MiR-503 promotes wound healing of diabetic foot ulcer by targeting FBN1

Ming-Li Wang¹, Jing Chen², Yue Zhou², Yu-Jie Zhao², De-Rong Sun², Qiang Wu³, Chang-Long Bi²

¹Department of Endocrinology, the Fourth Affiliated Hospital of Harbin Medical University, Harbin, China
²Comprehensive Second Department, the Fourth Affiliated Hospital of Harbin Medical University, Harbin, China
³Laboratory of Tropical Biomedicine and Biotechnology, School of Tropical Medicine and Laboratory Medicine, Hainan Medical University, Haikou, China

Objective: To highlight the relationship between miR-503 and wound healing of diabetic foot ulcer (DFU).

Methods: Microarray analysis was used to detect the dysregulated miRNAs between the DFU tissues and normal tissues. The expression of miR-503 in tissues and serum of patients with DFU was detected by qRT-PCR technique. Then, CCK-8 assay was applied to determine the cell proliferation. TUNEL assay was used for assessing the apoptosis of cells after treatment with miR-503. Possible correlation between miR-503 and fibillin1 (FBN1) was predicted according to data accessed on RNA22 website online, and was detected for confirmation by luciferase reporter assay.

Results: Microarray analysis showed that miR-503 was significantly decreased in the DFU tissues compared with normal tissues. While marked increase in the expression of miR-503 in tissues and serum of patients with DFU was confirmed by qRT-PCR technique. Then, CCK-8 assay indicated that transfection of miR-503 mimic obviously accelerated the cell proliferation. However, TUNEL assays suggested that miR-503 mimic inhibited the apoptosis of cells to improve the survival of fibroblasts. Besides, miR-503 AMO played a role in fibroblasts of DFU tissues exactly countering to miR-503 mimic treatment. It was predicted that MiR-503 is a complementary to the FBN1 by RNA22. Besides, SiRNA-FBN1 promoted the proliferation, but brought down the apoptosis of fibroblasts.

Conclusions: MiR-503 regulates the function of fibroblasts and wound healing of patients with DFU by targeting FBN1 directly which provides a novel and critical target for diagnosis and treatment of DFU.

1. Introduction

Diabetes mellitus is one of the universal and complex metabolic diseases characterized by dysfunctional glucose control and other syndrome of metabolism disturbance. Diabetes is accompanied by various types of side effects, which can affect the metabolism, immunity, urinary system, nervous system, vision and restorative function of patients during their whole lifetime. The number of adult patients with diabetes mellitus accounts for 9.4% in the American population on the basis of the statistical data [1]. According to
the statistics, the government investment on diabetes mellitus healthcare was more than 548 billion dollars in 2013[2]. Recently, the significant increase in the prevalence of diabetes mellitus leads to reduction in quality of life, serious financial burden and heavy social pressure of patients all over the world. Among the collective comorbidities, diabetic foot ulcers (DFUs) are the most prevalent and painful complications of patients afflicted with diabetes mellitus. In particular, DFU eventually leads to huge pain, chronic wound and repeated infection, even nontraumatic amputation and considerable mortality, which is devastating and fearful[3]. It is estimated that of those patients who are suffering from diabetes mellitus, about 25% are prone to develop DFU which is regarded as a severe worldwide problem and challenge. Current clinical therapies of DFU contain conventional medicine, physiatrics and operative treatments, which need a long period of hospitalization, high expense and considerable care, but it is not easy for the wound to recover thoroughly[4]. Nowadays, growing numbers of researchers focus on the fundamental research about the treatment, prevention and diagnosis of DFU and the mechanism of DFU wound healing requires high attention.

Recent reports have demonstrated that several genes play a vital role in the occurrence and development of wound healing in patients with DFU. For example, it is reported that vascular endothelial growth factor exhibited the ability to promote diabetic wound healing and improve wound closure in mouse models and vascular endothelial growth factor can be applied as a dressing for treatment of DFU[5]. Recent study has shown that hemoglobin A1c is closely associated with DFU and hemoglobin A1c can be used as a biomarker for predicting the wound healing time and denouement of DFU[6].

MicroRNAs (miRNAs) are a type of small single-strand noncoding RNAs which have the capacity of regulating gene expression and other biological function by either degrading mRNA or inhibiting translation. MiRNAs have been reported to serve a significant role in proliferation, apoptosis, growth, senescence, differentiation, invasion of cells and participant in numerous diseases, including multiple cancer, osteoporosis, hypertension, stroke, etc[7,8]. However, whether microRNA is involved in the process of wound healing of DFU is little explored at present. With this in mind, we identified the dysregulated miRNAs between the diabetic patients with DFU and healthy controls and investigated whether miRNA is connected with wound healing of DFU. In our study, we analyzed the reported microarray results which showed that miR-503 is visibly decreased in the tissues from patients with DFU and we continued to explore the function and mechanism of miR-503 in wound healing of DFU. The objective of this research is to confirm the relationship between miR-503 and wound healing of DFU and improve our understanding of DFU and wound healing. Our results suggest that miR-503 influences wound healing of diabetic patient with DFU by targeting fibillin1 (FBN1) gene. And this study provides a novel target and critical biomarker for treating DFU in clinical.

2. Materials and methods

2.1. Protocol approval

All the procedures in our study were approved by the ethics committee of our hospital. All the experiments have been carried out according to the research committee guidelines.

2.2. Participants and tissue collection

DFU tissue samples were obtained from 10 diabetic foot ulcers patients who received treatment in our hospital from January 2015 to July 2017 and agreed to participate. Altogether, five males and five females were enrolled with a mean age of (60.0±5.5) years. They were all accurately diagnosed with DFU according to the WHO recommendations and DFU was the leading cause of the hospitalization. The researcher took the tissues at the border of DFU by 4 mm biopsy punch. Besides, normal samples were collected from 10 age-matched healthy volunteers [5 males and 5 females; mean age, (61.0±6.0) years] who received reconstructive skin surgery at our hospital during the corresponding period. We also took the normal tissues from edge of wound area using biopsy punch. Tissues were treated with RNALater (Vazyme, China) and stored in −80°C refrigerator for further analysis. DFU tissues were regarded as the disease group, while normal samples were the control group. Each participant experienced oral glucose tolerance tests to quantify the diabetes status and diagnosis of diabetes mellitus was identified by two or more doctors.

Patients who suffered from dementia problems, mental problems, systemic musculoskeletal diseases, cardiovascular diseases, neurological disorders, skin tumor and other skin diseases were excluded from this study. In addition, these subjects have not taken toxic medicine, herbal medicine and were not receiving wound care, surgical operation or other treatments. All participants have written informed consent and participated in this study willingly.

2.3. Serum collection

We collected the blood in clinic in accordance with the previous study. A total of 15 mL blood samples of 21 patients with DFU and 20 healthy volunteers were collected in our hospital. The serum was stored in a −80°C refrigerator after centrifugal for further studies. The expression of miR-503 and FBN1 gene in serum from diabetic patients and control were detected by qRT-PCR analysis. Informed consents were written by all donors for the collection of blood samples willingly.

2.4. qRT–PCR

Total RNA was extracted from tissues samples by TRizol reagents (Invitrogen, USA), while miRNAs were isolated using miRNA isolation kit (Thermo, USA), chloroform (Tianjin Fine Chemical Company, China), isopropanol (Tianjin Fine Chemical Company, China) and anhydrous ethanol reagents (Tianjin Fine Chemical Company, China).
Chemical Company, China). After isolation, RNA was purified by RNase-free Dnase (Promega, USA). To quantify the level of gene transcription, the concentration and purity were measured by Nono Drop machine 8000 (Thermo, USA). Next, purified RNA samples were used to generate cDNA using ABI reverse transcription kit (ABI, USA) and double distilled water. Subsequently, the cDNAs were used for qRT-PCR using SYBR Green Master Mix (Roche, Switzerland) and specific primers according to the protocol. PCR procedures were denaturation at 95 °C for 2 min, then 30 cycles of 94 °C for 45 sec, 55 °C for 55 sec and 72 °C for 1 min, and extension at 72 °C for 10 min. The primers of miR-503 and FBN1 were designed and synthesized from GeenPharma company. The expression levels of miR-503 and FBN1 were detected by qRT-PCR. These experiments were repeated at least three times independently. The relative level of miR-503 and FBN1 was determined and calculated using the 2^-ΔΔCt comparative threshold method with 18S and GAPDH as internal reference respectively.

2.5. Cell isolation and culture

Skin fibroblasts were obtained from diabetic rats with DFU by a skinner. Skin fibroblasts were extracted from the full skin of the edge of ulcer wound as described in a previous study. First, the skin were dissected from tissues and cut into small pieces by an ophthalmic scissor. Subsequently, the skin was washed by PBS for 5 times and continued to be digested with trypsin (Beyotime, China) for 1 h with rapid shaking by hand. The fibroblasts were cultured in a 25 cm² culture flask with DMEM medium to be allowed to adhere overnight (Thermo, USA) containing 100 U/mL penicillin-streptomycin (Thermo, USA), 10% FBS (Sigma-Aldrich, USA) and maintained in a humidified incubator containing 5% CO₂ and 95% air at 37 °C. These cells were regarded as passage 0 and the medium was replaced every 4 d to remove non-adherent cells. After the cells reached 80%-90%, they were treated with 2 mL trypsin per time and subcultured by standard techniques. The cells were used at passages 5 to 7 in our experiments.

2.6. Transfection

For miRNAs, the cells were seeded in 24-well plates (Corning, USA) with normal culture medium and maintained for 24 h before experiments. Once the cells reached 50%-60% confluence, transfection was performed using X-treme transfection reagents (Vazyme, China) referring to the protocol. 50 nM of miR-503 mimic, 100 nM of miR-503 AMO, their corresponding negative control and optimal medium without FBS were used for transfecting the cells. The medium was changed after transfection for 6 h to improve the transfection efficiency. After 48 h transfection, the cells were harvested for additional experiments. We quantified the expression level of miR-503 of cells pretreated with miR-503 mimic and miR-503 AMO by qRT-PCR analysis.

To observe the effects of FBN1 overexpression on the skin fibroblasts, FBN1 overexpression plasmid vector was conducted. For FBN1 plasmid, the plasmid was conducted and obtained from GeenPharma company. When the cells were grown to 40%-50%, 0.5 ng plasmid was transfected into the cells using transfection reagents (Qiagen, Germany) and medium without FBS in 24-well plates and cultured for 48 h.

To investigate the role of knockdown FBN1, we obtained siRNA-FBN1 from GeenPharma to decrease the expression of FBN1 in fibrocytes. SiRNA-FBN and its negative control were used for transfection at a final concentration of 50 nM. The cells were used for further experiments after 48 h transfection.

2.7. CCK–8 assay

The proliferation ability of fibroblasts was measured using CCK-8 assay. The cells were cultured in 96-well plates and transfected with miR-503 inhibitor, miR-503 mimic and negative control for 24 h. The cells were incubated in 100 µL DMEM without FBS and 10 µL CCK-8 solution for 1 h to 4 h at a 37 °C incubator in the dark. The absorbance values were detected one per hour by a microplate reader (TECAN, Switzerland) to quantify the proliferation of cells per group.

2.8. TUNEL

The number of apoptotic cells was quantified by TUNEL Cell Death Detection Kit (Roche, Switzerland) corresponding to the standard procedures. At first, fibroblasts were harvested after transfection 48 h and rinsed with PBS for three times gently. Then, cells were fixed with 4% PFA and incubated with TUNEL reaction solution (viaL1:viaL2=1:9) for about 60 min in a dark place and the nuclei was counter-stained by DAPI solution (Solarbio, China) for 30 min at room temperature. The apoptotic cells were determined by the ratio of TUNEL-positive cells in total cells. Ten pictures at random were taken under a microscope (Olympus, Japan).

2.9. Luciferase reporter assay

The relationship between miR-503 and FBN1 was monitored using luciferase reporter assay. The 3′-UTRs of FBN1 were designed and synthesized by GeenPharma company. The wildtype and mutant 3′-UTRs of FBN1 were amplified and then inserted and cloned into the pMIR-reporter vector respectively, which was named FBN1-WT and FBN1-MUT. Bone marrow cells were co-transfected with FBN1-WT plasmid and miR-503, or FBN1-MUT plasmid and miR-503. After transfection of 24 h, these transfected cells were harvested and luciferase reporter assay was performed using the dual luciferase reporter kit (Promega, USA) to detect the relative luciferase activity.

2.10. Statistic analysis

All data were analyzed by SPSS software. Data were presented
as the Mean±Standard deviation. Comparisons between three or more groups were analyzed using ANOVA analysis, while contrast between two groups were performed by the t test. P<0.05, P<0.01 and P<0.001 indicated that the values were considered statistically significant.

3. Result

3.1. MiR–503 is markedly decreased in patients with DFU

Screening and analysis of GSE68185 microarray data showed that miR-503 was significantly decreased in the DFU tissues compared with normal tissues (Figure 1). We further determined the mRNA expression level of miR-503 in the DFU tissues by qRT-PCR. The analysis indicated that the mRNA level of miR-503 was also reduced in tissues from patients with DFU (Table 1). In order to confirm these results, we collected the serum of diabetic patients and normal volunteers. QRT-PCR analysis demonstrated that the level of miR-503 was decreased in the tissues and serum of patients with DFU. We further investigated whether miR-503 was closely correlated with DFU.

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative miR-503 expression level</th>
<th>Absorbance (λ =450 nm)</th>
<th>Apoptotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFU</td>
<td>0.285±0.100</td>
<td>0.1775±0.0950</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.030±0.160</td>
<td>1.0050±0.0600</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.0002</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

3.2. The effect of miR–503 on the skin fibroblasts isolated from DFU tissues

To further study the role of miR-503 in DFU, we extracted skin fibroblasts from the tissues of diabetic rats with DFU. First of all, we transfected fibroblasts with miR-503 mimic, miR-503 AMO and their corresponding negative control for 48 h. QRT-PCR results indicated that miR-503 was up-regulated after transfection of miR-503 successfully (Table 2). The proliferation ability of fibroblast was examined by CCK-8 assay. As shown in Table 2, CCK-8 assay confirmed that the miR-503 mimic promoted the growth of fibroblasts (Table 2). Then, the apoptosis of cells pretreated with miR-503 mimics was assessed by TUNEL staining. TUNEL staining demonstrated that the number of apoptotic fibroblasts was obviously reduced by miR-503 mimic (Table 2). These results suggested that miR-503 could promote the cell proliferation, but inhibited the apoptosis of cells to accelerate wound healing of DFU.

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<tbody>
<tr>
<td>miR-503 mimic</td>
<td>38.50±9.86*</td>
<td>0.975±0.150</td>
<td>3.50±1.12*</td>
</tr>
<tr>
<td>miR-503 AMO</td>
<td>0.35±0.11*</td>
<td>0.23±0.08*</td>
<td>30.00±4.12*</td>
</tr>
<tr>
<td>Negative control</td>
<td>1.03±0.13</td>
<td>0.450±0.110</td>
<td>16.00±4.06</td>
</tr>
</tbody>
</table>

3.3. MiR–503 AMO regulated the proliferation, apoptosis and invasion of fibroblasts

To further study whether miR-503 AMO affected the proliferation, apoptosis and invasion of fibroblast, we treated fibroblasts with miR-503 AMO and negative control respectively. The results revealed that the expression level of miR-503 was significantly decreased after transfection of miR-503 AMO (Table 3). In addition, CCK-8 assay suggested that miR-503 AMO suppressed the proliferation of fibroblasts. As shown in Table 3, miR-503 AMO accelerated the apoptosis of cells. Taken together, miR-503 has the capacity to regulate the proliferation and apoptosis of fibroblasts to affect wound healing.

<table>
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</table>

3.4. The effects of FBN1 on the fibroblasts from DFU tissues

To explore the mechanism that miR-503 regulates the wound healing, we used RNA22 database to identify the targets of miR-503. Among the candidate genes, FBN1, the key sector of elastin-associated microfibrils, encodes fibrin gene and is associated with elastic fiber sedimentation and cytokine regulatory roles[9,10]. FBN1 spreads over the connective tissues of the whole body and makes up the critical architectural part in extracellular microfibril regulatory networks[11-13]. However, whether FBN1 is involved in
DFU remains unclear. Therefore, we hypothesized that miR-503 may regulate wound healing by targeting FBN1.

First, prediction in RNA22 website showed that miR-503 has the binding sites with FBN1 (Figure 2). Luciferase reporter analysis predicted that overexpression of miR-503 decreased the luciferase activity of FBN1 3'-UTR reporter gene, while other group exhibited no changes (Table 4). Furthermore, we explored the level of FBN1 in the tissues and serum of patients with DFU. QRT-PCR analysis displayed that the expression of FBN1 notably increased in patients with DFU compared with healthy control (Table 4). Next, the effects of FBN1 plasmid on the proliferation, and apoptosis of fibroblasts were detected (Table 4). Taken together, FBN1 suppressed the proliferation and promoted the apoptosis of fibroblasts from diabetic with DFU.

3.5. SiRNA-FBN1 regulates the function of fibroblasts to affect the wound healing of DFU tissues

We also continued to study whether siRNA-FBN1 could regulate the proliferation and apoptosis of fibroblasts. The results demonstrated that siRNA-FBN1 promoted the proliferation, but brought down the apoptosis of fibroblasts (Table 5). Collectively, these results suggested that miR-503 regulate the function of fibroblast of patients with DFU by targeting FBN1 directly.

4. Discussion

As reported in the International Diabetes Federation, so far, 415 million patients worldwide (aging from 20 to 80 years old) are suffering the pain brought from diabetes mellitus, accounting for 8.8% of the total population[14]. Such a huge population with diabetes mellitus draws the attention of researchers and doctors. Other than the problems caused by diabetes mellitus, the side effects and complications of diabetes mellitus are terrible and serious. Among these complications, DFU is the most prevalent and common syndrome, which can even lead to amputation. Up to now, the basic foundation about the mechanism of DFU is little. Therefore, the aim of our study is to investigate the molecular mechanism about wound healing of patients with DFU.

Our bioinformatics analysis has chosen the dysregulated miRNAs between DFU tissues and normal tissues, and miR-503 was the most down-regulated genes in the DFU tissues. According to the previous reports, miR-503 is connected with cancer, nervous system disease, digestive system disease, infectious disease, etc[15-22]. At present, there was no report on the relationship between miR-503 and DFU. We also found that miR-503 was reduced in the tissues and serum of diabetic patients. This provided an important biomarker in the tissue and serum of diagnosis of diabetic patients with DFU. Our further experiments made clear that miR-503 can regulate the growth and apoptosis of fibroblasts. To further study how miR-503 regulated the wound healing of DFU tissues, we predicted the target genes of miR-503. It is reported in the previous study, mutations of FBN1 gene could lead to the occurrence of Marfan syndrome, a genetic disorder that affects connective tissue[23]. In the treatment of DFU, stably-coated EGF and basic fibroblast growth factor loaded onto a biocompatible hyaluronate-collagen dressing can accelerate wound healing. It is unclear whether the growth factor was regulated by the FBN1 gene and FBN1 can affect the DFU. Bioinformatic analysis and luciferae reporter assay showed that there is a negative regulation between miR-503 and FBN1. Besides, we found the expression level was notably increased in the tissues and serum of diabetic patients. In addition, FBN1 was related to the proliferation and apoptosis of fibroblasts.

In conclusion, our findings indicated that miR-503 and FBN1 regulate the wound healing. Our results found miR-503 was significantly increased in the serum and tissues of patients with DFU. These results provide a key and crucial molecular target for diagnosis and treatment of diabetic patients with DFU. This can improve the treatment of wound healing of DFU and its accompanying complications in clinical.

Conflicts of interest statement

We declare that we have no conflict of interest.
References


