signaling pathway,” “Glypican pathway,” “Syndecan-1-mediated signaling events,” “Nectin adhesion pathway,” “IGF1 pathway,” “ErbB receptor signaling network,” “IL5-mediated signaling events,” “Signaling events mediated by Hepatocyte Growth Factor Receptor (c-Met),” “vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) signaling network” and “Signaling events mediated by VEGFR1 and VEGFR2” (Fig. 3D). The differential expressed miRNAs associated with matched genes in VEGF/VEGFR pathway including down-regulated hsa-miR-1-5p and hsa-miR-130a-5p, and up-regulated hsa-miR-135a-5p, hsa-miR-665, hsa-miR-219a-1-3p, hsa-miR-212-3p, hsa-miR-34c-5p, hsa-miR-208b-3p, hsa-miR-9-3p and hsa-miR-873-5p, which may play an important...
regulatory role in promoting skin wound healing by promoting angiogenesis.

**Different mRNA profiles between DFS and NFS**

In this study, we downloaded the expression profile data of diabetic foot ulcers in diabetic foot ulcer patients through the GEO database. We analyzed the mRNA expression profiling by array data through the Limma package of R software, normalized the data of mRNA expression, and calculated the logFC, FDR, and P values of mRNA in the two groups. Screened mRNA with FDR < 0.05 and |logFC| > 2, the results showed that a total of 28 mRNAs were differentially expressed, of which 16 mRNAs including LCE6A, LCE1A, CACHD1, TXNIP, ANXA9, IFI44, CA6, SRGAP2B, DLEU2L, IFI44L, SRSF11, HMGN2, CDC14A, PALMD, EFCAB7, and LRG12 were down-regulated and 12 mRNAs, including SPRR3, SESN2, LCE3C, SPRR4, AJAP1, IVL, SPRR1B, SPRR1A, S100A7A, CDA, PRR9, and S100A9, were up-regulated (Fig. 4).

**Functional annotation and pathway enrichment analysis of DM mRNAs**

GO and KEGG pathway analyses were performed to understand better these DM miRNAs functions. BP analysis showed that “peptide cross-linking,” “keratinocyte differentiation,” “epidermal cell differentiation,” “skin development,” “epidermis development,” “keratinization,” and “cornification” terms were significantly enriched (Fig. 5A, Tab. 1). The CC analysis showed a list of genes was significantly enriched in “cornified envelope.” (Fig. 5A, Tab. 1). All these GO terms were closely related to skin generated. In KEGG analysis, we found that the enriched pathway was the IL-17 signaling pathway (Fig. 5B, Tab. 1).

**MicroRNA-mRNA network**

In this study, we predicted the target genes of miRNAs obtained by differential expression, and we selected miRDB, miRTarBase, and TargetScan as prediction tools to screen the target genes that existed in at least two databases.
Finally, we got 40868 target genes. After intersecting with 28 mRNAs differentially expressed in diabetic ulcer patients, 17 target genes were obtained. Since the regulation relationship between a miRNA and its target mRNA is negative, so we removed the unreasonable nodes in the network, and there are 30 miRNAs and 15 mRNAs left in the network. In the network, there are 8 miRNA regulating TXNIP, 5 miRNA regulating CDC14A, 5 miRNA regulating HMGN2, 4 miRNA regulating IFI44L, 4 miRNA regulating SRSF11, 3 miRNA regulating PALMD, 2 miRNA regulating LCE1A, 2 miRNA regulating LRIG2, and only one miRNA regulating ANXA9, CA6, EFCAB7, IFI44, S100A7A and SESN2 (Fig. 6).

The GO and KEGG analysis of core gene
We performed GO and KEGG enrichment analyses on the core genes CACHD1, CDC14A, HMGN2, IFI44L, LCE1A, LRIG2, PALMD, SRSF11, and TXNI in the network. The GO BP analysis results showed that LRIG2 and TXNIP was significant enrichment in “platelet-derived growth factor receptor signaling pathway” terms ($p < 0.05$). The KEGG results showed that CDC14A was significant enrichment in the “cell cycle”; TXNIP was significant enrichment in the “NOD-like receptor signaling pathway” (Figs. 7 and 8).

The GO and KEGG analysis of miRNAs and mRNAs
The GO and KEGG enrichment analyses on miRNAs-mRNAs (Tab. 2) showed that the top-3 enriched GO category “keratinization” is related to hsa-miR-125b-5p-IVL, hsa-miR-6813-5p-SPRR3 and hsa-miR-5698-SPRR1B, “peptide cross-linking” is related to hsa-miR-125b-5p-IVL, hsa-miR-6813-5p-SPRR3, and hsa-miR-5698-SPRR1B, and “keratinocyte differentiation” is related to hsa-miR-125b-5p-IVL, hsa-miR-6813-5p-SPRR3, hsa-miR-5698-SPRR1B. hsa-miR-130a-5p is also enriched in “mitochondrial DNA metabolic process,” “regulation of cAMP-dependent protein kinase,” “regulation of platelet-derived growth factor receptor signaling,” “regulation of gluconeogenesis involved in cellular glucose homeostasis,” and “regulation of response to reactive oxygen species.” hsa-miR-208b-5p is also enriched in “regulation of platelet-derived growth factor receptor signaling.” The enriched KEGG pathway “Nitrogen metabolism” is related to hsa-miR-6849-3p, hsa-miR-208b-5p, hsa-miR-548ac and CA6, “p53 signaling pathway” is related to hsa-miR-4474-3p, hsa-miR-1254, hsa-miR-130a-5p and SESEN2, and “Cell cycle” is related to hsa-miR-103a-2-5p, hsa-miR-873-5p, hsa-miR-4775 and CDC14A. These results further provided potential miRNAs-mRNAs and their pathways for wound healing of DM.

Discussion
In this study, we compared the expression of miRNAs between BMSCs exosomes from patients with diabetic ulcers and healthy adults. Because the exosomes have the function of mediating miRNAs vector, it can transport miRNAs to the target organ, which leads to the change of
FIGURE 4. Differential expression of mRNAs. Heatmaps demonstrate differential expression of mRNAs in the skin between diabetic ulcer patients (D, blue) and normal healthy adults (N, pink). The x-axis denotes differential expressed mRNAs, and the y-axis represents the samples. The expression values are shown in line with the color scale.

FIGURE 5. Enrichment analysis of mRNAs. A. The length of the histogram represents the number of enriched genes, and the saturation of the color represents the significance of the $q$-value. B. The larger the dot, the greater the number of genes. The lighter the color, the smaller the $q$-value.
microenvironment around the target organ, and then regulates the expression of mRNA in target organ cells. Previous studies have paid more attention to ulcer wound repair after MSCs transplantation (Kwon et al., 2008; Wan et al., 2013), but less attention has been paid to the role of exosomal miRNAs of BMSCs in regulating the mRNAs between diabetic ulcer patients and healthy adults on skin wound healing. We speculate that these DM miRNAs may affect the process of ulcer wound repair.

In patients with diabetic foot ulcers, vascular endothelial cells are damaged, their normal function is in disorder, and wounds are difficult to heal. Therefore, protecting endothelial cells and promoting angiogenesis may be a very effective way to repair diabetic ulcers (Mulder et al., 2014; Suresh et al., 2014). It had confirmed that BMSCs plays a good therapeutic effect in wound healing of diabetic ulcers (Motegi and Ishikawa, 2017). BMSCs can accelerate wound healing by differentiating into endothelial cells and paracrine angiogenesis factors, such as angiopoietin 1 and VEGF, after transplantation into the wound (Lee et al., 2016). The paracrine regulation of MSCs is mainly achieved by exosomes (Akyurekli et al., 2015), it promotes angiogenesis by growth factors and accelerates the reconstruction of damaged tissues and organs. Exosomes contain various proteins and miRNAs (Sato-Kuwabara et al., 2015; Valadi et al., 2007). miRNAs have been reported to play an important role in tissue repair and regeneration (Bjørge et al., 2017; Sahoo and Losordo, 2014). The KEGG analysis of differential expression miRNAs showed that the differentially expressed miRNAs were in "VEGF and VEGFR signaling network" and "Signaling events mediated by VEGFR1 and VEGFR2 pathway."

### TABLE 1

| GO Terms                  | p.adjust | Gene ID                                                                 | Count |
|---------------------------|----------|-------------------------------------------------------------------------|-------|
| BP peptide cross-linking  | <0.001   | SPRR1A/SPRR1B/SPRR3/SPRR4/IVL/PRR9/LCE1A/LCE3C                          | 8     |
| BP keratinocyte differentiation | <0.001 | SPRR1A/SPRR1B/SPRR3/SPRR4/IVL/PRR9/TXNIP/LCE6A/LCE1A/LCE3C              | 10    |
| BP epidermal cell differentiation | <0.001 | SPRR1A/SPRR1B/SPRR3/SPRR4/IVL/PRR9/TXNIP/LCE6A/LCE1A/LCE3C              | 10    |
| BP skin development       | <0.001   | SPRR1A/SPRR1B/SPRR3/SPRR4/IVL/PRR9/TXNIP/LCE6A/LCE1A/LCE3C              | 10    |
| BP epidermis development  | <0.001   | SPRR1A/SPRR1B/SPRR3/SPRR4/IVL/PRR9/TXNIP/LCE6A/LCE1A/LCE3C              | 10    |
| BP keratinization         | <0.001   | SPRR1A/SPRR1B/SPRR3/SPRR4/IVL/LCE6A/LCE1A/LCE3C                         | 8     |
| BP cornification          | <0.001   | SPRR1A/SPRR1B/SPRR3/IVL/LCE1A                                          | 5     |
| CC cornified envelope     | <0.001   | SPRR1A/SPRR1B/SPRR3/SPRR4/IVL/PRR9/LCE1A/LCE3C                         | 8     |

| KEGG terms                | p.adjust | gene ID                                                                 | Count |
|---------------------------|----------|-------------------------------------------------------------------------|-------|
| IL-17 signaling pathway   | <0.05    | S100A9/S100A7A                                                           | 2     |

FIGURE 6. miRNA-mRNA regulation network. The ellipse stands for mRNA, the triangle represents miRNAs, the red represents the upturn, and the green represents the downgrading.
hsa-miR-208b-3p, hsa-miR-9-3p and hsa-miR-873-5p are closely associated with matched genes in VEGF/VEGFR pathway. Among them, hsa-miR-130a-5p is involved in the “mitochondrial DNA metabolic process,” “regulation of cAMP-dependent protein kinase activity,” “regulation of gluconeogenesis involved in cellular glucose homeostasis,” “regulation of response to reactive oxygen species” and “p53 signaling pathway” with SESN2, “regulation of platelet-derived growth factor receptor signaling pathway” with LRIG2, and “cell cycle” with CDC14A. Hsa-miR-208b-5p is involved in the “regulation of gluconeogenesis involved in cellular glucose homeostasis” with LRIG2, and “nitrogen metabolism” with CA6. Has-miR-873-5p is involved in the “cell cycle” with CDC14A.

Wound healing caused by diabetic ulcers was delayed, due to a lack of angiogenesis factors (Pietramaggiori et al., 2010). It is found that VEGF treatment can significantly promote angiogenesis (Zeng et al., 2019) and shorten wound healing time in diabetic rats (Brem et al., 2009). In vivo, the miRNA in the exosome released by cells can be transported to neighboring cells and distant cells (Valadi et al., 2007). In breast cancer, it has been found that the
exosomal miR-105 released by MCF-10A and MDA-MB-231 can reduce the expression of the ZO-1 gene in endothelial cells and promote lung and brain metastasis (Zhou et al., 2014). The miR-214 of human microvascular endothelial cell line (HMEC-1) exosomes can stimulate the migration and angiogenesis of neighboring HMEC-1 cells (Van Balkom et al., 2013). The exosomal miR-92a of K562 cells was reported to significantly decrease the expression of integrin α5 in human umbilical vein endothelial cell (HUVEC) and promote endothelial cell migration and catheter formation (Umezu et al., 2013). In this study, GO analysis of DM miRNAs showed that the DM miRNAs play an important role in exosome-mediated intercellular signal transduction and communication, and vacuolar transport, suggesting that miRNAs in exosomes derived from BMSCs can affect the skin repair of diabetic ulcers through transport and VEGF pathways.

Functional analysis differential expression miRNAs of ulcer skin between diabetic ulcer patients and healthy adults showed that BP such as keratinocyte differentiation, epidermal cell differentiation, skin development, epidermis development, keratinization, cornification, and the cornified envelope was significantly enriched. It was found that the migration and proliferation of keratinocytes were closely related to skin wound healing and played an important role in wound repair of diabetic ulcer (Werner et al., 2007), suggesting those miRNAs play an important role in wound repair in patients with diabetic ulcers. They were closely associated with the IL-17-mediated signal pathway. IL-17 signal pathway is related to the function of keratinocytes (Ma et al., 2016; Wu et al., 2015). In psoriasis, a secreted intestinal antimicrobial protein REG3A promoted skin keratinocyte proliferation, can be induced by IL-17 (Lai et al., 2012). During wound repair of diabetic ulcers, the inhibition of IL-17 accelerates diabetic wound healing through the alteration of macrophage polarization (Lee et al., 2018).

In this study, we analyzed the miRNA-mRNA regulatory network. The GO analysis results showed that the core genes TXNIP and LRIG2 were significantly enriched in the “platelet-derived growth factor receptor signaling pathway” biological processes. The KEGG analysis results showed that the core genes CDC14A was significantly enriched in the “cell cycle” pathway and TXNIP was significantly enriched in the “NOD-like receptor signaling pathway.” The “platelet-derived growth factor receptor signaling pathway” plays an important role in wound healing (Peus et al., 1995). Human TXNIP is a protein with a molecular weight of 46kD

### Table 2

The GO and KEGG analysis for the interacted miRNAs-mRNA

| GO Terms                              | p.adjust | miRNA          | Gene ID |
|---------------------------------------|----------|----------------|--------|
| Keratinization/peptide cross-linking/keratinocyte differentiation | <0.001   | hsa-miR-125b-5p | IVL    |
|                                       |          | hsa-miR-6813-5p | SPRR3  |
|                                       |          | hsa-miR-5698   | SPRR1B |
| Cornified envelope                    | <0.01    | hsa-miR-125b-5p | IVL    |
|                                       |          | hsa-miR-5698   | SPRR1B |
| Isopeptide cross-linking via N6-(L-isoglutamyl)-L-lysine           | <0.05    | hsa-miR-125b-5p | IVL    |
| Epidermis development                 | <0.05    | hsa-miR-6813-5p | SPRR3  |
|                                       |          | hsa-miR-5698   | SPRR1B |
| Mitochondrial DNA metabolic process   | <0.05    | hsa-miR-4474-3p| SESN2  |
| Regulation of cAMP-dependent protein kinase activity             | <0.05    | hsa-miR-4474-3p| SESN2  |
| Regulation of platelet-derived growth factor receptor signaling pathway | <0.05    | hsa-miR-6514-3p| LRG2   |
|                                       |          | hsa-miR-548k   |         |
|                                       |          | hsa-miR-4775   |         |
| Protein binding, bridging             | <0.05    | hsa-miR-125b-5p| IVL    |
|                                       |          | hsa-miR-5698   | SPRR1B |
| Regulation of gluconeogenesis involved in cellular glucose homeostasis | <0.05    | hsa-miR-4474-3p| SESN2  |
| Regulation of response to reactive oxygen species                   | <0.05    | hsa-miR-4474-3p| SESN2  |

| KEGG terms                          | p.adjust | miRNA          | Gene ID |
|-------------------------------------|----------|----------------|--------|
| Nitrogen metabolism                 | <0.01    | hsa-miR-6849-3p| CA6    |
| p53 signaling pathway               | <0.01    | hsa-miR-4474-3p| SESN2  |
| Cell cycle                          | <0.05    | hsa-miR-103a-2-5p| CDC14A|

TABLE 2
composed of 39 amino acid residues, which can be expressed in a variety of tissues, it is located on chromosome 1q21.1 and highly conserved among species and genera (Van Greevenbroek et al., 2007). It has been found that TXNIP is associated with angiogenesis, the deletion of TXNIP can restore restorative angiogenesis in the obesity model induced by a high-fat diet (Elshaer et al., 2017). TXNIP overexpression in diabetic wound healing can directly lead to diabetic angiogenic dysfunction. Silencing the TXNIP gene can prevent endothelial cell migration disturbance and angiogenesis injury induced by high glucose, and animal experiments have also shown that siRNA silencing TXNIP prevents ischemia-mediated diabetic angiogenesis (Dunn et al., 2014), restores the production of VEGF, and promotes angiogenesis ability (Ng et al., 2007). The mRNA data that we downloaded from the GEO database are derived from diabetic ulcers patients’ foot skin. We infer that miRNAs in BMSCs exosomes regulates wound healing in diabetic ulcer skin mainly through TXNIP and promotes the increase of VEGF secretion by regulating TXNIP, thus increasing the ability of wound healing. In the miRNA-miRNA network, we can see that the miRNAs involved in regulating TXNIP were hsa-miR-4796-3p, hsa-miR-135a-5p, hsa-miR-665, hsa-miR-4632-5p, hsa-miR-4639-5p, hsa-miR-873-5p, hsa-miR-103a-2-5p, hsa-miR-5196-3p. It is found that hsa-miR-135a-5p is highly expressed in the scar tissue of patients with urethral stricture, and its high expression may be related to tissue fibrosis (Zhang et al., 2018). It has been found that hsa-miR-873-5p is an important multipotential miRNA, hsa-miR-873-5p and an important multipotential miRNA, may hinder the differentiation of human Embryonic Stem Cells (hESCs) into fibroblasts by targeting epithelial-to-mesenchymal transforming (EMT) (Sahu and Mallick, 2018). It has been found that the increase of hsa-miR-665 can inhibit coronary artery micro-angiogenesis mediated by CD34 (Fan et al., 2018). CDC14A is an important gene involved in cell cycle regulation (Vázquez-Novelle et al., 2010). The healing of skin wounds is closely related to the division of epidermal cells. Studies have shown that CDC14A is involved in the G2/M phase of the mitotic cell cycle of epidermal cells (Zanet et al., 2010). In the study of wound repair, many studies have shown that the "NOD-like receptor signaling pathway" plays an important role in wound repair, in the process of intestinal epithelial cell repair, the NOD-like receptor signaling pathway participates in the repair of wound surfaces (Parlati and Yeretssian, 2014). In cardiovascular disease, NOD-like receptors are involved in the repair of cardiovascular damage (Bracey et al., 2015), and studies have found that in type 2 diabetic patients and mice, abnormal NOD-like receptor signaling pathway in macrophages can damage wound healing (Mirza et al., 2014).

To sum up, we found that there are differences in the expression of miRNAs between BMSCs exosomes from patients with diabetic ulcers and healthy adults. Part of the specific miRNAs such as hsa-miR-130a-5p was transported to the ulcer site through the exosomes and participated in wound healing by regulating genes such as SESN2, LRIG2, and CDC14A. However, by limitation of only 5 patients were collected in our study, few differential miRNAs with significant FDR were obtained. The effect of gender on DM should be considered with a larger sample, and other miRNAs might be identified in the future. In addition, we will verify the core genes in the regulatory network by in vivo and in vitro experiments.

Data Availability Statement: The research data used to support the findings of this study are currently under embargo. Requests for data, 12 months after publication of this article, will be considered by the corresponding author.

Funding Statement: Supported by the National Natural Science Foundation of China (81571910), Science and Technology key Project of Guangdong province (2014B020212010), the National Key Research and Development Plan of China (2017YFC1103301), and Medical Scientific Research Foundation of Guangdong Province of China (B2018026).

Conflict of Interest: None.

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