miR-15a-5p up-regulates TLR4 and induces the formation of hypertrophic scars and keloids

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ABSTRACT

The formation of hypertrophic scar and keloid is considered to be a very complex pathological process. Our previous studies have shown that mir-15a-5p is an important miRNA in HTS tissues, and its expression level is significantly increased. Therefore, the potential mechanism of action of miR-15a-5p in scarring arouses our interest. This study preliminarily investigated the expression level of miR-15a-5p in HTS tissues and normal skin tissue and further explored the molecular mechanism. The results of this study once again confirmed that the expression level of miR-15a-5p was increased in HTS tissues and cells, and the closely related mRNA and protein levels of MyD88 and TGF-β were also highly expressed. The relative expression levels of fibrosis-related indicators in HTsFb cells were up-regulated, such as collagen-I, collagen-III and α-SMA. We constructed the HTS cell model and BALB/c nude animal model, and down-regulating miR-15a-5p, the HTsFb cells proliferation was inhibited, and qRT-PCR results showed that the fibrosis index mRNA was also reduced, and significantly reduce the pathological state of scar tissue. In conclusion, miR-15a-5p may participate in the formation and development of HTS through TLR/MyD88 signaling pathway and TGF-β1 signaling pathway.

Introduction

The process of cutaneous wound healing is believed to be regulated by many factors, and the deposition and degradation of extracellular matrix (ECM) have an important effect on the healing outcome. When abnormal matrix deposition occurs, abnormal ECM accumulates at the wound site, resulting in keloid and hyperplastic scars (HCS) (1,2). Ghahary found significant increases in the expression of ECM components such as collagen-I, collagen-III, and fibronectin in scar tissue (3). In fibroblasts, the expression level and activity of collagenase were significantly lower than normal cells (4). Recently, more and more scholars have proposed specific methods to reduce scar formation, which can inhibit matrix deposition through the topical application of targeted inhibitors to the wound (5-7).

Hypertrophic scars (HTS) are considered to be complications caused by the poor prognosis of burns or wounds, characterized by local redness, itching, sclerosis and contracture, resulting in impaired joint motion, repeated scar rupture and even cancer (3,8). At present, the pathological mechanism of HTS formation has been elaborated by some scholars. Fibroblasts abnormal proliferation plays an important role, especially myoblast fibroblasts transformed by these cells, which are considered to be an important component of hypertrophic scar (3,4). With the deepening of research, many hypotheses have been proposed, but they still need to be supplemented for the pathogenesis of HTS, which has important clinical significance.

In the phenomenon of life, epigenetics is a universal regulation of gene expression, the most important feature is not to change the DNA sequence, but can produce genetic changes. Such as methylation, hydroxylation, histone modification, chromosome remodeling, and non-coding RNA (5). MicroRNAs (miRNAs) are a class of non-coding single-stranded RNA molecules encoded by endogenous genes that are about 22 nucleotides in length. Initially, it was widely believed that miRNA had no biological function, but in recent years, miRNA has gradually become a research hotspot with the research of various biological processes such as epigenetics, transcription process and post-transcriptional gene expression, chromosome silencing and nuclear transmission, etc. (6-8). However, the important role of miRNA in the process of HTS and fibrosis has aroused our interest and attention (9-11). Our previous study found that the expression level of miR-15a-5p was significantly increased in hypertrophic scar tissue, which was the result of bioinformatics analysis of public databases. At present, studies have focused on the role of miR-15a-5p in adenovirus infection, placental invasion, and osteoporosis (12-14), but its main biological effects in the process of scar formation need to be supplemented.

Toll-like receptors (TLRs), located on the surface of various cell membranes, are transmembrane proteins involved in non-specific immune processes, including lipopolysaccharides (LPS), bacterial lipoproteins, hyaluronic acids, heat shock proteins, etc. (15). Among them, TLR4 can recognize the components involved in fibrosis forma-
tion, such as fibridenin, hyaluronic acid, heat shock protein 60 (HSP60), HSP70 and high mobility group protein B1 (HMGB1) (16-18). Some researchers regulate the expression level of BAMBI family members by stimulating TLR4 on hepatic stellate cell membranes. BAMBI family members have a similar structure to the extracellular region of transforming growth factor-β1 (TGF-β1) receptor, thus activating TGF-β1 and promoting the formation of liver fibrosis (19).

For scleroderma patients, abnormal TLR4 in the damaged tissues activated the Smad signaling pathway, which acts on fibroblasts, increasing cell sensitivity, and increasing the effect of TGF-β1 on fibroblasts, resulting in increased collagen-I and collagen-III synthesis. (20). In the latest studies, up-regulation of TGF-β1 expression levels, connective tissue growth factor (CTGF) and collagen can effectively activate TLR4, and TLR4 expression is significantly increased in hypertrophic scar tissue, which will accelerate the progression of HTS (21-26). This is consistent with our previous bioinformatics analysis results, and provides a reliable theoretical basis for us, to explore the biological effects of TLR4 in the course of TSH.

Our previous experiments have found that miR-15a-5p can bind to TLR4 in scar tissue, but the mechanism of action of both is still unclear, which is the main purpose of our study. First, in order to find differentially expressed genes in keloid tissue and hypertrophic keloid tissue, statistical difference analysis was performed between the expression profile data of these two parts of the data set and normal tissue. Subsequently, we will screen out meaningful differentially expressed genes in keloid tissues for correlation analysis, and explore the potential biological functions and the role of hsa-miR-15a-5p in the development of keloid scars.

Materials and Methods

Bioinformatics analysis of scar tissue and normal skin tissue

The miRNA and mRNA expression profile data analyzed in this study were downloaded from the GEO database, and the GSE7890 contained sequencing data of 3 keloid scar tissue and 3 normal samples. Dataset GSE151240 contained 14 hypertrophic scar tissue and 14 normal controls. Sequencing results from these two data sets were used in follow-up studies. R software was used for screening of all data after standardizing and was screened for differentially expressed genes (DEGs) between scar tissue and normal tissue. Setting the filter condition as follows: \[ | \log_{\text{FC}} | > 2, \text{the adjusted value} < p - 0.05 \] (27,28), and map the volcano figure. Thus, the heatmap package in R software performs correlation analysis of differential genes between miRNA and mRNA co-expression patterns. Finally, the clusterProfiler package was used for enrichment analysis.

Raising animals and cell proliferation

The mice used in the experiment were purchased from Beijing Life River Experimental Animal Technology Co., LTD. 48 BALB/c nude mice were selected as experimental animals in this study. The mice were aged at 6 weeks and weighed 21.5±1.5. The mice were randomly divided into six groups with three females and three males in each group. The mice were kept in a good environment, where they could freely move, eat, and drink, temperature of 24-26°C and relative humidity of 50%-60%. In addition, the cells used in this study were purchased from Pronosai Biotechnology Co., LTD. Normal human skin fibroblasts (NsFb) and Human hypertrophic scar fibroblast (HTSFb) were collected, and cultured at 37°C in an incubator containing 5% carbon dioxide for proliferation, requiring DMEM medium and 10% fetal bovine serum (FBS).

Plasmid transfection process

Lipofectamine 3000 (5 μL, Invitrogen) was used to transfect vectors (2.5 μg). All plasmids used in this study were aided by HonorGene Company in Changsha, China. When HTsFb cells grew well, they were spread in 6-well plates and given intervention measures. Low expression of miR-15a-5p: Short hairpin RNA (shRNA) targeting miR-15a-5p; Negative control: Negative control sequence (sh-NC). High expression of miR-15a-5p: transfected pcDNA3.1-miR-15a-5p vector; Negative control: transfected with pcDNA3.1-NC. High expression of TLR4: transfected pcDNA3.1-TLR4 vector; Negative control: pcDNA3.1-NC. High expression of MMP3: over-MMP3 transfection; Negative control: sh-NC. Low expression of MMP3: transfection sh-MMP3; Negative control: over-NC. Incubate for 48 h in the incubator.

Lentivirus transfection and mouse infection

Lentivector plLVX-IREStomo carrying sh-NC, sh-mir-15a-5p #1, sh-mir-15a-5p #2, pcDNA3.1-NC and pcDNA3.1-TLR4 was transfected into HTsFb. After 48 hours, virus particles were collected by ultracentrifugation and injected into BALB/c nude mice.

qRT-PCR experimental operation

The NsFb cells, HTsFb cells and BALB/c nude mice were collected. Trizol reagents are used to treat cells and tissues to extract total RNA. The RNA was then reverse-transcribed into cDNA using the kit. Finally, according to the kit instructions, the UltraSYBR mixture as a qRT-PCR reaction system, and the PCR procedure was run. The primer sequence is shown in Table 1. The test kits used are as follows: Thermo Fisher, no. 15596026; CWbiotech, no. CW2569; CWbiotech, no. CW2601.

Western blot (WB)

The NsFb cells and the HTsFb cells were collected, total proteins were extracted from cells using RIPA lysis buffer (Cell Signaling Technology, Boston, USA), and subsequent Western blot experiments were performed. Under the action of current, the total protein was separated by 15% SDS-PAGE adhesive according to protein molecular weight, and then the protein on the adhesive was slowly transferred to the PVDF membrane for the convenience of subsequent operation. After incubation with 5-10% BSA solution for 1-2 hours, the PVDF membrane was cut into many bands according to the molecular size and then incubated with primary antibodies for 2 hours. All primary antibodies were purchased from Abcam and were mixed with antibody diluent at 1:1000 and incubated with PVDF membranes. After washing, the secondary antibodies labeled with horseradish peroxidase (1:3,000, Abcam) were incubated for 1-2 hours. Finally, the blots were completed with ECL reagent and exposed to Amersham Imager 600.
Establishment of the scarred nude mouse model

The lesion tissues of clinical scar patients were collected and cut into 2.0cm × 1.5cm slices. Human scar tissue was implanted on the back of the BALB/c nude. The sutures were removed 15 days after surgery, and the scar tissue of the mice was examined. If tissue necrosis occurred, the mice were no longer included in the study. Subsequently, all eligible BALB/c nude mice were anesthetized by nasal inhalation of 3% isoflurane (tsbiochem, no. T19651). Finally, all the BALB/c nude mice were killed by intravenous injection of barbiturates (150 mg/kg).

H&E staining

Scar tissue was isolated from the focal tissue of the experimental BALB/c nude mouse. The following operations were performed. After a few hours of washing with PBS, the gradient alcohol was dehydrated at 70%, 80%, and 90%, respectively. Then ethanol and xylene were incubated for 15min and finally embedded with paraffin wax. The wax blocks were cut into 4μm tissue slices, and the nucleus and cytoplasm were colored with hematoxylin and eosin dye solution, respectively. Observe and preserve picture under a microscope. (Olympus, Tokyo, Japan).

Statistical analysis

For all data, at least three replicated measurements were taken. One-way ANOVA was then performed with GraphPad Prism 8 to compare differences in the results between multiple groups. Results were considered to be statistically significant for P-values < 0.05 were considered as.

Results

Screening of miR-15a-5p by bioinformatic analysis

The datasets GSE7890 and GSE151240 were analyzed by bioinformatics. Our analysis indicated that MMP3 showed a high expression tendency in keloid and hypertrophic scar tissue. In addition, 364 differential genes were screened, of which 285 genes were down-regulated, it was noteworthy that 79 genes, including TLR4, MyD88 and TGF, were up-regulated (Figure 1a-b). Subsequently, to continue exploring the interactions between these differential genes, we conducted a correlation analysis between miRNA and mRNA. The correlation coefficients between miR-15a-5p and TLR4, MyD88 were all >0.5 (Figure 1c-d). Finally, 79 genes with relatively high expression were selected for enrichment analysis. GO enrichment analysis results showed that mRNA was involved in secondary metabolite biosynthesis, melanin biosynthesis, melanin metabolism, extracellular matrix, extracellular matrix containing collagen, melanosomal membrane, extracellular matrix structural components, and heparin-bound extracellular matrix structural components. KEGG analysis showed that miRNA was mainly related to pathways in cancer, cytokine-cytokine receptor interaction signaling pathway and TNF signaling pathway (Figure 1e-f).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>F: TGGCACCCAGCACAAGGAA</td>
</tr>
<tr>
<td></td>
<td>R: CTAAGTCATAGTCCGCTAGAAGCA</td>
</tr>
<tr>
<td>U6</td>
<td>F: CATGATGACCCAGTGCCGACC</td>
</tr>
<tr>
<td></td>
<td>R: TCTTCTCGTCGTACGCAC</td>
</tr>
<tr>
<td>Hsa-miR-15a-5p</td>
<td>F: TGTTACATCTACTCCTCAGC</td>
</tr>
<tr>
<td></td>
<td>R: CTTTGCTGATTTCGATCCAT</td>
</tr>
<tr>
<td>TLR4</td>
<td>F: GGTAAATACGCGGTAAAGG</td>
</tr>
<tr>
<td></td>
<td>R: AAAGAGTAAAGGTGTAGGT</td>
</tr>
<tr>
<td>MyD88</td>
<td>F: GGGCTTGTGTTGCGTAGAT</td>
</tr>
<tr>
<td></td>
<td>R: GGAGACGGAATAACAGGGC</td>
</tr>
<tr>
<td>TGF-β</td>
<td>F: AGAAGACGGAACGACAGC</td>
</tr>
<tr>
<td></td>
<td>R: GCTCCAGCCTCCTTCAT</td>
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Table 1. Primer sequences of qRT-PCR.
The expression level of miR-15a-5p was verified

The relative expression level of miR-15a-5p in HTS-Fb cells was verified by qRT-PCR test, and statistical analysis was conducted, which indicated that the expression level of miR-15a-5p in HTSFB cells was significantly increased (Figure 2a, **p < 0.01). In addition, we also detected the expression levels of MyD88, TGF-β1 and other related proteins in the downstream signaling pathway of TLR4 by WB test. The results suggested that the expression levels of these cytokines showed a significant up-regulation trend in HTS-Fb cells (Figure 2c-f, **p < 0.01).

Effect of miR-15a-5p on fibrosis status in HTS mice

Human scar tissue was transplanted into mice, and different treatments were given to the lesion site. As shown here, we observed a significant decrease in the number of capillaries and abnormal proliferation of fibroblasts in the sh-miR-15a-5p#1 group compared to the sh-NC group, and epithelial thickness was also reduced, while the opposite was true in the sh-miR-15a-5p #1 + TLR4 group (Figure 3a). TLR4, TGF-β1 and α-SMA were significantly decreased in HTS tissues (** p < 0.01), while the relative expression levels of TLR4, TGF-β1 and α-SMA were significantly increased in sh-mir-15a-5p #1 + pcDNA-TLR4 groups (Figure 3b-d).

Discussion

At present, with the continuous development of bioinformatics technology, miRNA has gradually become a research hotspot. It can regulate the expression level of other proteins, and then participate in the process of various diseases, such as malignant tumors, fibrosis and autoimmune diseases (29-34). Some scholars have proposed that THBS1 can induce the growth of hypertrophic scar (HTS) fibroblasts in scar tissue (35). In fact, the formation of HTS tissue is a very complex pathological process, that requires the involvement of multiple cell signaling pathways, such as miRNA FAM225B, which can promote the progression of HTS (37). In this study, we used miRNA expression profile data to screen for differentially expressed miRNAs between the HTS sample and normal skin samples. By analyzing the HTS tissues in the public database, the expression level of miR-15a-5p was significantly increased. In particular, miR-15a-5p was significantly correlated with the expression level of other miRNAs, which is the most important miRNA suggested by bioinformatics. This was verified in our subsequent cell studies. Studies have suggested that collagen is secreted by fibroblasts, excessive proliferation of fibroblasts leads to the increase of collagen, and the accumulation of extracellular matrix, thus becoming the main trigger for the hypertrophic scar tissue, and involved in the formation process (38,39). Subsequently, in this study, we cultured HTS-Fb cells as the experimental group and Ns-Fb cells as the control group. miR-15a-5p was inhibited in the experimental group, and the proliferation ability of HTS-Fb cells was decreased. In addition, the expression level of related proteins was also down-regulated. Our results suggest that miR-15a-5p is potentially related to the HTS-Fb cells proliferation and fibrosis, and participate in and regulate this process. In addition, in vivo experiments of BALB/c nude mice confirmed that lentiviral granules containing shRNA targeting miR-15a-5p could reduce the pathological status of HTS tissue, and inhibit the expression level of fibrosis markers. Based on the above results, it is reasonably suspected that the up-regulation of miR-15a-5p is closely related to the development of HTS. However, in the process of HTS formation, the downstream molecules regulated by miR-15a-5p still need to be explored. Therefore, we...
continue to conduct in-depth mining through bioinformatics and construct cell models and mouse models to further study the action mechanism of miR-15a-5p, in the formation and development of HTS.

In many reports on fibrosis studies, TLR4/MyD88 signaling pathway is regarded as a research hotspot, which mainly activates inflammatory pathways through mediating various cytokines, thus promoting the fibroblast fibrosis process (40). This signaling pathway can regulate the NF-κB signaling pathway to secrete a variety of inflammatory factors, and excessive expression of inflammatory cytokines can induce fibrosis generation (41, 42). In addition, this signaling pathway can also activate TGF-β, thus inducing the formation of ECM and inhibiting its degradation. TGF-β can also inhibit the production of fibroblasts into fibrotic myofibroblasts. Therefore, TGF-β is considered to be an important growth factor involved in the fibrosis process (43). In addition, studies have found that TGF-β can activate the Smad signaling pathway (44) while inhibiting the expression level of TGF-β can reduce fibrosis formation (45, 46). We found that the signaling pathway mediated by TLR4/MyD88 and TGF-β can be very important for HTS fibroblasts cells. These two pathways are activated, and the expression levels of related proteins are significantly increased. and when we down-regulated the expression level of miR-15a-5p, TLR4 and TGF-β were also decreased. Therefore, based on the above experimental results, we have reason to believe that the correlation between miR-15a-5p and TLR4 is not accidental. miR-15a-5p may inhibit TLR4/MyD88 and TGF-β signaling pathways to be involved in the formation of the HTS. Of course, our research can't stop there. The molecular mechanisms involved in TLR4 are complex and this research can't fully reveal the process. To explore the important biological role of TLR4 in HTS, more researchers are still needed to work together. However, there are still limitations to this study. In future work, the formation of HTS will still be the focus of our research. We will conduct in-depth analysis and verify our ideas through experiments, and continue to explore the specific action mechanism of various molecules in the TLR4/MyD88 and TGF-β signaling pathway. The results of all our current studies were integrated and compared separately with TLR4 and its synergistic function with miR-15a-5p. Note: the object of our study was BALB/c nude mice, in which xenograft HTS tissue from clinical sources was transplanted into the mice, and this is our independent animal model. No complete clinical trials have been conducted, so only prospective data are provided.

Through bioinformatics analysis results and laboratory data, we demonstrated that miR-15a-5p may be involved in the formation and development of HTS, through TLR4/MyD88 signaling pathway and TGF-β signaling pathway.

Conflict of Interests
The authors declared no conflict of interest.

References
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