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# Examining the connection between small non-coding RNAs(sncRNAs)204-3p and the healing process of diabetic foot ulcers

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**Abstract**

**Background and aim:** the present study was conducted with the aim of determining the association of small non-coding RNA-204-3p with wound healing in diabetic foot ulcers.

**Method:** 100 patients with type 2 diabetes with diabetic foot ulcers between April 2022 and March 2024, who had a diabetic foot ulcers period of more than 4 weeks, were included in this study (DFUs group). 100 newly diagnosed type 2 diabetes patients (type 2 diabetes group) and 100 people were considered as control group. Quantitative PCR (qRT-PCR) method was used to determine the expression level of miR-204-3p in peripheral blood and wound margin tissue. SPSS Statistics 23 software was used for data analysis; All data values were reported as an average at a significance level of less than 0.05.

**Result:** The level of plasma miR-204-3p expression in the DFUs group and the T2DM group is significantly lower than the control group ($p<0.01$). a statistically significant difference was observed between miR-204-3p level and fasting plasma glucose and HbA1c in two DFUs and T2DM groups ($p<0.05$). a higher risk was associated with diabetes duration (OR: 4.8; 95% CI 1.2-9.9; $p<0.01$) and low plasma miR-204-3p expression (OR: 3.1; 95% CI 1.1-9.4; $p<0.01$).

**Conclusion:** miR-204-3p can be used as a biomarker to detect the onset and healing of diabetic foot ulcer in type 2 diabetic patients.

**Key words:** Wound healing, MicroRNAs, Healing process, type 2 Diabetes, Diabetic foot ulcers

## Introduction

Diabetes mellitus is one of the most common metabolic disorders in the world, which is caused by a role in the secretion or action of insulin or a combination of both factors(1). According to the International diabetes federation report in 2021, 537 million adults aged 20 to 79 years had diabetes and it is estimated that this amount will increase to 629 million people in 2045(2, 3). Diabetic foot ulcers (DFUs) are a major complication of diabetes mellitus and are associated with high morbidity, mortality, and associated costs(4). The estimated overall prevalence of DFUs was 53.2%(5).

Wound repair is a complex, dynamic and regular physiological process in which the skin repairs itself after injury and includes several steps, including inflammatory response, wound contraction and wound covering, re-epithelialization(6).

microRNAs are small regulatory molecules with a length of 18 to 25 nucleotides and are considered a central factor in post-transcriptional regulation in animals and plants(7). microRNAs bind to the untranslated region (3' UTRs) in the target molecules, i.e. mRNAs, and negatively regulate their expression by increasing the rate of degradation or inhibiting their translation(8). microRNAs exist in body tissues and fluids such as serum, blood, urea and saliva(9-11). These molecules are stable in the blood environment and are protected from RNAse activity and can be measured by methods such as real time PCR(12). Studies have shown that miRNAs play an important role in the development of the epidermis and maintaining its function(13-16). The study showed that the expression of miR-204 changes during wound healing, however, there is no evidence for the role of miR-204 in the regulation of wound healing(17). An in vitro study has also shown that inhibiting the expression of miR-204 can suppress the proliferation of rat corneal epithelial cells and promote apoptosis(18).

Evidence that miR-204-3p has a protective role in glucose-induced podocyte apoptosis and dysfunction has been recently published(19, 20). However, many studies have not been conducted to investigate the role of miR-204-3p on the healing of diabetic foot ulcers, and more studies are needed to confirm the evidence. Therefore, the present study was conducted with the aim of determining the association of small non-coding RNA-204-3p with wound healing in diabetic foot ulcers.

## Method

In the current study, 100 patients with type 2 diabetes with DFUs between April 2022 and March 2024, who had a period of DFUs for more than 4 weeks, were included in the study and

included in the first group (DFUs group). 100 newly diagnosed type 2 diabetes patients were included in the second group (T2DM group). 100 people as a control group, who were healthy people and underwent physical examination, were placed in the third group (control group). A written consent form was obtained from all participants in the study. If a person was not able to give consent due to low literacy, an informed consent form was obtained from his/her supervisor or first grade teachers. All participants were informed that they will receive all routine hospital treatment if they withdraw from the study.

***Inclusion criteria***

DFUs group:

1. the Ankle-brachial index of 0.8 to 1.3
2. Wagner classification in grade II-III
3. Wound area in 2 to 20 square centimeters

T2DM group:

1. One week to six months without DFUs
2. no diabetic peripheral neuropathy
3. no lower extremity atherosclerosis disease

Control group:

The results of 75 g oral glucose tolerance test (OGTT) were normal.

***Exclusion criteria***

1. None of the participants in all three groups have severe heart, liver or kidney disorders.
2. None of the participants in all three groups had cancerous wounds.

***collect wound tissue samples***

All 100 patients underwent wound debridement after hospitalization. During the debridement process, skilled surgeons performed the sampling and the complete skin tissue was cut at a distance of 0.5 cm from the wound margin according to the sampling protocol. All patients in this group received anti-infective, hypoglycemic, anti-hypertensive, neuroprotective treatment and hypoproteinemia was corrected. The wound was treated with negative pressure and drainage, and after eight weeks of treatment, complete healing of the wound was observed in these patients.

Venous blood was taken from all participants in the study in the fasting state from the elbow at 7 to 8 in the morning and the blood sample was collected in anti-coagulant tubes to determine fasting plasma glucose. HbA1c, WBC, CRP and ESR, lipid, glucose and blood lipid indices, miR-204-3p of plasma were analyzed using P800 biochemical analyzer made by Roche,

Switzerland. fasting blood sugar was measured using the glucose oxidase method; Total cholesterol and high concentration triglyceride, low-density lipoprotein and high-density lipoprotein were differentiated using oxidized enzyme-linked colorimetry. High pressure liquid chromatography was used to determine HbA1c, CRP level was evaluated by immunoturbidometric method and ESR was measured by Westergren method.

RPMI 1640, also known as RPMI medium, is a growth medium used in cell culture, which in the present study was used with 10% Sigma-Aldrich fetal bovine serum. Human keratinocytes were inoculated into a culture flask containing 10 ml medium and cultured in a humidified incubator at 37°C in an atmosphere of 5% CO2 and 95% air. It should be noted that the environment was changed every two to three days.

Human keratinocytes were simultaneously collected and treated with D-glucose. The obtained cells were randomly divided into five groups: Normal glucose group (D-glucose 5 mmol/L), high glucose (D-glucose 30 mmol/L), miR-204-3p overexpression, KLF6 expression-inhibition, miR-204-3p and KLF6 overexpression. GenePharma was used for the synthesis of Hsa-miR-204-3p lentivirus (miR-204-3p) and negative control lentivirus (miR-NC).

***Detection of miR-204-3p expression using real-time quantitative PCR assays***

PCR was used to detect the expression of miR-204-3p in wound margin tissue. According to the instructions of the miRcute extraction and isolation kit, RNA was obtained from peripheral blood, and RNA was extracted from wound tissue. The primer sequence of miR-204-3p is reported in Table 1.

Table 1. primer sequence of miR-204-3p

| miR-204-3p | forward primer | reverse primer |
|---|---|---|
| | 5′-AGC TGT ACA AGT AAG CCT GAT CAT GTA CCC ATA GG-3′ | **5′-GGG AGA GGG GCT TAG CTT ATG GGA CAG TTA TGG GC-3** |

***Data analysis***

SPSS Statistics 23 software was used for data analysis; All data values were reported as an average at a significance level of less than 0.05. The difference between the two groups was analyzed using the t-test, and the analysis of variance test was used for the three groups. Correlation was evaluated with Spearman's test. Multivariate unconditional logistic regression method was also used.

**Result**

In the comparison of the three groups, there was no statistically significant difference between the participants' age, gender distribution, diastolic and systolic blood pressure, low-density lipoprotein cholesterol and total cholesterol ($p>0.05$), while in terms of fasting plasma glucose, high-density in lipoprotein cholesterol and C-reactive protein there was a significant difference between the three groups ($p<0.01$). A significant difference was observed in the comparison of the duration of diabetes between DFUs group and T2DM group($p<0.01$). The mean of ulcer duration in DFUs group was 7.05±0.75 weeks and ulcer area was 10.50±0.59 $cm^2$ (Table 1). The level of plasma miR-204-3p expression in the DFUs group and the T2DM group is significantly lower than the control group ($p<0.01$) (Table 2).

Table 2. Demographic and clinical characteristics of patients

| **Variables** | **DFUs group n=100** | **T2DM group n=100** | **control group n=100** | **p-value** |
|---|---|---|---|---|
| **Age (mean±SD)** | 64.24±4.05 | 64.20±4.16 | 63.59±4.37 | 0.518 |
| **sex** | | | | |
| female | 34 (34.0%) | 34 (34.0%) | 33 (33.0%) | 0.985 |
| Male | 66 (66.0%) | 66 (66.0%) | 67 (67%) | |
| **diastolic blood pressure** | 7.31 ±0.56 | 7.31 ±0.52 | 7.28 ±0.49 | 0.915 |
| **systolic blood pressure** | 12.44±0.92 | 12.41±0.92 | 12.01±0.69 | 0.967 |
| **fasting plasma glucose (mmol/L)** | 9.2±0.49 | 8.3±0.42 | 7.4±0.59 | 0.000 |
| **LDL-C (mmol/L)** | 2.64±0.56 | 2.62±0.54 | 2.65±0.53 | 0.921 |

| | | | | |
|---|---|---|---|---|
| **HDL-C (mmol/L)** | 0.92±0.04 | 1.21±0.21 | 1.37±0.14 | 0.000 |
| **C-reactive protein** | 30.30±0.55 | 8.49±0.55 | 6.28±0.35 | 0.000 |
| **TCH (mmol/L)** | 4.4±0.26 | 4.4±0.25 | 4.4±0.24 | 0.922 |
| **duration of diabetes (Years)** | 13.50±2.24 | 0.51±0.24 | - | 0.00 |
| **HbA1c** | 8.01±0.78 | 7.89±0.93 | 5.6±1.19 | 0.000 |
| **Ulcer duration (week)** | 7.05±0.75 | | | |
| **Ulcer area (cm2)** | 10.50±0.59 | | | |
| **Plasma-miR-204-3p** | 1.14 (0.61-2.94) | 2.18 (1.08-5.01) | 3.11 (1.54-7.1) | 0.000 |
| **miR-204-3p expression in the wound margin tissue** | 2.13 (1.21-4.84) | - | - | - |

***Association between miR-204-3p expression level and wound margin tissue***

According to Table 3, a statistically significant difference was observed between miR-204-3p level and fasting plasma glucose and HbA1c in two DFUs and T2DM groups ($p<0.05$); The findings showed that there was no significant difference between miR-204-3p level and Ulcer duration and Ulcer area in DFUs group ($p>0.05$). In the control group, there was no significant difference between miR-204-3p level and fasting plasma glucose and HbA1c($p>0.05$).

Table 3. Association between miR-204-3p expression level and wound margin tissue

| **Variables** | **DFUs group n=100** | **T2DM group n=100** | **control group n=100** |
|---|---|---|---|
| **fasting plasma glucose (mmol/L)** | -0.36 p-value= 0.013 | -0.41 p-value= 0.043 | -0.184 p-value= 0.094 |
| **HbA1c** | -0.24 p-value= 0.001 | -0.31 p-value= 0.012 | -0.132 p-value= 0.183 |
| **Ulcer duration (week)** | -0.112 p-value= 0.14 | - | - |
| **Ulcer area (cm2)** | -0.102 | - | - |

| | |
|---|---|
| | p-value= 0.28 |

***Risk factors for DFU***

Based on the findings of the multivariate logistic regression test in diabetic patients with DFU, observed that low plasma miR-204-3p expression, HbA1c, diabetic duration and C-reactive protein are independent risk factors for DFU (Table 4); a higher risk was associated with diabetes duration (OR: 4.8; 95% CI 1.2-9.9; p<0.01) and low plasma miR-204-3p expression (OR: 3.1; 95% CI 1.1-9.4; p<0.01) (Table 3).

Table 4. Risk factors for DFU

| Variable | β | SE | Wald | OR | 95% CI | p-value |
|---|---|---|---|---|---|---|
| HbA1c | 0.44 | 0.20 | 3.15 | 1.9 | 1.1-6.9 | 0.004 |
| Plasma miR-204-3p expression | 0.34 | 0.13 | 5.13 | 3.1 | 1.1-9.4 | 0.007 |
| Diabetic duration (year) | 0.63 | 0.21 | 8.18 | 4.8 | 1.2-9.9 | 0.005 |
| C-reactive protein | 0.21 | 0.10 | 2.16 | 1.62 | 1.03-11.1 | 0.021 |

**Discussion**

In the present study, it was observed that in patients who were newly diagnosed with type 2 diabetes, the expression level of miR-204-3p in their peripheral blood was significantly lower than that of the control group, and on the other hand, in diabetic patients with DFU, the expression levels of miR -204-3p in peripheral blood was significantly lower than T2DM group and control group. The findings of the present study showed that the lower expression of miR-204-3p can be considered as an independent and important risk factor for DFU. Therefore, it can be stated that low levels of miR-204-3p are closely related to wound healing. As a result, decreased expression of miR-204-3p, apart from being a strong risk factor for the onset of DFU, can be considered as a potential biomarker for predicting diabetic foot ulcer healing. Consistent with the results of the present study, Zhao et al.,2023 showed that the expression level of peripheral blood miR-204-3p in the T2DM group was lower than the control group [2.38 (1.31-5.04) vs. 3.27 (1.51-6.98)] (P<0.05). Similarly, the peripheral blood miR-204-3p expression

level in the DFU group was significantly lower than that in the T2DM group [1.15 (0.78-2.89) vs. 2.38 (1.31-5.04)] ($P < 0.01$)(19). In the present study, it was observed that miR-204-3p expression level was negatively correlated with fasting plasma glucose, HbA1c levels in both T2DM and DFU groups. Based on this, the low expression of miR-204-3p in peripheral blood can be related to hyperglycemia. However, more studies are needed to explain these results and it has been reported in the study that increased blood sugar can effectively affect the expression of miR-24 and reduce its expression(21). It is suggested that studies investigate the relationship between the environment with high glucose and the change in the expression of miR-204-3p.

Based on the findings, the independent risk factors for the occurrence of diabetic foot ulcers were HbA1c, Diabetic duration and C-reactive protein, which previous studies have also confirmed these findings(19, 22, 23). Evidence has shown that the treatment of DFU in diabetic patients is more challenging and difficult compared to non-diabetic chronic skin wounds, and hyperglycemia is considered an unfavorable factor. Since the decrease in the expression of miR-204-3p can be related to hyperglycemia and any decrease in the expression of miR-204-3p can affect the occurrence and recovery of DFU, it is concluded that hyperglycemia can cause a harmful effect on DFU.

Few studies have been done to confirm the results of the present study, so more studies with a larger sample size are needed. According to our knowledge, few studies have investigated the relationship between miR-204-3p and diabetic foot ulcer healing. It is suggested that more laboratory studies be conducted in different geographical areas.

## Conclusion

Expression of low levels of miR-204-3p is an independent and significant risk factor for diabetic foot ulcers; miR-204-3p can be used as a biomarker to detect the onset and healing of diabetic foot ulcer in type 2 diabetic patients.

## References

1. Kumar R, Saha P, Kumar Y, Sahana S, Dubey A, Prakash O. A Review on Diabetes Mellitus: Type1 & Type2. World Journal of Pharmacy and Pharmaceutical Sciences. 2020;9(10):838-50.
2. Nanditha A, Chamukuttan S, Raghavan A, Ramachandran A. Global Epidemic of Type 2 Diabetes Mellitus: An Epidemiologist's Perspective. Current Trends in Diabetes. 2020:36.
3. Olatidoye OP. Diabetic Care Center and Nutrition/Dietetics in Nigeria. Medical Entrepreneurship: Springer; 2023. p. 287-310.
4. Armstrong DG, Tan T-W, Boulton AJ, Bus SA. Diabetic foot ulcers: a review. Jama. 2023;330(1):62-75.
5. Maldonado-Valer T, Pareja-Mujica LF, Corcuera-Ciudad R, Terry-Escalante FA, Chevarría-Arriaga MJ, Vasquez-Hassinger T, et al. Prevalence of diabetic foot at risk of ulcer development and its components stratification according to the international working group on the diabetic foot (IWGDF): A systematic review with metanalysis. PLoS One. 2023;18(11):e0284054.
6. Han S-K. Basics of wound healing. Innovations and Advances in Wound Healing: Springer; 2023. p. 1-42.
7. Ergin K, Çetinkaya R. Regulation of microRNAs. miRNomics: MicroRNA Biology and Computational Analysis. 2022:1-32.
8. Navarro E, Mallén A, Hueso M. Dynamic variations of 3′ UTR length reprogram the mRNA regulatory landscape. Biomedicines. 2021;9(11):1560.
9. Gayosso-Gómez LV, Ortiz-Quintero B. Circulating MicroRNAs in blood and other body fluids as biomarkers for diagnosis, prognosis, and therapy response in lung cancer. Diagnostics. 2021;11(3):421.
10. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA. MicroRNAs in body fluids—the mix of hormones and biomarkers. Nature reviews Clinical oncology. 2011;8(8):467-77.
11. Weber JA, Baxter DH, Zhang S, Huang DY, How Huang K, Jen Lee M, et al. The microRNA spectrum in 12 body fluids. Clinical chemistry. 2010;56(11):1733-41.
12. Etheridge A, Lee I, Hood L, Galas D, Wang K. Extracellular microRNA: a new source of biomarkers. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2011;717(1-2):85-90.
13. Andl T, Murchison EP, Liu F, Zhang Y, Yunta-Gonzalez M, Tobias JW, et al. The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. Current Biology. 2006;16(10):1041-9.
14. Yi R, Pasolli HA, Landthaler M, Hafner M, Ojo T, Sheridan R, et al. DGCR8-dependent microRNA biogenesis is essential for skin development. Proceedings of the National Academy of Sciences. 2009;106(2):498-502.
15. Lee A-Y. The role of microRNAs in epidermal barrier. International Journal of Molecular Sciences. 2020;21(16):5781.
16. Dopytalska K, Czaplicka A, Szymańska E, Walecka I. The Essential Role of microRNAs in Inflammatory and Autoimmune Skin Diseases—A Review. International Journal of Molecular Sciences. 2023;24(11):9130.
17. Etich J, Bergmeier V, Pitzler L, Brachvogel B. Identification of a reference gene for the quantification of mRNA and miRNA expression during skin wound healing. Connective Tissue Research. 2017;58(2):196-207.
18. Yu Sy, Dong B, Fang Zf, Hu Xq, Tang L, Zhou Sh. Knockdown of lnc RNA AK 139328 alleviates myocardial ischaemia/reperfusion injury in diabetic mice via modulating miR-204-3p and inhibiting autophagy. Journal of cellular and molecular medicine. 2018;22(10):4886-98.

19. Zhao X, Xu M, Tang Y, Xie D, Deng L, Chen M, et al. Decreased expression of miR-204-3p in peripheral blood and wound margin tissue associated with the onset and poor wound healing of diabetic foot ulcers. International Wound Journal. 2023;20(2):413-29.
20. Han X, Li Q, Wang C, Li Y. MicroRNA-204-3p attenuates high glucose-induced MPC5 podocytes apoptosis by targeting braykinin B2 receptor. Experimental and Clinical Endocrinology & Diabetes. 2019;127(06):387-95.
21. Xiang Y, Cheng J, Wang D, Hu X, Xie Y, Stitham J, et al. Hyperglycemia repression of miR-24 coordinately upregulates endothelial cell expression and secretion of von Willebrand factor. Blood, The Journal of the American Society of Hematology. 2015;125(22):3377-87.
22. Noor S, Zubair M, Ahmad J. Diabetic foot ulcer—a review on pathophysiology, classification and microbial etiology. Diabetes & Metabolic Syndrome: Clinical Research & Reviews. 2015;9(3):192-9.
23. Ladurner R, Küper M, Königsrainer I, Löb S, Wichmann D, Königsrainer A, et al. Predictive value of routine transcutaneous tissue oxygen tension (tcpO2) measurement for the risk of non-healing and amputation in diabetic foot ulcer patients with non-palpable pedal pulses. Medical Science Monitor. 2010;16(6):CR273-CR7.