

Original Article

Quantification of the differential expression levels of microRNA-203 in different degrees of diabetic foot

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Abstract: Background: As a common and important complication of diabetes, foot ulcers are characterized by high incidence, poor prognosis and variation in the clinical presentation. The current methods for classification of the diabetic foot are many, but few of them are validated owing to the lack of specific and accurate laboratory index. Thus, the development of new bio-markers to assess and manage diabetic foot is of high importance. Methods: 46 patients who had undergone wound repairing operation were included in this study and skin tissue samples around the ulcers were collected during the operation. In accordance with The Wound Score of Strauss, all subjects were divided into four groups, such as normal skins group, healthy wounds group, problem wounds group and futile wounds group, and respectively, there are 6, 4, 22, 14 individuals in each group. For each group, we assessed the expression profile of microRNAs (miRNAs) in each skin tissue sample by TaqMan probe-based qRT-PCR assay. Result: Skin-enriched microRNA-203 (miR-203) was readily detected in skin tissue samples, and, in contrast to normal skin tissue, samples from patient with diabetic foot ulcers significantly have a higher expression level in miR-203. Moreover, our study demonstrated the first time that expression profile of miR-203 was positively correlated with the severity of diabetic foot ulcers. Compared with other parameters in wound scoring systems for the assessment of severity of diabetic foot ulcers, the determination for miR-203 was more accurate and validated. Conclusion: Our results demonstrated that expression profile of miR-203 in diabetic foot had a positive correlation with the severity of diabetic foot ulcers, which indicated that miR-203 can be served as a new, accurate and validated bio-marker for evaluating the severity of diabetic foot ulcers in clinic. The significant finding of the study: Quantification of miR-203 in different degrees of diabetic foot. This study adds a new bio-marker for evaluation and management of diabetic foot.

Keywords: Foot Ulcer, diabetes, severity, evaluation, miR-203, bio-markers

Introduction

Foot ulcers, as a serious complication of diabetes mellitus, are characterized by its high morbidity, poor prognosis and variation in the clinical presentation. They not only have a significant impact on quality of life, but also bring a great burden on the family and the society. Until now, more than 10 different systems have been developed to classify diabetic foot ulcers for daily clinical practice. However, none has gained widespread acceptance because of the lack of specificity and accuracy. Consequently, it is difficult for the surgeon to evaluate and manage the diabetic foot ulcers.

miRNAs are ~22-nucleotide-long endogenously expressed non-coding RNAs that act as a new

class of gene regulators at the posttranscriptional level. Accumulating evidence demonstrates that miRNAs play pivotal roles in skin development and pathologies regulating a lot of different cellular processes, such as proliferation, differentiation, death and wound healing. Dysregulation of specific miRNA is critical in derailing the healing sequence in chronic problem wounds. Therefore, miRNAs are currently regarded as new potential diagnostic bio-markers and potential therapeutic targets by researchers worldwide [1-3]. Also, studies have demonstrated that miR-203 is the most abundant keratinocyte-specific miRNA in the epidermis [4] and plays a great role in keratinocyte functions in healthy and psoriasis-affected skin.

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Table 1. Comparison between control group and experiment group

Group	Sex		Age (years)	DD (months)	DDFU (months)
	F	M			
CG	3	3	38-62	-	-
EG	16	24	45-65	12-24	3-6

CG: Control group; EG: Experimental group; DD: Duration of diabetes mellitus; DDFU: Duration of diabetic foot ulcers.

In this study, we pose the hypothesis that the levels of specific skin miRNA species can be used to detect and monitor the pathological development associated with diabetic foot ulcers. Quantification of its differential expression by quantitative PCR with reverse transcription (qRT-PCR) in various different degrees of diabetic foot ulcers is used to develop the existence of correlation between the levels of miRNAs and various different degrees of diabetic foot ulcers. These findings are likely to contribute to a novel, special and accurate bio-marker for the evaluation and management of diabetic foot.

Materials and methods

Ethics statement

All subjects signed a consent form before surgery and gave written informed consent prior to study participation. The surgeries were reported to the medical department and the study was approved by the local ethics committee of the First Affiliated Hospital, Sun Yat-sen University.

Sample harvest and screening criteria

A total of 46 rectangles, full-thickness skin samples (about 2 cm²) on the edge of the wound were taken from the individuals who undergone wound repairing operation during the period from November 2013 to April 2014. Among them, 6 were normal and healthy skin tissues, and were performed as control group (CP); the left 40 were diagnosed as type 2 diabetes with foot ulcers according to the international standards, and were performed as experimental group. Each group characteristics were shown in **Table 1**. In accordance with The Wound Score of Strauss [5], the experiment group was divided into healthy wounds group (HW), problem wounds group (PW) and futile

wounds group (FW), and respectively, each group has 4, 22, 14 specimens. With the sequence group, diabetic foot ulcers were getting worse. After surgical removal, each sample was frozen in liquid nitrogen and stored at -70°C for further studies.

The inclusion criteria were as follows: patients with acute wound but no diabetes mellitus or patients with diagnoses of type 2 diabetes mellitus and diabetic foot ulcers.

The exclusion criteria were as follows: patients with type 1 diabetes mellitus and diabetic foot ulcers; patients with skin tumors and other skin diseases.

Quantitative RT-PCR

Total RNA was isolated from tissue samples with Trizol (Invitrogen) and RNA was reverse transcribed by M-MLV Reverse Transcriptase (Promega, WI, USA) according to the manufacturer's protocol. Sequence-specific primers for miR-203 Gene were, respectively, showed in **Table 2**. Real-time PCR was performed with GoTaq® qPCR Master Mix (Promega, WI, USA) on a MiniOpticon™ Real-Time PCR detection instrument (Bio-Rad) using the SyBr Green detection protocol as outlined by the manufacturer. Briefly, the amplification mixture consisted of 0.5 μM primers, 10 μl of GoTaq® qPCR Master Mix, and 1.5 μl template DNA in a total volume of 20 μl. Samples were amplified with the following program: initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation for 15 seconds at 95°C and annealing/elongation for 60 seconds at 60°C. All PCRs were run in triplicate, and control reactions without template were included. After the reactions, the cycle threshold (Ct) data were determined using default threshold settings, and the mean Ct was determined from the triplicate PCRs.

Statistically analysis

A comparative ΔCt method was used to compare each condition with controls, and values were expressed as 2ΔCt. The data shown are represented as tendency chart and means ± SD for at least three independent experiments. The collected data were analyzed by using GraphPad Prism 6.

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Table 2. Sequence of primers for miR-203 gene

hsa-U6-F	CTCGCTTCGGCAGCACA
hsa-U6-R	AACGCTTCACGAATTTGCGT
hsa-mir203-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACCTAGTG
hsa-mir203-F	GCGGTGAAATGTTTAGGAC

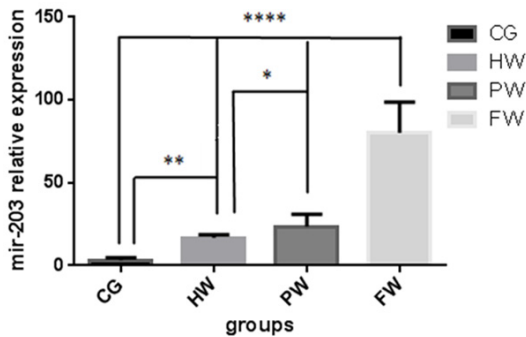


Figure 1. Alteration of the skin-specific miR-203 levels in each group. The skin-specific miR-203 levels in each group were measured using TaqMan probe-based qRT-PCR assay. The difference between groups was analyzed using GraphPad Prism 6. Data was presented as mean \pm SEM. * P <0.05; ** P <0.01; **** P <0.0001.

Results

Relative expression of miR-203 in CG, HW, PW, FW group were assessed as 3.47 ± 2.89 , 16.68 ± 4.63 , 24.01 ± 4.35 , 80.25 ± 6.02 . Compared with the normal skins group, individuals with diabetic foot ulcers had a 4.81- to 23.11-fold more expression of miR-203 in skin tissue. The highest level of miR-203 was the futile wounds group; the level of miR-203 in problem wounds group was higher than that in healthy wounds group; the least was the normal skins group. These dates were also analyzed by using GraphPad Prism 6. Consequently, we can obtain the changing trend chart (**Figure 1**) of the expression profile of miR-203 with the degree of diabetic foot ulcers. Differences between groups were statistically significant (**** P <0.0001, ** P <0.01 or * P <0.05). The results suggest that with foot ulcers getting more serious, the expression profile of miR-203 in skin was higher.

Discussion

Foot ulcers are a serious complication of diabetes mellitus that are associated with high morbidity, poor prognosis and variation in the clinical

presentation. They not only have a significant impact on quality of life, but also bring a great burden on the family and the society. Currently, to evaluate

accurately is considered as the first step in treating this kind of chronic wounds. Classification systems for diabetic foot ulcers not only help to assess the wound, but also can be used to predict the outcome of diabetic foot ulcer in the early stage, dynamically monitor the changes in the wound, and rationally direct the filler therapeutic schedule. Until now, more than 10 different systems have been developed to evaluate diabetic foot ulcers for daily clinical practice, but none has gained widespread acceptance. The Wound Score of Strauss [5] was popularized widely at present because of its simplicity for clinical application and its validity for wound studies. 10 criteria which judged to be the most important and effective were included in the system. They were as follows: management, including objectivity, versatility, ability to measure progress, validity and reliability, and appearance of the wound base, size, depth, bio-burden and perfusion. The last five was used as grading parameters that were selected for their usefulness in describing the wound and helping with making of treatment decisions. Each assessment was graded on a 0 to 2 scale and the goal was summated to generate the Wound Score. Healthy wounds scores 8 to 10. Problem wounds grades 4 to 7. Futile wounds are defined as with scores of 0 to 3. From the score three levels of seriousness become apparent. This method meets the requirements of being easy to use and quick to obtain, and was adapted to clinical activities. Those criteria were considered as a paradigm for wound evaluation [6]. However, presentation of diabetic foot ulcers in clinic was so various, but grading criteria are highly subjective. At present, because of the lack of a specific and accurate index for clinical practice, we fail to make intervention for preventing and healing in the early. It is of great importance for us to develop a new, direct, and accurate laboratory index.

miRNAs are ~22-nucleotide-long endogenously expressed non-coding RNAs that regulate the expression of gene products by inhibition of

translation and/or transcription in animals. They play important role in diverse physiological and pathological processes, and are linked to a variety of diseases [7, 8]. Recent evidence suggests that dysregulation of specific miRNA is critical in derailing the healing sequence in chronic problem wounds. Studies have shown that some specific miRNAs play pivotal roles in several developmental and physiological processes, especially in skin development and the changes of morphology and function [9-11]. Through studies in mice with null *Dicer* or *dgcr8* keratinocytes, Teta et al. and Yeo et al. have revealed depletion of epidermal stem cell, skin barrier dysfunction, disorder of hair growth and development, and hyperplasia of basal follicular keratin cells [12, 13]. Also, several miRNAs, such as miR-203, miR-21, miR-146a/b, and miR-34, participate in regulation of cell differentiation and proliferation in skin development [3, 14-16]. Further studying has shown that miR-203 is the most abundant keratinocyte-specific miRNA in the epidermis and plays vital role in cell differentiation and proliferation. MiR-203 is induced in the skin concomitantly with stratification and differentiation [17]. Overexpression of miR-203 in the basal layer makes cells lose the proliferative capacity earlier and initiate cell-cycle existence. On the contrary, Depletion or knockout of miR-203 can regulate the transition of keratinocytes from the basal layer to the upper layers. Expression of miR-203 is upregulated in patients with chronic inflammatory skin disease such as psoriasis and atopic eczema, which indicates miR-203 plays a role in regulating inflammatory reaction, cell apoptosis and protease activity [18-20]. Otherwise, miR-203 was expressed during cells differentiating and was described as a 'stemness' repressor and an indirect promoter of differentiation process [21, 22]. Therefore, miR-203 have been confirmed that acts at least in part by targeting and negatively regulating suprabasal expression of basal genes, thereby acting as a switch between proliferation and differentiation. However, miR-203 is absent in proliferating and migrating keratinocytes at the edge of a wound, while being heavily detectable in the wound's surrounding [24]. Reasonably, we can put forward hypothesis that dysregulation of this specific miRNA is critical in derailing the healing sequence in chronic problem wounds. In our study, we have the first time that expression

profile of miR-203 was positively correlated with the severity of diabetic foot ulcers.

In conclusion, our results have suggested a new, accurate and validated laboratory index to evaluate and monitor diabetic foot ulcers dynamically. These findings can contribute to make early diagnosis for the severity of diabetic foot ulcers in clinic, so as to take measures for the early prevention, the prompt treatment. Moreover, there are reasons to hypothesize that miR-203 may play an important role in the impairment in diabetic wound healing. At the next step, we will confirm our conjecture in the animal level and do the research mechanism on cell and molecular level.

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Disclosure of conflict of interest

None.

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