

miRNA Expression Profiles in Keloid Tissue and Corresponding Normal Skin Tissue

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Abstract

Background Because the molecular mechanism behind keloid pathogenesis is still largely unknown, the clinical management of keloids remains problematic. miRNA (microRNA) is a novel class of small regulatory RNA that has emerged as post-transcriptional gene repressors and participants in diverse pathophysiological processes of skin disease. In the present study we aimed to investigate expression profiles of miRNA in keloid tissue and to develop a further understanding of the molecular mechanism involved in the pathogenesis of keloids.

Methods miRNA expression profiles in 12 pairs of keloid tissue and corresponding normal skin tissue were analyzed through a mammalian miRNA microarray containing established whole human mature and precursor miRNA sequences. Real-Time quantitative PCR was performed to confirm the array results. The putative targets of differentially expressed miRNA were functionally annotated by bioinformatics approaches.

Results miRNA microarray analysis identified 32 differentially expressed miRNAs, and a total of 23 miRNAs exhibited higher expression, while 9 miRNAs demonstrated lower expression in keloid tissue than in normal skin tissue. Functional annotations of differentially expressed miRNA targets revealed that they were enriched in several signaling pathways important for scar wound healing.

Conclusion This study showed that the expressions of many miRNAs were altered in keloid tissue, and their

expression profiling may provide a useful clue for exploring the pathogenesis of keloids. miRNAs might partly contribute to the etiology of keloids by affecting several signaling pathways relevant to scar wound healing.

Keywords miRNA · Keloid tissue · Normal skin tissue

Keloids are a common disease in dermatology and plastic surgical practice and have proven to be very resistant to treatment. The classic feature of a keloid is that the scar tissue progressively invades the surrounding normal skin and beyond the original wound area [1]. Patients with keloid scars often experience major discomfort, such as itching, aching, and cosmetic disfigurement. Many different treatments have been used in the past but the clinical management of keloids remains problematic. The therapeutic results are inconsistent and sometimes even ineffective. Hence, alternatives are needed. A good way of treating keloids depends on a good understanding of their molecular processes. A better understanding of the pathophysiology of keloid scarring holds great promise for developing novel therapeutic strategies. In this regard, keloids are the result of unbalanced cellular dynamics caused by overabundant fibroblast proliferation and the lack of fibroblast apoptosis [2]. MicroRNA (miRNA) is a short noncoding RNA regulating gene expression in many biological processes, including proliferation, apoptosis, and differentiation [3, 4]. There is no such report about miRNAs' effects on the expression of any mRNA, which is differentially expressed in keloid tissue versus control tissue, but there are many studies showing that miRNAs impact on many skin diseases [5]. For example, it was reported that miR-125b can regulate the expression of fibroblast growth factor receptor 2 (*FGFR2*) [6]. The

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expression of miR-125b and *FGFR2* was inversely correlated in both transfected keratinocytes and in psoriatic skin. Loss of miR-125b in psoriatic skin may contribute to hyperproliferation and aberrant differentiation of keratinocytes. *Krt16*, *Krt17*, *Dlx3*, and *Fgf10* were verified as direct miR-31 targets. MiR-31 is involved in establishing an optimal balance of gene expression in the hair follicle required for its proper growth and hair fiber formation [7]. It is also suggested that miRNAs may be potential diagnosis biomarkers and are likely to be involved in the pathogenesis of systemic scleroderma [6, 7]. Let-7a is involved in human papilloma virus related to non-melanoma skin cancer [8].

miRNAs are recently discovered small, highly conserved, 18–25-nucleotide long, single-strand RNA molecules. miRNAs are involved in the post-transcriptional regulation of gene expression through the binding to 3'-untranslated mRNAs, which are then degraded or translationally inhibited. Coding sequences for many miRNAs are conserved between very distantly related organisms, suggesting that they do indeed play important roles in cellular processes, and for some miRNAs there is specific evidence for their regulatory role. Hence, it is not surprising to find that miRNAs have roles in some disease development through the control of aspects of cellular growth and differentiation [8–10]. Recently, investigations into the biological significance of miRNA in skin morphogenesis and disease pathogenesis have been carried out. Various skin defects, including disturbed epidermal organization, compromised barrier function, and evaginated hair germs, have been observed when miRNAs were globally ablated by *Dicer* conditional knockout in embryonic skin progenitors [11, 12]. Dysregulated miRNAs have been identified in skin inflammatory disorders and malignant lesions [13, 14].

The essential roles of miRNAs in skin development and pathophysiology have been taken into consideration; however, the functional roles of miRNAs in the pathogenesis of keloids remain largely unknown. These findings may logically lead to the hypothesis that miRNAs play a crucial role in the form and growth of keloids. We hypothesized that different expression patterns of miRNAs might contribute to the formation and growth of keloids. To address this question, we performed comprehensive miRNA profiling and made a comparative miRNA analysis between keloid tissue and normal skin tissue. Bioinformatics analysis of the putative targets of these miRNAs proved that several signaling transduced pathways modulated by miRNAs may contribute to the pathogenesis of keloids, which helps elucidate the etiology of keloids and reveal therapeutic targets. This is the first report of differentially transcribed miRNAs in keloid samples.

Materials and Methods

Keloid and Normal Tissue Samples

Keloids and the corresponding normal skin tissues were obtained from 12 patients treated at The Second Affiliated Hospital of Harbin Medical University. This study abides by the Helsinki Declaration on ethical principles for medical research involving human subjects. All subjects gave informed consent to the work. All patients' diagnosis of keloids was confirmed by histology tests. No patients received any treatment before the surgical procedure. Keloid tissue and normal tissue were carefully excised, then 4-mm punch biopsies were taken from every sample. The sample tissue was flash frozen using liquid nitrogen and immediately placed in liquid nitrogen and stored at -80°C .

Total RNA Purification and Analysis

Keloid tissue and normal skin tissue were ground in liquid nitrogen, immediately transferred to lysis/binding buffer (Applied Biosystems, Foster City, CA, USA), and homogenized with a rotor stator (IKA, Staufen, Germany). RNA was isolated using standard RNA extraction methods with chloroform, isopropanol, and EtOH. Total RNA from the biopsies was isolated using the one-step mirVanaTM miRNA Isolation Kit (Applied Biosystems) following the manufacturer's instructions. The RNA quality was assessed by Agilent RNA 6000 NanoAssay (Agilent Technologies, Santa Clara, CA, USA) accepting an RNA integrity number higher than 7.5 and the RNA concentration was determined using a NanoDropTM 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

miRNA Microarray Analysis

In this study we performed the experiment using the in situ oligonucleotide microarray. miRNA was labeled by the mirVana microRNA Labeling Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The monoreactive Cy3 dye (Amersham Pharmacia Biotech, Uppsala, Sweden) was used in the dyeing step. The fluorescent probes were lyophilized then resuspended in 15 μl DEPC water and 5 μl of 4 \times hybridization buffer, denatured by heating for 5 min at 65°C , then snap-cooled on ice for 15 min. The hybridization was carried out for 20 h at 42°C in a rotating hybridization oven. After hybridization, slides were washed and then scanned by a Generation III array scanner (Amersham Pharmacia). Hybridization was carried out three times on three different days.

Signal intensities for each spot were analyzed and calculated by the Image Quant 5.0 (Amersham Pharmacia

Biotech) and Array Vision 6.0 (Imaging Research, Ltd.). Signal intensities for each spot were scanned and calculated by subtracting local background (based on the median intensity of the area surrounding each spot) from total intensities. The median value of the four spots (duplicate probes in every chip, two chips used for hybridization) for each miRNA was chosen. After data transformation, normalization was performed by using a per-chip 50th percentile method that normalizes each chip on its median, allowing comparison among chips. To highlight miRNAs that characterize each group, a per-gene on median normalization was performed, which normalizes the expression of every miRNA on its median among samples.

Quantitative Real-time PCR

To confirm the reproducibility of the data obtained from the miRNA microarray, the three most significantly upregulated miRNAs and three downregulated miRNAs were analyzed. Quantitative RT-PCR (qRT-PCR) was performed using the TaqMan MiRNA Assay System (Applied Biosystems). The miRNA-enriched fraction was used instead of the total RNA. Briefly, about 2 ng of the miRNA-enriched fraction (<200 nt) was subjected to a real-time reaction using a miRNA-specific looped primer, according to the manufacturer's protocol, to make cDNA. Subsequent PCR used miRNA-specific forward and reverse primers along with an appropriate quantity of RT cDNA product and TaqMan universal mix. The PCR reaction for each miRNA–cDNA was run in quadruplicate. A negative control without template was included in parallel to assess the specificity of the PCR reaction. PCR was carried out in an ABI 7900 (Applied Biosystems) in a 20- μ l volume with the following thermal cycling parameters: enzyme activation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60 s. All other conditions used the manufacturer's values.

Data acquired from the PCR reactions were analyzed using SDS 2.3 software (Applied Biosystems). Each miRNA from the keloid samples was compared with the corresponding normal skin sample. Comparisons are described as log values of the ratio of miRNA expression in keloid samples versus that in the corresponding normal tissue. For comparison of keloid and normal skin tissue, we performed statistical analysis by Student's *t* test.

Prediction of microRNA Targets and Categorization of Signaling Pathway Including microRNA Targets

Three publicly available databases, TargetScanHuman 5.1 (<http://www.targetscan.org/>), DIANA-microT v3.0 (http://www.diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi), and

MicroCosm Targets version 5 (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>), were used for miRNA target gene prediction. The predicted gene was considered a putative target candidate when it was predicted by three databases. Predicted target genes in combination with miRNA and whole-genome microarray data were used to visualize possible biological miRNA/mRNA processes correlating to keloid development and/or progression. The bioinformatics' annotations of all putative miRNA targets, including categorization of biological processes and signaling pathways, were performed using the online DAVID Bioinformatics Resources (<http://david.abcc.ncifcrf.gov/>). A modified Fisher exact test with a *p* value was performed to determine whether the proportions of genes falling into each category could have been due to random chance.

Results

Differential Expression of miRNA in Keloid Tissues

The miRNA expressions for the keloid samples and the matched normal skin tissues were analyzed using a paired *t*-test analysis. The miRNA patterns were found to be significantly different between the two groups, as shown in Fig. 1. To eliminate false-positive results and to improve the precise degree of results, we abided by the following criteria. When miRNA reached or exceeded the following strict criteria, it was considered a differential expression candidate. First, only miRNAs with alternations of at least twofold were included. Second, abnormal expression of miRNA must be observed in all the paired keloid and normal tissues (12 paired tissues). The total number of miRNAs in the miRNA microarray in this study was 1,200, which represents all the human miRNAs known to date. Among them, 32 miRNAs were identified as listed in Table 1. Compared with the corresponding normal skin, 23 upregulated miRNAs and 9 downregulated miRNAs were found in keloid tissues. miRNA-21 was revealed to have the highest fold change (6.87-fold) among the 23 upregulated miRNAs, and miRNA-203 had the lowest expression level (10.19-fold) in the 9 downregulated miRNAs.

Validation of the Microarray Data by qRT-PCR

To validate the results from the miRNA microarray, we further employed quantitative RT-PCR to measure the abundance of the miRNA, including upregulated miRNA-21, miRNA-4269, and miRNA-382 and downregulated miRNA-203, miRNA-205, and miRNA-200c. The qRT-PCR data indicated that the transcriptional level of miRNA-21, miRNA-4269, and miRNA-382 and miRNA-203, miRNA-205, and miRNA-200c coincided perfectly with

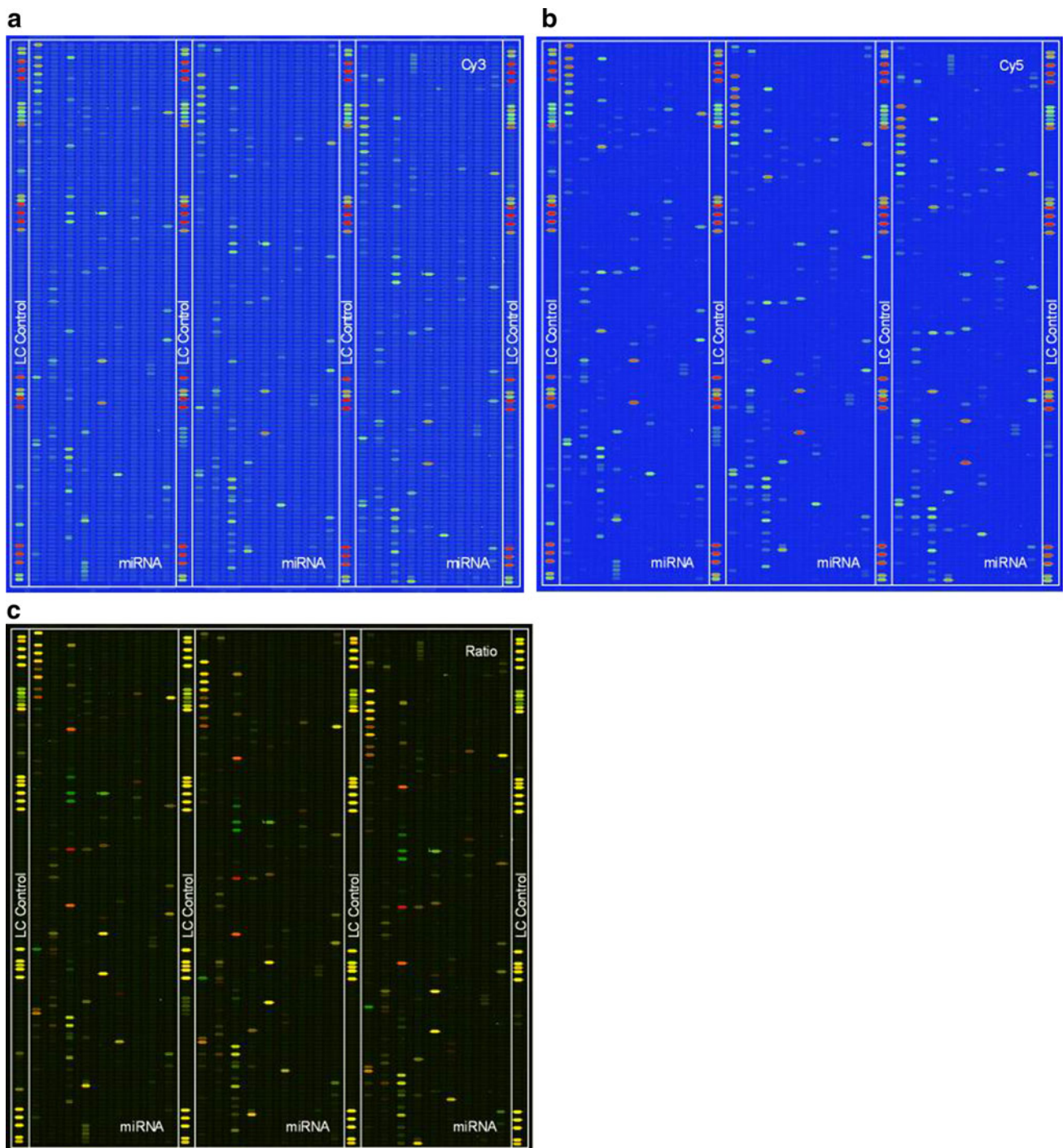


Fig. 1 Representative regions of chip images. From the Cy3 and Cy5 images one may read miRNA profiles directly, and from the ratio images one may get a quick sense of differential expressions between the corresponding samples. The images are displayed in pseudocolors so as to expand the visual dynamic range. In the Cy3 and Cy5 intensity images, as signal intensity increases from 1 to 65,535, the corresponding color changes from *blue* to *green*, to *yellow*, and to *red*. In the Cy3/Cy5 ratio image, when the Cy3 level is higher than the Cy5 level, the color is *green*; when the Cy3 level is equal to the Cy5

level, the color is *yellow*; and when the Cy5 level is higher than the Cy3 level, the color is *red*. In this section, a list of differentially expressed transcripts is also provided following the chip images. From the list, one can have a quick overview of the difference between the normal skin samples and keloid sample on the chip. **a** (Cy3) represented the result of miRNA in the normal skin. **b** (Cy5) represented the result of miRNA in the keloid. **c** Cy3/Cy5 which showed the different expression of miRNAs between normal skin and keloid

Table 1 Differentially expressed miRNA with $P < 0.01$

No.	miRNA-ID	Normal skin signal	Keloid signal	Log ₂ (normal skin/keloid)
1	hsa-miR-203	3,609.51	3.14	-10.19
2	hsa-miR-205	5,008.10	6.87	-9.30
3	hsa-miR-21	92.10	9,767.63	6.87
4	hsa-miR-200c	2,315.02	20.74	-6.83
5	hsa-miR-200b	145.76	3.61	-5.37
6	hsa-miR-4269	23.54	937.90	5.32
7	hsa-miR-1246	4,914.19	194.60	-4.66
8	hsa-miR-382	26.26	385.64	4.10
9	hsa-miR-487b	11.60	163.89	4.02
10	hsa-miR-155	32.18	447.22	3.80
11	hsa-miR-98	13.43	203.42	3.53
12	hsa-miR-127-3p	34.32	378.57	3.40
13	hsa-miR-31	23.90	251.89	3.37
14	hsa-miR-939	20.60	184.88	3.24
15	hsa-miR-134	21.92	178.47	3.19
16	hsa-miR-432	22.35	161.09	3.14
17	hsa-miR-152	101.25	829.77	3.08
18	hsa-miR-455-3p	178.97	1,469.07	2.97
19	hsa-miR-424	20.10	129.64	2.83
20	hsa-miR-379	33.36	219.78	2.78
21	hsa-miR-181b	18.09	123.77	2.77
22	hsa-miR-222	955.60	154.71	-2.65
23	hsa-miR-132	58.38	309.93	2.56
24	hsa-miR-494	16.17	88.98	2.55
25	hsa-miR-199a-5p	43.76	335.66	2.42
26	hsa-miR-199a-3p	3,285.49	17,315.58	2.40
27	hsa-miR-214	3,394.17	16,457.09	2.28
28	hsa-miR-3613-3p	9,746.96	2,430.09	-2.24
29	hsa-miR-3652	158.59	729.12	2.20
30	hsa-miR-221	334.39	76.24	-2.13
31	hsa-miR-30b	238.02	59.52	-2.11
32	hsa-miR-181c	54.17	325.83	2.10

microarray results. The expression level of upregulated miRNA-21, miRNA-4269, and miRNA-382 increased up to 6.92-, 5.71-, and 4.20-fold, respectively, and the downregulated miRNA-203, miRNA-205, and miRNA-200c decreased 10.23-, 9.35-, and 6.92-fold, respectively (Fig. 2).

Putative Targets of miRNAs and Functional Analysis by Bioinformatics

We carried out the computational predictions of target genes for all differentially expressed miRNAs. Each miRNA potentially regulates many targets. To decrease the total number of false-positive targets, the targets were considered as putative candidates by three programs. After

carefully analyzing the results of putative targets, we identified the targets for each miRNA. A total of 839 potential targets were predicted and the number of potential targets of one miRNA varied from 9 to 97. To elucidate target pathways of miRNAs, we carried out analysis of GO (gene ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway, which helps to further study the biological process and corresponding metabolic network regulated by potential miRNAs. The DAVID platform was used to identify GO and KEGG signaling pathways for the putative targets of miRNAs. We found that many targets played significant roles in several important pathways that have been closely associated with keloids, including TGF- β , MAPK, apoptosis, and cell cycle.

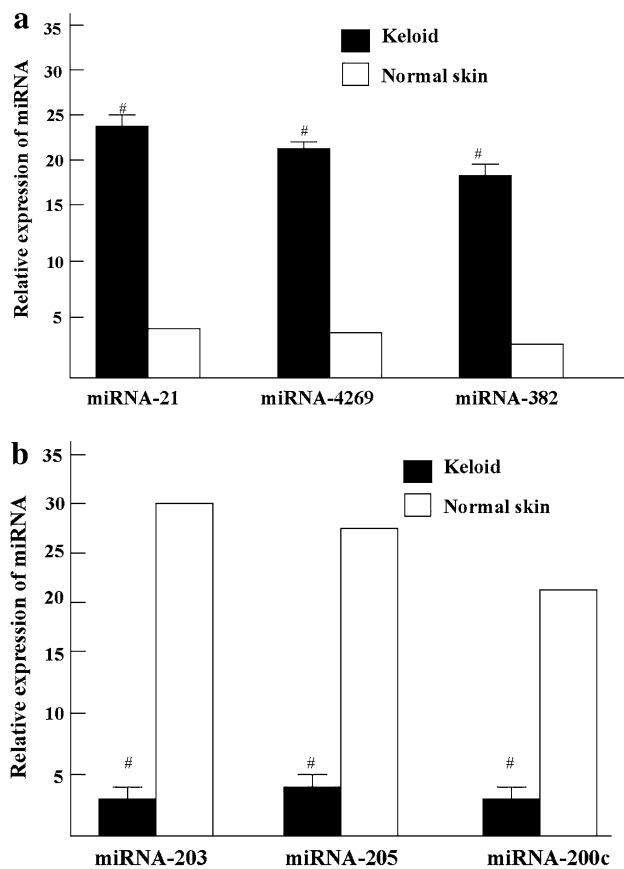


Fig. 2 Confirmation of miRNA expression by quantitative real-time PCR. **a** miR-21, miR-4269, and miRNA-382 were upregulated. **b** miR-203, miR-205, and miRNA-200c were downregulated in keloid tissue. The experiment was conducted in triplicate. # $P < 0.01$ versus normal

Discussion

In this study we identified 32 potential miRNAs that were differentially expressed between keloid tissue and corresponding normal skin tissue. Twenty-three of them were highly expressed in keloid samples and the remaining nine were lightly expressed. To date, nearly nothing is known about the roles of most of these miRNAs in the pathology of keloids. We inferred that these miRNAs probably contributed to the etiology of keloids due to their different expression patterns in keloid tissue. Our results revealed that miRNAs probably modulate several key signaling pathways that have been previously related to the pathogenesis of keloids by binding to the 3' untranslated region (UTR) of target genes. To the best of our knowledge, this study is the first to explore the functional significance of miRNAs in the pathogenesis of keloids.

To date, the causes of keloid formation are fundamentally unknown, and it is necessary to further explore the pathogenesis of keloids. Previous studies proved that some

genes were abnormally expressed in transcribed and/or translated levels in keloid tissues [15–18]. The fundamental mechanism of miRNAs' regulation probably exists in keloid tissue. The data of the miRNA microarray identified some distinct expression patterns of miRNA between keloid tissue and corresponding normal skin tissue. Such differences might be relevant to the pathological characteristics of keloids. In addition, we validated six differentially expressed miRNAs, including three upregulated miRNAs and three downregulated miRNAs, by quantitative real-time PCR. The reliability of the profiling results was verified because the qRT-PCR data were consistent with the miRNA microarray results. Therefore, we believe that our profiling results may provide a novel and significant beginning for exploring the potential roles of miRNA in the pathogenesis of keloids.

Some miRNAs identified in this study had been shown to play important roles in some biological procedures of cellular mechanisms [19]. Previously, Sonkoly et al. [20] observed that miR-203 has a unique expression pattern among all the miRNAs and can be termed a skin-specific miRNA. This suggested that miR-203 has a skin and/or keratinocyte-specific function. Results of the study by Sonkoly et al. [20] showed that miR-203 expression patterns distinguish psoriasis from healthy skin and from atopic eczema skin. The psoriasis-specific and disease-specific miR-203 expression identified might be potential therapeutic targets for chronic inflammatory skin diseases. The observation became more precise and scientific as recent evidence [21] demonstrated that miR-203, as the most upregulated miRNA during keratinocyte differentiation, could depend on p53. This may explain how expression of miR-203 could keep the balance between proliferation and differentiation, as well as the response to DNA damage, in keratinocytes [22]. The antiproliferative effect of miR-203 in epidermal stem cells is mediated through the direct repression of p63 expression [23]. Thus, miR-203 may be a general inhibitor of progenitor and stem cell proliferation. Silencing of miR-203 contributes to uncontrolled proliferation in certain cancers [24]. The latest results showed that elevated levels of miR-203 lead to increased secretion of MMP-1 and IL-6 via the NF- κ B pathway [24].

Recent evidence has shown that miR-21 plays critical roles in the differentiation of normal cells [25] and may be a suitable molecular target for therapies aimed specifically at reducing vestibular schwannoma growth [26]. Upregulation of miR-21 is concurrent with downregulation of potent tumor suppressor proteins PTEN and programmed cell death [27]. These proteins control aspects of apoptosis, proliferation, invasion, and migration. PTEN is also regulated by miR-221/222 and miR-214 [28, 29]. Silencing

miR-21 in some cells results in increasing apoptosis, which further suggests a functional role for miR-21 [30].

MiR-205 was found in all examined squamous epithelium [31]. It was demonstrated [32] that the lipid phosphatase SHIP2 is a target of miRNA-205 in epithelial cells. More importantly, the corneal epithelia-specific miR-184 can interfere with the ability of miR-205 to suppress SHIP2 levels. Some data [33] describe miR-205 as a new onco-suppressor gene in breast cancer, able to interfere with the proliferative pathway mediated by the HER receptor family, and these data also provide experimental evidence suggesting that miR-205 can improve the responsiveness to specific anticancer therapies [33].

The miR-200 family (containing miR-200a, miR-200b, and miR-200c) is preferentially expressed in the epidermis, whereas the family of miRNA is expressed exclusively in hair follicles [34]. The miRNA microarray showed [35] that the miR-200 family members were upregulated in endometrial endometrioid carcinomas as compared with those in normal endometrial tissues. When endometrial cancer cells were treated with specific anti-miRs, including anti-200a, 200b, and 200c, the results showed that anti-miR-200a, -200b, and -200c significantly inhibited the growth of cells. miR-200 family members can be self-regulated by Zeb1/2 [36, 37]. It was found [37] that TGF- β 1 was induced by miR-200b/c upregulation in mesangial cells. Apart from the fact that some miRNAs have been implicated in several human diseases, even skin cancers, there are still some miRNAs that have not been reported on.

To elucidate the significance of miRNAs, we performed a global analysis of miRNA-regulated signaling pathways and related genes on the basis of miRNA expression profile and bioinformatics interpretation by using GO and KEGG. We found that these miRNAs were associated with many signaling pathways, including the cell cycle, MAPK, p53, focal adhesion, TGF- β , and biosynthesis of collagen protein. It had already been reported that these pathways take part in cell activation and even keloid fibrogenesis. For example, MAPK mediated mitosis and the synthesis of collagen and matrix metalloproteinases in scar generation [38]. One of the main reasons for keloid formation is collagen overdeposition. miRNAs have been reported in many collagen-related diseases such as liver fibrosis, osteoarthritis, systemic sclerosis, and diabetic nephropathy [deposition of collagen IV in proximal tubule cells (PTCs)] [39]. miRNA can also regulate early chondrocyte differentiation by influencing collagen [13, 39]. It has also been reported that the use of miRNA treatment to modulate TGF- β expression holds great promise in preventing tendon adhesion formation [18]. According to the previous finding, we logically made the hypothesis that miRNA may participate in the mechanism for deposition of collagen in

keloid formation. We will next perform the experiment to explore this question.

To obtain a better understanding of the functional significance of miRNA, it was important to identify and validate the miRNA targets [40]. In this study, hundreds of target genes were predicted, including several key mediators of cellular signaling pathways and some ECM proteins. All the putative targets were analyzed by the DAVID bioinformatics platform to obtain further functional annotation. According to the results of DAVID bioinformatics, these targets were highly gathered and involved in several signaling pathways, e.g., MAPK, cell cycle, apoptosis, and TGF- β . These findings strengthened the notion that multiple signaling cascades participated in the pathogenesis of keloids and a sophisticated regulatory network composed of miRNAs' modulation. miRNAs might contribute to the pathogenesis of keloids by regulating their targets and influencing multiple signaling pathways.

Of course, we note the findings of our study have some limitations. Our results are preliminary and we will perform the next experiment to elucidate the issue. In this phase of the study, we could hardly elucidate the different distributions of miRNAs in different parts of the body; it is the focus of the next study. Different expression patterns of miRNAs between keloid tissue and normal skin tissue were caused by complicated reasons. However, this study could not give a full explanation of the reasons. Therefore, more work is needed to further explore the molecular networks connected to miRNAs.

Conclusion

In this study, we identified differentially expressed miRNAs through a miRNA microarray between keloid tissue and normal skin tissue. These data strongly suggest that some miRNAs take part in the molecular network of keloid pathogenesis. Differentially expressed miRNAs affect multiple signaling pathways relative to keloid pathology through regulating the expression of its target genes. More studies are needed to further elucidate the functional roles of every miRNA and relevant signaling pathway in the pathogenesis of keloids. These results open a new page for studying the hitherto unexplored roles of miRNAs in the pathogenesis of keloids.

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Disclosure The authors declare that they have no conflicts of interest to disclose.

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