European Review for Medical and Pharmacological Sciences 2020; 24: 8117-8126

Serum and exosomal hsa_circ_0000907 and hsa_circ_0057362 as novel biomarkers in the early diagnosis of diabetic foot ulcer

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Abstract. – OBJECTIVE: Diabetic foot ulcer (DFU) is a serious chronic complication leading to disability and death in patients suffering from diabetes. Currently, there is no effective marker for its early diagnosis. The aim of this study is to analyze the difference of circRNA expression profiles between DFU and normal human wounds (NHW) and to screen serum biomarkers for the early diagnosis of DFU.

MATERIALS AND METHODS: Differentially expressed circRNAs were screened by bioinformatics analysis, using GSE114248 chip data downloaded from GEO database, including 5 pairs of tissue samples from DFU patients and NHW cases. Accordingly, 20 cases of DFU (Wagner grade 0~2), 20 non-DFU diabetes and 20 healthy controls were selected in the screening test, and the total RNAs of serum and serum-derived exosomes were extracted. The screened circRNAs were verified in the third largest cohort, and the ROC curves were drawn to assess the diagnostic efficiency.

RESULTS: As discovered by experiment, there were a total of 67 circRNAs presented differential expressions between the two groups, with 28 circRNAs upregulated and 39 circRNAs downregulated in DFU group. Two types of circRNAs, hsa_circ_0000907 and hsa_circ_0057362, were selected as candidate biomarkers in current study and validated in a large cohort. The AUCs of serum hsa_circ_0000907 and hsa_circ_0057362 to diagnose early DFU were 0.9389 and 0.8792, respectively, and the AUCs of exosomal hsa_circ_0000907 and hsa_circ_0057362 to diagnose early DFU were 0.8783 and 0.8481, respectively. Furthermore, the expressions of serum hsa_circ_0000907 and hsa_circ_0057362 were negatively correlated with ankle brachial index (ABI) and transcutaneous oxygen pressure (TcPO2) in DFU patients.

CONCLUSIONS: Serum and exosomal hsa_circ_0000907 and hsa_circ_0057362, especially hsa_circ_0000907, have novel diagnostic capabilities in the early diagnosis of DFU.

Key Words:
Diabetic foot ulcer, Circular RNA, Hsa_circ_0000907, Hsa_circ_0057362, Diagnosis, Exosome.

Introduction

DFU is a serious chronic complication that may lead to disability and even death in patients suffering from diabetes mellitus[1]. Scholars[2] have shown that diabetes accounts for 40%-60% of all non-traumatic lower limb amputations. Among diabetes-related distal limb amputations, 85% occur following foot ulcers, and the prevalence of foot ulcers in diabetes patients ranges from 4% to 10%, whereas the proportion of lower limb arterial disease in patients over 50 years old in China is 19.5%[2]. A single-center study[3] has shown that the proportion of lower limb arterial disease in diabetic patients over 60 years of age was 35.4%. The incidence of new ulcers within one year was 8.1% in diabetic patients and is 31.6% in DFU patients in China[4]. It is noteworthy that early diagnosis and treatment serve as important methods in reducing the incidence of DFU, which thereby is expected to reduce the amputation rate and mortality.

Circular RNA (circRNA) is a class of coding/non-coding RNA molecules that covalently bind at the 3' end and 5' end to form closed loop composed of exons and/or introns[5,6]. They are widely expressed in mammalian cells[7-10], and have tissue-cell specificity, structural stability and sequence conservation. CircRNA contains more abundant transcription than linear mRNAs, which are widely involved in intracellular RNA-mediated regulatory networks, and plays a role in regulating gene expression at the tran-

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scriptional and post-transcriptional levels[11,12]. Studies[13-15] have confirmed that circRNA is a component of competitive ceRNA and can regulate gene transcription, translation and other functions by inhibiting the activity of miRNA, which plays an important role in physiological processes such as the cell cycle or senescence. CircRNA is closely related to the occurrence and progression of a variety of diseases, such as diabetes and other metabolic diseases were reported by recently[16-18]. Xu et al[19] found that circRNA-Cdr1as acts as a RNA sponge and inhibits its activity by binding to miR-7 in islet cells, increasing insulin synthesis and release at both the transcriptional and translational levels. Currently, very few existing studies have investigated circRNA and DFU. Wang et al[20] found that patients with DFUs had higher levels of hsa_circ_0084443 than patients with NHWs, and overexpression of hsa_circ_0084443 was found to promote the growth of keratinocyte.

Exosome is a kind of membranous vesicle with a diameter of 30-150 nm, which is released into the extracellular matrix after intracellular polyvesicles fuse with the cell membrane and is widely distributed in various body fluids[21-23]. Exosome contains proteins, nucleic acids (mRNA, miRNA, lncRNA, and DNA) and lipids, which are important mediators of cell-to-cell communication[24-26]. Furthermore, the concentration of miR-3976 in the serum exosome of patients with Wagner grade 3-4 diabetic foot was observed to be significantly higher than that of patients with Wagner grade 0-2 diabetic foot[27]. In current study, in order to screen the serological biomarkers for the early diagnosis of DFU, bioinformatics methods were used to analyze the differences in circRNA expression profiles between DFUs patients and NHWs patients, and the top 11 significantly upregulated circRNAs in different cohorts were screened and tested. In addition, our study traced the performances of serum and exosomal hsa_circ_0000907 and hsa_circ_0057362 in the early diagnosis of DFU.

Materials and Methods

Study Population

A total of 85 patients with DFU (Wagner grade 0-2) from the First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology from December 2018 to August 2019 were collected. Another 85 diabetic patients without DFU and 90 healthy subjects were collected as controls. Their serums were collected and frozen at -80˚C immediately. Additionally, 10 pairs of DFU and NHW tissue samples were collected for microarray correctness verification, and another 10 DFU tissue samples were collected as a screening cohort. This study was approved by the Ethics Committee of our hospital, and informed consent form was taken from the patients. The DFU stage is based on the Wagner grade[28].

The exclusion criteria for this study were as follows: subjects with malignancy, dysfunction of liver and kidney, autoimmune disease, any other clinically systemic acute or chronic inflammatory disease(s), untreated hypertension, gestational diabetes mellitus, type 1 diabetes, and diabetes with other complications.

Study Design

The proposed study's design was shown in Figure 1. The differently expressed circRNAs were analyzed and identified based on bioinformatics analysis, and the results were validated with 10 pairs of DFU and NHW tissues by qRT-PCR. The screened circRNAs were then validated in an independent cohort using tissue, serum and exosomal samples (DFU group, tissue sample n = 10, serum sample n = 20; healthy control group, n = 20; non-DFU controls with diabetes, n = 20). The significantly expressed top 2 circRNAs with the largest AUCs were selected as biomarkers, and their diagnostic values were validated in another independent cohort (healthy control group, n = 70; non-DFU controls with diabetes, n = 65; and DFU group, n = 65).

Circular RNA Expression Analysis

To investigate the expression profiles of circRNAs in healthy individuals and DFU patients, the GSE114248 chip data from GEO database were download. The original data were analyzed and obtained based on circRNA microarray with 5 pairs of DFU and NHW tissue samples included. Limma R package was used to analyze the differential expression, with a filter condition of ($|logFC| > 2$, adj. p-value < 0.05). The bidirectional hierarchical clustering was carried out to plot the Cluster map of differential genes based on the pheatmap R package (https://cran.r-project.org/web/packages/pheatmap/).

Vesicle Isolation and Morphological Identification

ExoRNeasy Serum/Plasma Midi Kit (QIAGEN) was used to extract serum exosomes, as

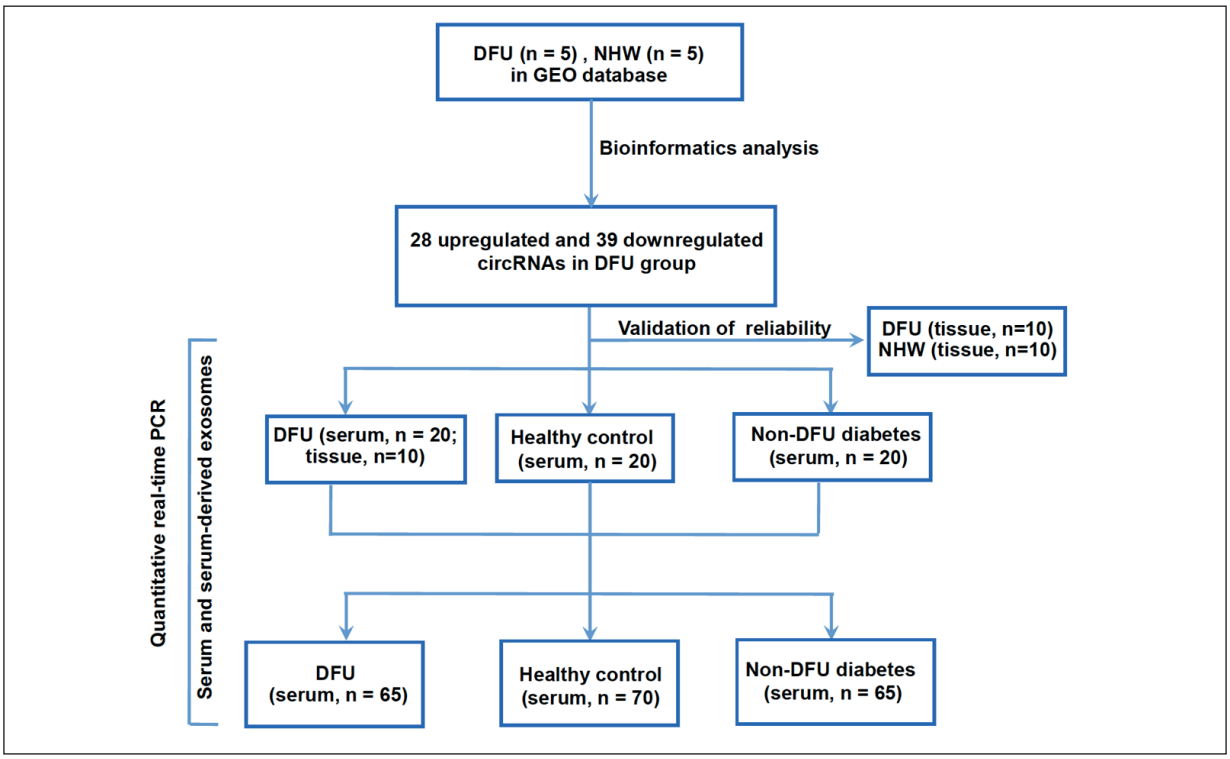

Figure 1. Schematic diagram of the study design.

outlined by the extraction process in www.qiagen.com/HB-1179. The extracted exosome was identified using the transmission electron microscope (TEM) (FEI TECNAI G2, Philips), which was then resuspended with PBS, diluted 10 times and had 20 µl absorbed on the carrier copper mesh with a pore size of 2 nm. It was then placed at room temperature for 10 min, after which 30 µl phosphotungstic acid (3%) was used for negative staining for 3 min. Last, TEM observation was done after naturally drying it at room temperature.

Immunoblotting

After the total protein was extracted from the exosome eluent by RIPA lysate, it was separated by 12% gel at a constant pressure of 80 V and transferred to a polyvinylidene difluoride (PVDF) membrane at a constant current of 250 mA. TBST containing 5% skimmed milk powder was used for blocking overnight. Rabbit anti-human monoclonal antibody CD9 (Abcam, Cambridge, MA, USA, 1:300) and rabbit anti-human monoclonal antibody CD63 (Abcam, Cambridge, MA, USA, 1:300) were added, and mouse anti-human TSG101 monoclonal antibody (Abcam, Cambridge, MA, USA, 1:500) was incubated at room temperature for 4 h. Horseradish peroxidase (HRP) labeled goat anti-mouse/rabbit IgG (1:10000) secondary antibody solution was then incubated at room temperature for 2 h, and ECL luminescent solution was added for imaging.

Quantitative Real Time-PCR (qRT-PCR)

The miRNeasy Kit (Qiagen, Hilden, Germany) was used to extract the total RNA from serum, as specifically shown in X. The extracted serum and exosome total RNA were reverse transcribed into cDNA using a Reverse Transcription System (Promega, Madison, WI, USA) according to the instructions. The sequences of all primers used are listed in Table I. The reaction system was amplified by a ABI7500 fluorescence quantitative PCR detector under the following conditions: denaturation at 95°C for 10 min, then 95°C for 15 s for 40 cycles, followed by 60°C for 1 min. $2^{-\Delta\Delta CT}$ was calculated for relative quantification, and the product was also sent to Sanger sequencing.

Statistical Analysis

SPSS 20.0 (IBM Corp., Armonk, NY, USA) was used to analyze the data. The measured data

Table I. Primer sequences of all top 11 circRNAs and GAPDH for qRT-PCR.

CircRNA name	Sequence	Product length (bp)
hsa_circ_0000907	F: 5'-ATCCTGGGTGCTGGCC-3'; R: 5'-CTTTCACACTCATCCTGCCG-3'	98
hsa_circ_0059104	F: 5'-GAGAGTGTGGCTTCATTCGC-3'; R: 5'-CCACTGGGTTCATCTTGGGG-3'	93
hsa_circ_0072697	F: 5'-CAGATTTGTAACTGGAAAACTCATGA-3'; R: 5'-CATGGATGCACTGGGGAGAT-3'	99
hsa_circ_0000967	F: 5'-AGCAAGAAGAGGACCTGTGC-3'; R: 5'-GAAGAGGCCGCTGTGTAGAG-3'	89
hsa_circ_0057362	F: 5'-CCTGGTGGTAAAGGCGAAAT-3'; R: 5'-TAGCCTGCGAGTCCTCCTAC-3'	89
hsa_circ_0067301	F: 5'-TAACACGCTGGCCCATTACA-3'; R: 5'-GAAGGATGTAGCTCTGTGTGC-3'	58
hsa_circ_0081069	F: 5'-CTGGTGCCCCTGGTGAAAAT-3'; R: 5'-ACGTGGTCCTCTATCTCCGG-3'	80
hsa_circ_0089762	F: 5'-AGGGCGTGATCATGAAAGGT-3'; R: 5'-ACGGCGGACTAATCTTCAACT-3'	142
hsa_circ_0080968	F: 5'-AGGAATAACTGCGTGGGGTT-3'; R: 5'-TGTCTCCCATTATGCACCCG-3'	72
hsa_circ_0024731	F: 5'-TCAAATCCTCAGTGATGCTCCT-3'; R: 5'-TGAGGGTCTGCGTGGTGTA-3'	106
hsa_circ_0089763	F: 5'-ACGGTAGTATTTAGTTGGGGCA-3'; R: 5'-TCTTCCCACTCATCCTAACCCT-3'	61
GAPDH	F: 5'-GACAGTCAGCCGCATCTTCT-3'; R: 5'- GCGCCCAATACGACCAAATC-3'	104

were expressed as means ± SD, and the differences between the two groups were compared using the Student's *t*-test. The correlation between circRNA expression and clinicopathological features was analyzed by the Spearman correlation test. $p<0.05$ was considered to be statistically significant, and Graph Prism 5.0 (La Jolla, CA, USA) was utilized to draw statistical pictures.

Results

Profiles of CircRNA Expression in DFU and Validation

A total of 67 differentially expressed circRNAs were obtained, of which 28 were upregulated and 39 were downregulated (Figure 2A and 2B). In order to obtain the most clinically applicable biomarkers, stricter screening criteria, i.e., the fold change is more than 2.0; and the value of *p* is less than 0.0001, were followed to select candidate biomarkers from the 28 upregulated circRNAs. 11 upregulated circRNAs complied with criteria were finally obtained. We confirmed these obtained circRNAs as candidate biomarkers in later validation using tissue samples (n=10), which is consistent with the data of bioinformatics analysis (Figure 2C). Thus, it was demonstrated that the circRNA profile was reliable.

Identification of Isolated Vesicles

The transmission electron microscope showed that the size of the serum-derived exosome was 80-120 nm, which was a small vesicle with a membranous structure, consistent with the morphological characteristics of the exosome (Figure 3A). The expression of exosome iconic proteins CD9, CD63, and TSG101 were also detected by WB to determine whether the isolated and purified exosome vesicles were successful. The results from the aforementioned experiment documented well that the expressions of CD9, CD63, and TSG101 could be detected in exosome eluent (Figure 3B), indicating that the exosomes were successfully extracted.

Validation of Differentially Expressed CircRNAs in Serum and Exosomes

The expression of 11 kinds of upregulated circRNAs in tissues, serum and serum-derived exosome were further examined by qRT-PCR in an independent cohort. According to the Figure 4A to 4C, the expression of serum and exosomal hsa_circ_0000907 and hsa_circ_0057362 were found to be highly consistent with their expression status in tissues, and showed higher AUCs than the remaining 9 circRNAs in diagnosing early DFU (Figure 4D to 4G) (ROC curves of the remaining 9 circRNAs were not shown).

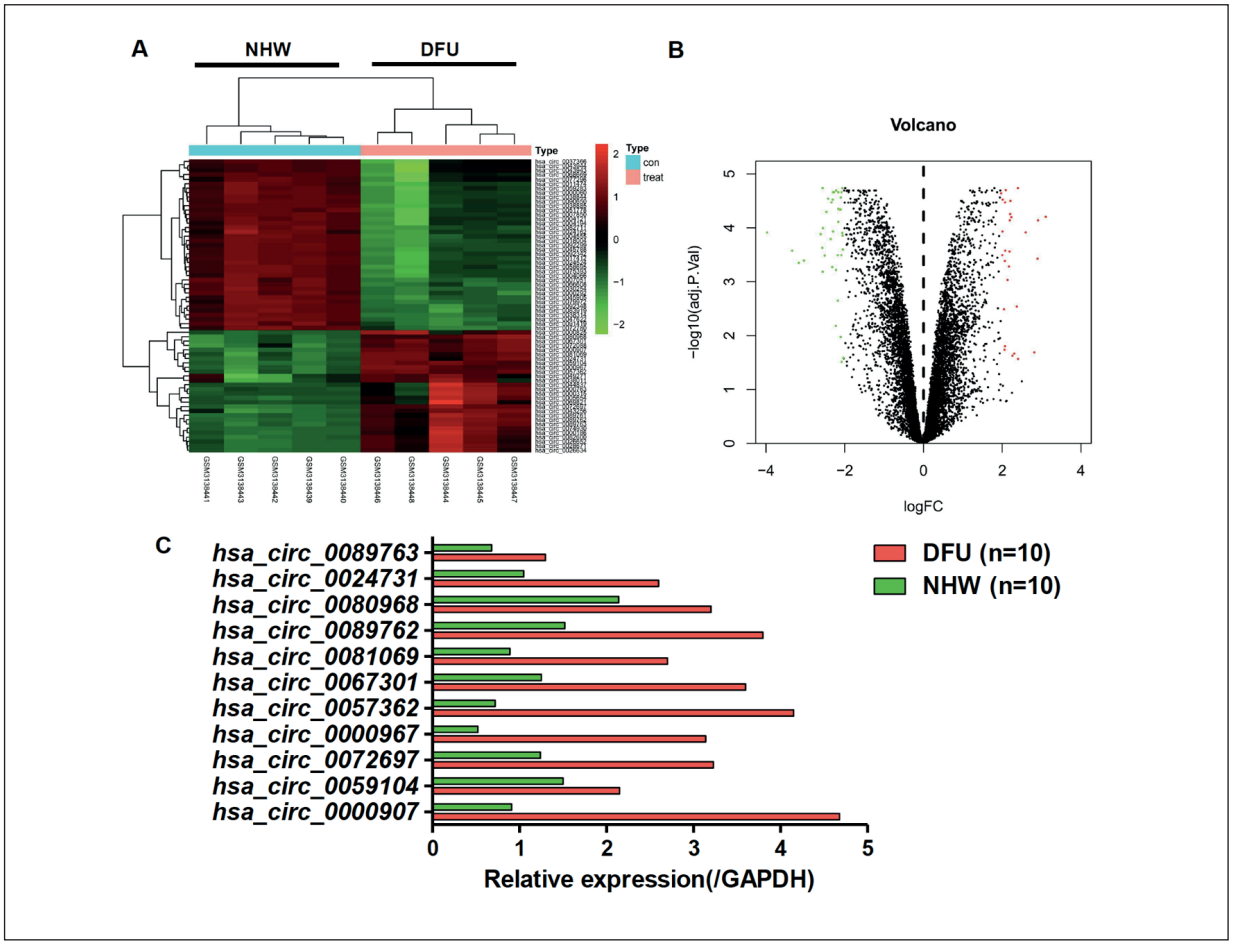

Figure 2. Differential expressions of circRNAs in DFU patients and NHW cases. **A,** Heatmap of the circRNA expression profiles in DFU and NHW. **B,** Volcano plots of the differentially expressed circRNAs, consisting of fold-change values (logFC, x-axis) and statistically significant differences (*p*-values, Y-axis). The red points showed the significantly up-regulated circRNAs, and differentially up-regulated ones were represented by green points. **C,** Verification of the top 11 up-regulated circRNAs in tissues by qRT-PCR to corroborate the reliability of the circRNA profile.

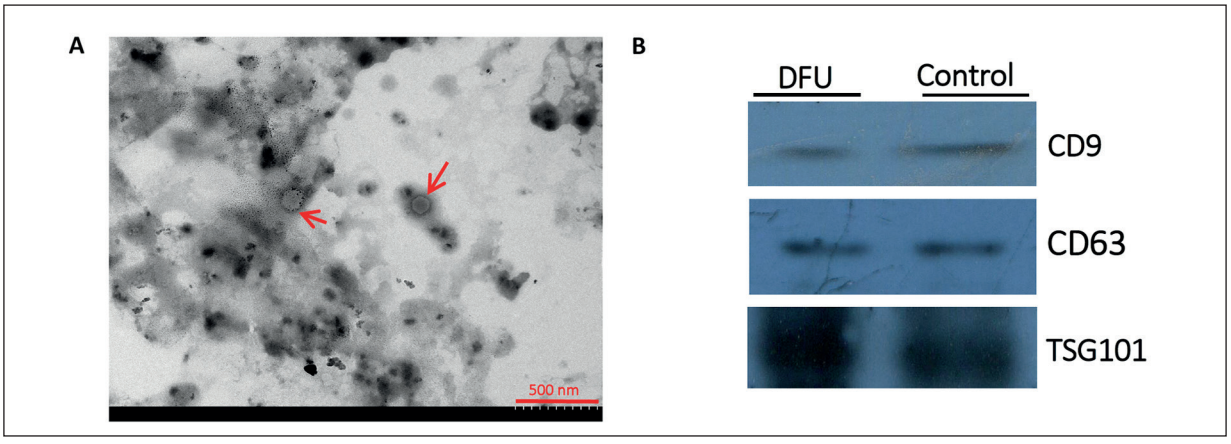

Figure 3. Identification of the vesicles isolated from serum of the DFU patients. **A,** Extracted vesicles were identified by transmission electron microscope at an accelerating voltage of 80 kV. The red arrows indicated the isolated vesicles. **B,** Expressions of the exosome marker proteins CD9, CD63 and TSG101 were further quantitated by Western immunoblotting.

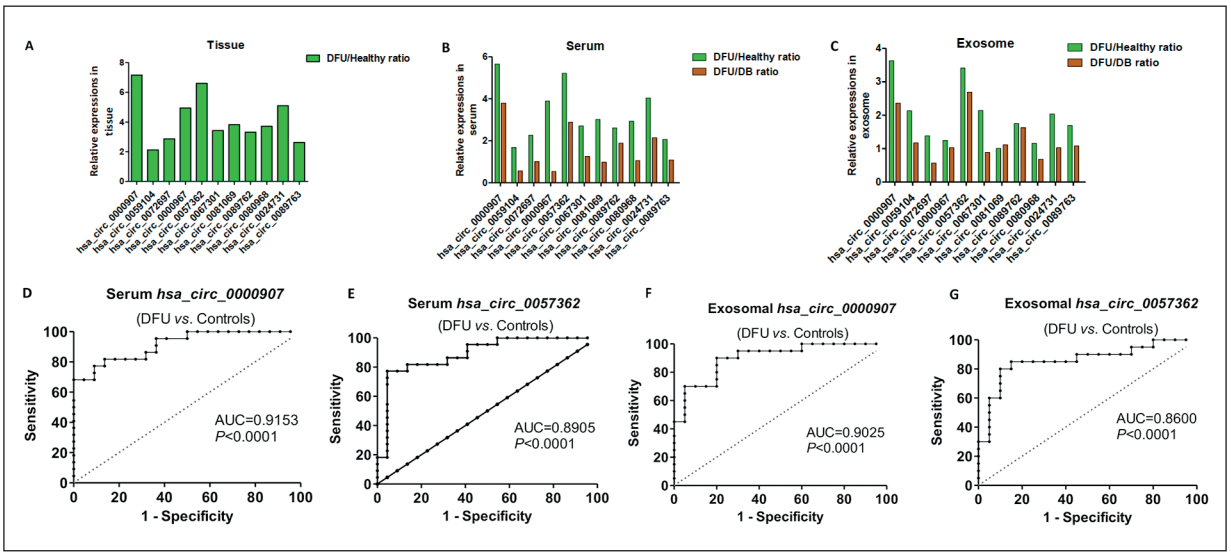

Figure 4. Expressions of the upregulated circRNAs in (**A**) tissues, (**B**) serum, and (**C**) serum-derived exosomes were further validated in DFU patients and controls using qRT-PCR. **D-G,** The plotted ROC curves of circRNAs with high AUCs. Serum and exosomal hsa_circ_0000907 and hsa_circ_0057362 showed the highest AUCs in an independent screening cohort (healthy control group, n = 20; non-DFU controls with diabetes, n = 20; and DFU group, n = 20) than other circRNAs (data not shown).

As illustrated by Figure 4B, the kurtosis of serum hsa_circ_0000907 expression was 5.65 times higher than that in healthy subjects and 3.8 times higher than that in non-DFU patients with diabetes. Besides, the kurtosis of serum hsa_circ_0057362 expression was 5.22 and 2.89 times higher than that in healthy subjects and non-DFU patients with diabetes, respectively. Similarly, the kurtosis of exosomal hsa_circ_0000907 expression was 3.63 times higher than that of healthy subjects and 2.36 times higher than that in non-DFU patients with diabetes, while the kurtosis of exosomal hsa_circ_0057362 expression was 3.41 times and 2.69 times higher than that of healthy subjects and non-DFU patients with diabetes, respectively (Figure 4C).

Serum and Exosomal Hsa_Circ_0000907 and Hsa_circ_0057362 as a Promising Biomarker for the Early Diagnosis of DFU

In order to evaluate the expressions and diagnostic performances, the corresponding efficacy of serum and exosomal hsa_circ_0000907 and hsa_circ_0057362 were validated in another independent cohort. The expression of serum and exosomal hsa_circ_0000907 and hsa_circ_0057362 in early DFU (Wagner grade 0-2) were discovered to be obviously higher than that in healthy subjects and non-DFU controls with diabetes (Figure 5A to 5H). The ROC curve analysis revealed that the AUCs of early DFU (Wagner grade 0-2) diagnosed by serum and exosomal hsa_circ_0000907 and healthy patients were 0.9389 (SEN and SPE were 87.14% and 90.77%, respectively. Youden index=0.7791) and 0.8783 (SEN and SPE were 80.00% and 80.85%, respectively. Youden index=0.6085) (Figure 5I to 5L), while the AUCs of early DFU and non-DFU diabetes distinguished by serum and exosomal hsa_circ_0000907 were 0.7912 and 0.8298, respectively. Moreover, the performance of hsa_circ_0057362 in the early diagnosis of DFU was simultaneously evaluated. The results based on the evaluation indicated that the AUCs of serum and exosomal hsa_circ_0057362 in the diagnosis of early DFU and healthy patients was estimated to be 0.8792 (SEN and SPE were 81.42% and 78.46%, respectively. Youden index=0.5989) and 0.8481 (SEN and SPE were 86.005 and 70.22%, respectively. Youden index=0.5621) (Figure 5M and 5N). As revealed in Figure 5O and 5P, the AUCs for distinguishing DFU from non-DFU diabetes was 0.7564 and 0.8327, respectively. Collectively, the above data suggested that serum and exosomal hsa_circ_0000907 and hsa_circ_0057362 are capable of being utilized as promising biomarkers for the early diagnosis of DFU.

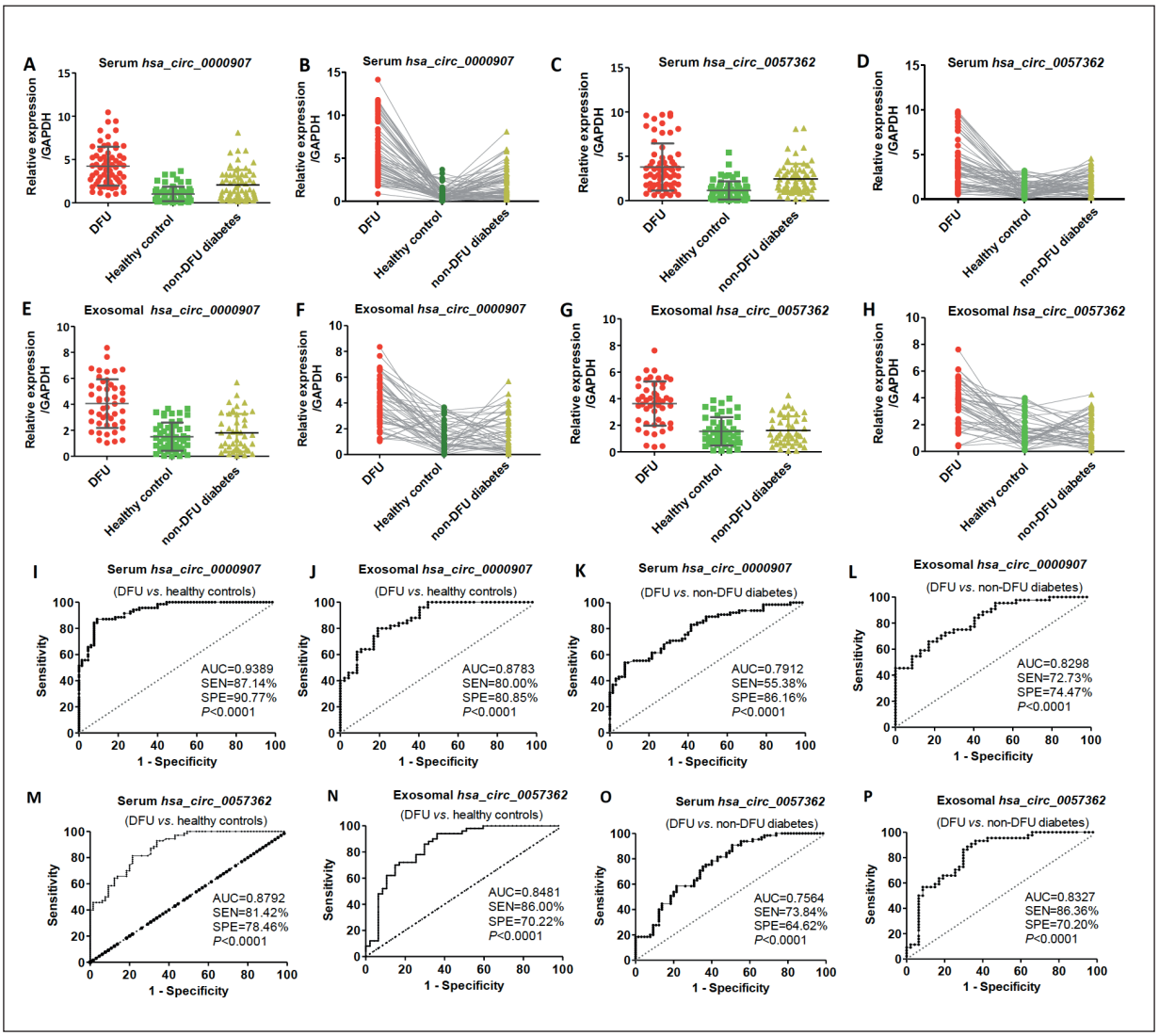

Figure 5. The diagnostic efficacy of serum and exosomal hsa_circ_0000907 and hsa_circ_0057362 of early DFU. Expressions of (**A, B**) serum hsa_circ_0000907 and (**C, D**) hsa_circ_0057362 significantly increased in DFU cases compared with controls, and expressions of (**E, F**) exosomal hsa_circ_0000907 and (**G, H**) hsa_circ_0057362 were also elevated in DFU cases. **I-L,** The ROC curves of serum and exosomal hsa_circ_0000907 in distinguishing DFU patients from healthy controls or non-DFU diabetes. **M-P,** The ROC curves of serum and exosomal hsa_circ_0057362 in discriminating DFUs from healthy controls or non-DFU diabetes.

Correlation Between the Expression of Serum/Exosomal Circrnas and the Clinicopathological Features of Patients

As shown in Figure 6A to 6D, the expressions of serum hsa_circ_0000907 and hsa_circ_0057362 were negatively correlated with ankle brachial index (ABI) and transcutaneous oxygen pressure ($TcPO_2$), suggesting that serum hsa_circ_0000907 and hsa_circ_0057362 may be involved in the progression of DFU. Nevertheless, the point which can be figured out from Figure 6E to 6H is the expressions of exosomal hsa_circ_0000907 and hsa_circ_0057362 have no significant correlation with ABI or $TcPO_2$.

Discussion

DFU is a serious chronic complication that leads to disability and death in diabetic patients across the world[1,4,28,29]. It is characterized by its high incidence, difficulty in treatment and huge expense[29]. Early diagnosis and treatment are still important methods of reducing the incidence of

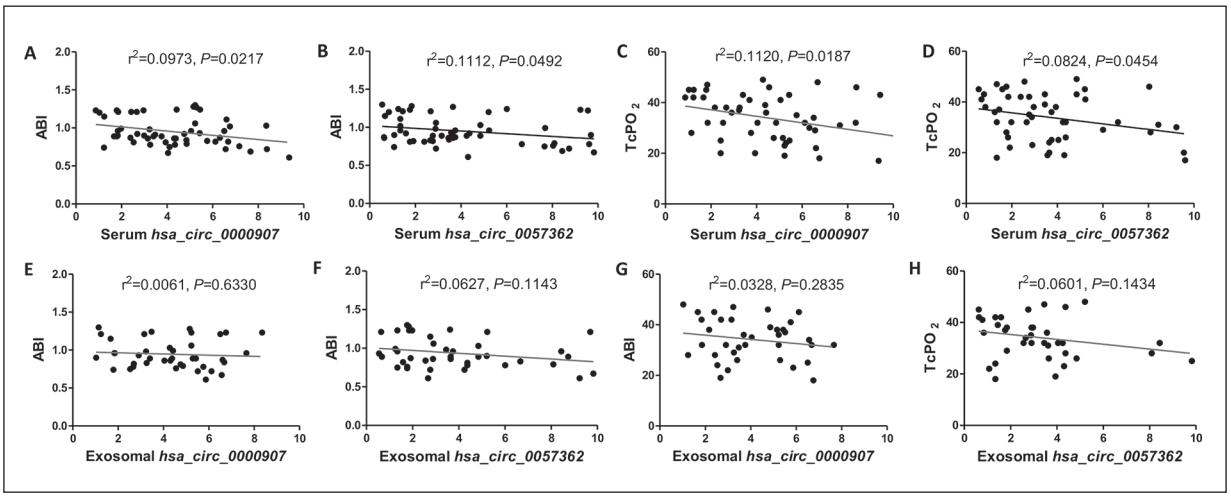

Figure 6. Correlations between serum and exosomal expressions of hsa_circ_0000907 and hsa_circ_0057362 and the two predictors ABI and $TcPO_2$ of the DFU cases. **A-D,** The expressions of serum hsa_circ_0000907 and hsa_circ_0057362 were negatively correlated with ABI values and $TcPO_2$ in DFU cases. **E-H,** exosomal hsa_circ_0000907 and hsa_circ_0057362 showed no significant correlations with ABI values and $TcPO_2$ in DFU cases.

diabetic foot, amputation rate and mortality[29]. However, there is still a lack of effective serological markers for the early diagnosis of DFU. CircRNA is a type of RNA molecule with a closed ring structure, which has been disregarded as noise for a long time[5,6]. Recently, due to the rapid development of sequencing technology, circRNA has been discovered on a large scale[7-10]. Currently, no studies have documented the clinical utility of serum and exosomal circRNAs for the early detection of DFU. To the best of our knowledge, this is the first study that found and reported the value of serum and exosomal circRNAs for the early diagnosis of DFU.

At present, few reports exist on circRNA(s) and DFU. Wang et al[20] found that the expression of hsa_circ_0084443 in DFU tissue was significantly higher than that in NHW's, and overexpressed hsa_circ_0084443 was observed to promote the growth of keratinocyte. Here, the difference of the circRNA expression profiles between DFUs and NHWs in GEO database was analyzed, and the database fed back 67 kinds of differentially expressed circRNA, of which 28 were up-regulated. Moreover, 28 kinds of upregulated circRNA were screened, after which 11 kinds of circRNA were selected with the highest differential expression for preliminary verification. Finally, hsa_circ_0000907 and hsa_circ_0057362 were screened from serum and exosomes for subsequent verification. For the first time, serum hsa_circ_0000907 was found to have the largest AUC in diagnosing DFU and healthy patients, followed by serum hsa_circ_0057362. Importantly, hsa_circ_0000907 and hsa_circ_0057362 were also discovered to be effective in distinguishing DFU from non-DFU diabetic patients, with a higher AUC of 0.7912 for serum hsa-circ-0000907 than for serum hsa-circ-0057362 (AUC=0.7564). In this regard, serum hsa_circ_0000907 and hsa_circ_0057362 were expected to be useful as non-invasive and accurate molecular markers for the early diagnosis of DFU.

According to Zhao et al[17], microarray analysis was used to analyze and compare the expression of serum circRNA in 6 patients with T2DM and 6 normal volunteers, and it was found that there were differences in the expression of 489 kinds of circular RNA, of which 78 kinds of circular RNA were up-regulated while 411 were down-regulated in the type 2 diabetes group; by screening and qRT-PCR, the peripheral blood of 247 normal participants, patients with prediabetes and diabetic patients were compared, where human peripheral blood has_circ_0054633 was discovered to be able to be used as a diagnostic marker for prediabetes and type 2 diabetes, with a SEN of 75% and a SPE of 79%, respectively[17].

As illustrated in previous studies[21-23], exosome is a type of cystic vesicle, which is essentially a lipid bilayer produced and excreted by cells through active secretion. It is known that exosome plays an important role in cell-to-cell information exchange and substance transmis-

sion[24-26]. Due to the improvement of medical technology, exosomes-derived nucleic acids have been noted to play an indispensable role in the early diagnosis, treatment and prognosis of certain diseases, including cancer and metabolic diseases[30-32]. For instance, the concentration of PCX in urine extracellular vesicles of patients with diabetic nephropathy was also found to be significantly increased, which may serve as a potential marker for the clinical detection of diabetic nephropathy[33]. Additionally, it has been reported that the concentration of miR-3976 in serum-derived exosomes was significantly higher in patients with Wagner grade 3-4 DFU than in patients with Wagner grade 0-2[27]. In terms of this effect, exosomes from the serum of DFU patients and healthy people were isolated respectively and further verified *via* ETM and immunoblotting. Moreover, hsa_circ_0000907 and hsa_circ_0057362, with high diagnostic value, were demonstrated to be derived from exosomes. The AUC of exosomal hsa_circ_0000907 and hsa_circ_0057362 in diagnosing DFU and healthy participants were 0.8783 and 0.8481, respectively. However, the diagnostic efficacy of hsa_circ_0000907 and hsa_circ_0057362 from exosomes was lower than that from serum, and insufficient evidence is presented as to whether the free circRNA in serum is derived from the release of exosomes. Intriguingly, exosomal hsa_circ_0000907 and hsa_circ_0057362 shown a higher diagnostic efficacy in distinguishing between DFU and non-DFU patients suffering from diabetes than in serum, and it may be of great significance for clinical differential diagnosis.

ABI is an important indicator of blood circulation of the limbs[33]. In addition, $TcPO_2$ is a key indicator of insufficient blood supply around the body[34]. Accordingly, the expression levels of serum hsa_circ_0000907 and hsa_circ_0057362 were negatively correlated with ABI and $TcPO_2$, suggesting that hsa_circ_0000907 and hsa_circ_0057362 may be involved in the progression of DFU. Intriguingly, as exosome-derived hsa_circ_0000907 and hsa_circ_0057362 were not significantly correlated with ABI or $TcPO_2$, more investigations are needed to confirm the findings.

Conclusions

In summary, this is the first study to report the value of hsa_circ_0000907 and hsa_circ_0057362 in serum and exosomes in the early diagnosis of DFU, suggesting that serum and exosomal hsa_circ_0000907 and hsa_circ_0057362, especially serum hsa_circ_0000907, may serve as important markers in the early diagnosis of DFU. Many limits are covered in this study, this investigation is a single-center study with a wide range and few samples, hence, the results obtained may be biased. In the future, high-quality large-sample multicenter researches are required to confirm the corresponding results.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Authors' Contribution

Zengjun Chen designed the study; Xiaojuan Shi and Ke Shi conducted the bioinformatics analysis; Liujun Fu and Jie Liu collected the samples. Zengjun Chen, Xiaojuan Shi and Wenbo Zhang conducted the experiments and analyzed of the data; Wenbo Zhang and Kepan Su conducted part of the analysis of the data; Zengjun Chen proofread, revised and final approved the manuscript; all authors have approved the version to be published.

References

1) Lim JZ, Ng NS, Thomas C. Prevention and treatment of diabetic foot ulcers. J R Soc Med 2017; 110: 104-109.
2) Guan H, Liu ZM, Li GW, Guo XH, Xu ZR, Zou DJ, Xing HL, Liu W, Sheng ZY, Tian HM, Zhu DL, Yu DM, Zhuang WT, Chen LL, Weng JP. Analysis of peripheral arterial obstructive disease related factors among diabetic population aged > or = 50. Zhonghua Yi Xue Za Zhi 2007; 87: 23-27.
3) Wirsing P, Andriopoulos A, Böttticher R. Arterial reconstructions of the lower limbs in diabetics and nondiabetics. Comparative late results. J Mal Vasc 1986; 11: 185-189.
4) Jiang Y, Wang X, Xia L, Fu X, Xu Z, Ran X, Yan L, Li Q, Mo Z, Yan Z, Ji Q, Li Q. A cohort study of diabetic patients and diabetic foot ulceration patients in China. Wound Repair Regen 2015; 23: 222-230.
5) Patop IL, Wüst S, Kadener S. Past, present, and future of circRNAs. EMBO J 2019; 38: e100836.
6) Salzman J. Circular RNA expression: its potential regulation and function. Trends Genet 2016; 32: 309-316.
7) Chen LL, Yang L. Regulation of circRNA biogenesis. RNA Biol 2015; 12: 381-388.
8) Li X, Yang L, Chen LL. The biogenesis, functions, and challenges of circular RNAs. Mol Cell 2018; 71: 428-442.

9) Vo JN, Cieslik M, Zhang Y, Shukla S, Xiao L, Zhang Y, Wu YM, Dhanasekaran SM, Engelke CG, Cao X, Robinson DR, Nesvizhskii AI, Chinnaiyan AM. The landscape of circular RNA in cancer. Cell 2019; 176: 869-881.e813.

10) Meng S, Zhou H, Feng Z, Xu Z, Tang Y, Li P, Wu M. CircRNA: functions and properties of a novel potential biomarker for cancer. Mol Cancer 2017; 16: 94.

11) Kristensen LS, Andersen MS, Stagsted LVW, Ebbesen KK, Hansen TB. The biogenesis, biology and characterization of circular RNAs. Nat Rev Genet 2019; 20: 675-691.

12) Ebbesen KK, Hansen TB, Kjems J. Insights into circular RNA biology. RNA Biol 2017; 14: 1035-1045.

13) Qi X, Zhang DH, Wu N, Xiao JH, Wang X, Ma W. CeRNA in cancer: possible functions and clinical implications. J Med Genet 2015; 52: 710-718.

14) Zhong Y, Du Y, Yang X, Mo Y, Fan C, Xiong F, Ren D, Ye X, Li C, Wang Y, Wei F, Guo C, Wu X, Li X, Li Y, Li G, Zeng Z, Xiong W. Circular RNAs function as ceRNAs to regulate and control human cancer progression. Mol Cancer 2018; 17: 79.

15) Cortés-López M, Miura P. Emerging functions of circular RNAs. Yale J Biol Med 2016; 89: 527-537.

16) Jiang G, Ma Y, An T, Pan Y, Mo F, Zhao D, Liu Y, Miao JN, Gu YJ, Wang Y, Gao SH. Relationships of circular RNA with diabetes and depression. Sci Rep 2017; 7: 7285.

17) Zhao Z, Li X, Jian D, Hao P, Rao L, Li M. Hsa_circ_0054633 in peripheral blood can be used as a diagnostic biomarker of pre-diabetes and type 2 diabetes mellitus. Acta Diabetol 2017; 54: 237-245.

18) Wang T, Pan W, Hu J, Zhang Z, Li G, Liang Y. Circular RNAs in metabolic diseases. Adv Exp Med Biol 2018; 1087: 275-285.

19) Xu H, Guo S, Li W, Yu P. The circular RNA Cdr1as, via miR-7 and its targets, regulates insulin transcription and secretion in islet cells. Sci Rep 2015; 5: 12453.

20) Wang A, Toma MA, Ma J, Li D, Vu M, Chu T, Wang J, Li X, Xu Landén N. Circular RNA hsa_circ_0084443 is upregulated in diabetic foot ulcer and modulates keratinocyte migration and proliferation. Adv Wound Care (New Rochelle) 2020; 9: 145-160.

21) He C, Zheng S, Luo Y, Wang B. Exosome theranostics: biology and translational medicine. Theranostics 2018; 8: 237-255.

22) Sun Z, Shi K, Yang S, Liu J, Zhou Q, Wang G, Song J, Li Z, Zhang Z, Yuan W. Effect of exosomal miRNA on cancer biology and clinical applications. Mol Cancer 2018; 17: 147.

23) Soares Martins T, Catita J. Exosome isolation from distinct biofluids using precipitation and column-based approaches. PLoS One 2018; 13: e0198820.

24) Zhang J, Li S, Li L, Li M, Guo C, Yao J, Mi S. Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics Proteomics Bioinformatics 2015; 13: 17-24.

25) Skotland T, Hessvik NP, Sandvig K, Llorente A. Exosomal lipid composition and the role of ether lipids and phosphoinositides in exosome biology. J Lipid Res 2019; 60: 9-18.

26) Mathieu M, Martin-Jaular L. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat Cell Biol 2019; 21: 9-17.

27) Liu Jing CH, Liu Xingzhou, Wu Yuxi, Lan Biyun, Mai Lifang, Yang Chuan, Yan Li, Ren Meng. Serum exosomal miR-3976 in patients with diabetic foot of different Wagner grades: expression and possible mechanism involved. Chin J Clinicians (Electronic Edition) 2019; 13: 401-408.

28) Wagner FW, Jr. The dysvascular foot: a system for diagnosis and treatment. Foot Ankle 1981; 2: 64-122.

29) Zhang P, Lu J, Jing Y, Tang S, Zhu D, Bi Y. Global epidemiology of diabetic foot ulceration: a systematic review and meta-analysis. Ann Med 2017; 49: 106-116.

30) Jiang S, Hu C, Liu P, Lu M. Tumor-derived exosomes in cancer metastasis risk diagnosis and metastasis therapy. Clin Transl Oncol 2019; 21: 152-159.

31) Sharma A, Khatun Z, Shiras A. Tumor exosomes: cellular postmen of cancer diagnosis and personalized therapy. Nanomedicine (Lond) 2016; 11: 421-437.

32) Castaño C, Novials A. Exosomes and diabetes. Diabetes Metab Res Rev 2019; 35: e3107.

33) Eleftheriadou I, Tentolouris A, Grigoropoulou P, Tsilingiris D, Anastasiou I, Kokkinos A, Perrea D, Katsilambros N, Tentolouris N. The association of diabetic microvascular and macrovascular disease with cutaneous circulation in patients with type 2 diabetes mellitus. J Diabetes Complications 2019; 33: 165-170.

34) Zubair M, Ahmad J. Transcutaneous oxygen pressure (TcPO(2)) and ulcer outcome in diabetic patients: Is there any correlation? Diabetes Metab Syndr 2019; 13: 953-958.