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Keloid microRNA expression analysis and the influence of miR-199a-5p on the proliferation of keloid fibroblasts

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ABSTRACT. The purpose of this study was to identify microRNAs (miRNAs) involved in keloid formation and determine their influence on the proliferation of keloid fibroblasts (KFs). Eight specimens each of resected keloid tissue and normal skin tissue were collected. miRNAs that are differentially expressed in keloid tissue and normal skin were detected using an miRNA microarray and verified by quantitative real-time polymerase chain reaction (RT-PCR). Seventeen differentially expressed miRNAs, including miR-199a-5p, were identified by microarray hybridization. qRT-PCR analysis confirmed the decrease in miR-199a-5p expression in keloid *vs* normal tissue that was detected by the microarray analysis. Mimics of differentially expressed miRNAs were then transfected into a KF cell line, and the effect of miRNA

overexpression on the proliferation of KFs was assayed using the EdU assay. Compared with mock-transfected cells, KFs transfected with a miR-199a-5p mimic showed significantly lower cell proliferation and an altered cell cycle, with cells having significantly longer S and G2/M phases. The significantly lower expression of miRNA-199a-5p in keloids likely influences the cell cycle of KFs and restrains their proliferation, suggesting that miR-199a-5p probably plays a role in the regulation of KF proliferation.

Key words: Keloid; Expression profile; miR-199a-5p; Keloid fibroblast; KF Proliferation

INTRODUCTION

A keloid is the result of a dysregulated wound healing process and is characterized by the excessive formation of scar tissue, which proliferates beyond the boundaries of the original wound. In addition to being clinically invisible, keloids cause pain or itching and can even lead to dysfunction, Such as restrictions of joint movement, and some on activities limitations. Furthermore, keloids seriously endanger the physical and psychological health of patients. Presently, several methods are used to prevent and cure keloids, such as surgical excision, steroid injections, radiation therapy, and compression therapy. However, the efficacy of these methods is variable. This is primarily because the processes underlying keloid pathogenesis are not well understood. Several cellular biology and molecular biology studies have shown that aberrant gene structure and function may underlie the formation of keloids, providing a new direction for keloid research. Studies confirm that genes related to tumor formation and cytokine function may participate in the formation of keloids and influence their development (Ohtsuru et al., 2000; Satish et al., 2006).

MicroRNAs (miRNAs) are short (22 nucleotides) non-coding single-stranded RNAs that negatively regulate target messenger (m)RNA expression in a post-transcriptional manner. miRNAs, as part of the RNA-induced silencing complex (RISC), bind to the 3' untranslated region (3'UTR) of their target mRNAs, and cause either the degradation or translational inhibition of these mRNAs (Lu et al., 2005; Cho, 2007). miRNAs exist in almost all eukaryotic cells and are extensively involved in cell proliferation, differentiation, development, metabolism, apoptosis, and other biological processes. The abnormal expression of miRNAs may therefore be closely associated with the occurrence, development, and prognosis of certain diseases. Of the known miRNA genes in humans, approximately 50% are located in tumor-associated chromosomal regions (Liu et al., 2008). miRNAs have been shown to play a significant role in tumorigenesis (Calin et al., 2004). In 2005, Iorio et al. used an miRNA chip to detect miRNAs differentially expressed in breast cancer samples as compared to normal breast tissue. They found that the expression of miR-145, miR-125b, miR-21, and miR-155 is significantly lower in breast cancer tissue than in normal breast tissue. The degree of decline in expression was closely related to the tumor stage and pathological type of the cancer (Iorio et al., 2005). Similarly, miR-15 and miR-16 expressions were found to be reduced in chronic B-cell lymphoma (Calin et al., 2002). In addition, restraining the expression of miR-21 is commonly and markedly upregulated in human glioblastoma

and that inhibiting miR-21 expression leads to caspase (caspase-3 and caspase-7)activation and associated apoptotic cell death in multiple glioblastoma cell lines. These findings suggest that overexpressed miR-21 may function as a micro-oncogene in glioblastomas cell lines (U373, A172, LN229, U87, LN428, and LN308) by blocking expression of key apoptosis-enabling genes. (Chan et al., 2005). Due to the idea that miRNAs may function as proto-oncogenes or tumor suppressor genes in tumor cells, the study of miRNA function in tumorigenesis is an active field of medical research.

In this study, using miRNA microarray analysis, we investigated the miRNA expression profile of keloid tissue in order to identify miRNAs that may be involved in the etiology of keloid formation.

MATERIAL AND METHODS

Specimens and cells

KF cell lines were purchased from Shanghai Aiyan Biotech Co. Ltd., Shanghai, China. Specimens were collected from inpatients and outpatients of the Guangdong Medical College Hospital between January 2010 and March 2011. Exclusion criteria included the presence of tumors, genetic or infectious diseases, and treatment such as radiation therapy or chemotherapy prior to surgery. Specimens were divided into 2 groups: keloid group (the experiment group) and normal skin group (control group). Each group had 8 samples. The youngest sample donor was 6 years old and the oldest was 59 years old. Tissue in both the keloid and control groups was obtained from 3 males and 5 females.

Total RNA extraction

Three hundred milligrams specimen was weighed and grounded into powder form in liquid nitrogen. Next, 3 mL Trizol reagent was added to the specimen and it was homogenate for 1 to 2 min with a mortar and pestle. Next, the sample was incubated at room temperature for 5 min to make it Complete dissociation. It was then centrifuged at 12,000 rpm for 5 min and the pellet was discarded. Next, 600 μL chloroform was added to the supernatant, mixed well, and then the sample was incubated at room temperature for 15 min. Next, it was centrifuged at 12,000 rpm for 15 min at 4°C. The material in the tube separated into 3 layers. The upper layer was transferred to another eppendorf tube. Isopropanol was added to this layer (0.5 mL isopropanol/mL Trizol) and the mixture was incubated for 10 min. Then the sample was centrifuged at 12,000 rpm for another 10 min at 4°C and the supernatant was discarded. The pellet was washed with 1 mL 75% ethanol and then centrifuged at 7500 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was air-dried at room temperature for 10 min. The pellet was then resuspended in 30-50 μL diethylpyrocarbonate (DEPC) water and then incubated in a water bath for 10 min at 55°-60°C in order to promote RNA solubility.

The NanoDrop ND-1000 (NanoDrop, Mailing and Shipping Address 3411 Silverside Rd Bancroft Building Wilmington, DE) was used to determine the concentration and purity of RNA. Agarose gel electrophoresis was used to detect RNA degradation.

miRNA separation

The Ambion (Ambion, 2130 Woodward Street, Austin, TX, USA) miRNA Isolation Kit was used to separate miRNA. Fifty to 100 µg of total RNA was added to an eppendorf tube with 5 times as much volume of Lysis/Binding buffer and the mixture was mixed well. Next, 1/10th the volume of the miRNA Homogenate Additive was added and the mixture was mixed by vortexing. The eppendorf tube was then placed on ice for 10 min. Next, 1/3 the volume of 100% ethanol was added and the sample was mixed well by inverting the tube. The mixture was then transferred to a filtration column and centrifuged at 10,000 rpm for 15 s. The filtrate was discarded and the filtration column was placed in a collecting tube and the filtration membrane was washed separately with Washing Solution 1 and Washing Solution 2. The filtration column was then transferred to a new collecting tube and the miRNA was eluted with 100 µL preheated (95°C) RNase free water. The column was centrifuged at 10,000 rpm for 30 s, and the eluate with the miRNAs was collected. RNA quality was tested as described for total RNA.

miRNA microarray chip detection

Four samples of keloid tissue and 3 samples of normal skin tissue were used for the detection of differentially expressed miRNAs. The miRCURYTM LNA Array (v14.0) (Exiqon, Denmark) was used according to the manufacturer protocol. Each probe was repeated four times in the chip. After chip hybridization, the microarray was scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Inc., Union City, CA, USA). The GenePix pro 6.0 image analysis software was used to analyze the chip image and transform the image signal into a digital signal. Background noise was subtracted from the original value. The mid-value was used as the standard factor to standardize the entire chip. The signal value of each gene was computed to obtain the average. Cluster 3.0 was used for hierarchical clustering analysis (CapitalBio, Beijing, China). This experiment was conducted with the assistance of the Shanghai Kangcheng Sheng Bio-Engineering Co., Ltd. (Shanghai, China).

Quantitative real-time polymerase chain reaction (qRT-PCR) validation

All 8 keloid and normal specimens were used for qRT-PCR analysis. Based on the literature, miR-199a-5p was selected for qRT-PCR validation. After reverse transcription of total RNA, single-stranded cDNA was used as the template for PCR amplification. The PCR cycle was as follows: 95°C pre-denaturation for 20 s, 95°C denaturation for 10 s, 60°C for 20 s, and 70°C for 10 s. The denaturation and amplification cycles was repeated 40 times. U6 snRNA was used as the internal reference.

EdU (5-ethynyl-2'-deoxyuridine) proliferation test

EdU is a novel thymidine analogue. Ethynyl can replace the deoxy thymine ring, which is connected with the C5-methyl. It can be incorporated into replicating DNA in place of thymidine. Using this method, the amount of time spent by a cell in the S phase of mitosis can be determined.

KF cells were cultured in 5% CO_2 at 37°C in medium containing high glucose Dulbecco's Modified Eagle Medium (DMEM) and 10% FBS. KF cells in the logarithmic growth phase were used to seed 96-well plates at a density of 2 x 10^3. Cells were plated in a total volume of 100 µL per well and incubated overnight in 5% CO_2 at 37°C. A mimic of miR-199a-5p was transfected into KFs using Lipofectamine™ 2000 according to manufacturer instructions. An mi-199a-5p mimic Negative Control (NC) was also transfected in KFs. At 46 h after transfection, cells were serially subjected to EdU product specification, EdU marking, fixing, Apollo staining, and DNA staining. Acumen and BD high content screening instruments were used for image acquisition and analysis immediately after the completion of staining. In 3 high-power fields (3 wells, 1 image/well), the total number of cells (Hoechst 33342 positive cells) and the number of proliferating cells (EdU positive cells) were recorded. These numbers were averaged and the labeling index (EdU-positive cells/total cells) was calculated.

Statistical analysis

The SPSS 17.0 software (SPSS Inc. Chicago, USA) was used to analyze the qRT-PCR and EdU detection data. Measurement data are reported as means ± standard deviation (SD). An independent *t*-test was used to test statistical significance because of homogeneity of variance in both groups. An independent sample *t*-test was used to analyze. Statistical significance was accepted at $P < 0.05$.

RESULTS

Quality control of the RNA

Optical density measurements were obtained for each RNA sample. All RNA samples had an A260/A280 ratio at about 1.8-2.0 and an A260/A230 ratio > 2. The RNA samples did not show degradation upon electrophoresis. The ratio of the intensity of the 28S ribosomal (r) RNA band to that of the 18S rRNA band was approximately 2:1. Thus, the RNA samples met the quality requirements for miRNA microarray analysis.

miRNA chip detection

The miRNAs differentially expressed in normal skin and keloid tissue were identified by scanning the fluorescence intensity of the microarray chip using the Axon GenePix 4000B microarray scanner (Figure 1). The Genepix Pro 6.0 software was used to analyze the raw data. Of the 293 miRNAs identified as differentially expressed, 168 miRNAs were upregulated and 125 miRNAs were downregulated compared with normal tissue. Cluster 3.0 was used for the cluster analysis of differentially expressed miRNAs (Figure 2).

Based on the 2 sample *t*-test ($P < 0.05$) statistical analysis in both keloid and normal skin tissue, the threshold for calling miRNAs differentially expressed was established as a fold-change ≥1.50 for upregulation and a fold-change ≤0.67 for downregulation. Seventeen miRNAs showed differential expression in all samples tested. hsa-miR-516b, hsa-miRPlus-E1106, and hsa-miRPlus-E1247 were upregulated in keloid tissue whereas hsa-miR-214, hsa-miR-645, hsa-miR-338-5p, hsa-miR-934, hsa-miR-199a-5p, hsa-miR-21*, hsa-

miR-122*, hsa-miR-186*, hsa-miR-495, hsa-miR-412, hsa-miR-551b*, hsa-miRPlus-F1158, hsa-miRPlus-E1038, and hsa-miR-1038 were downregulated in keloid tissue (Table 1).

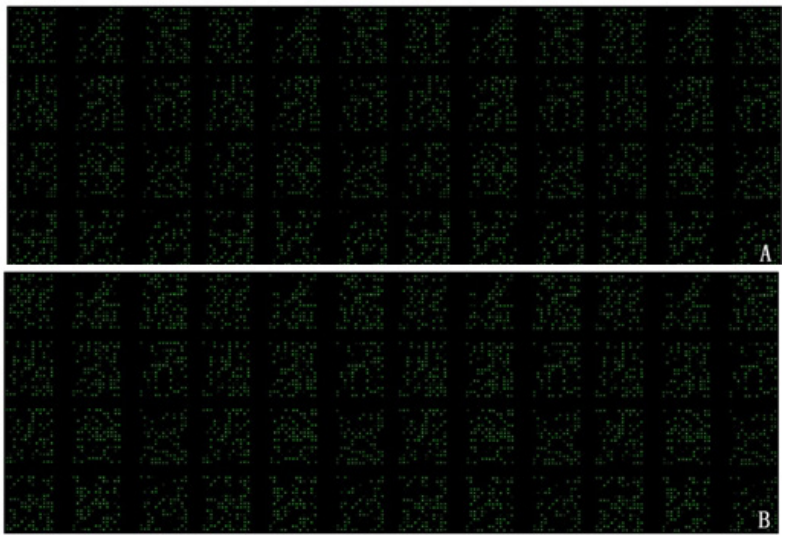

Figure 1. Microarray hybridization fluorescence. **A.** Keloid tissue, **B.** normal skin tissue.

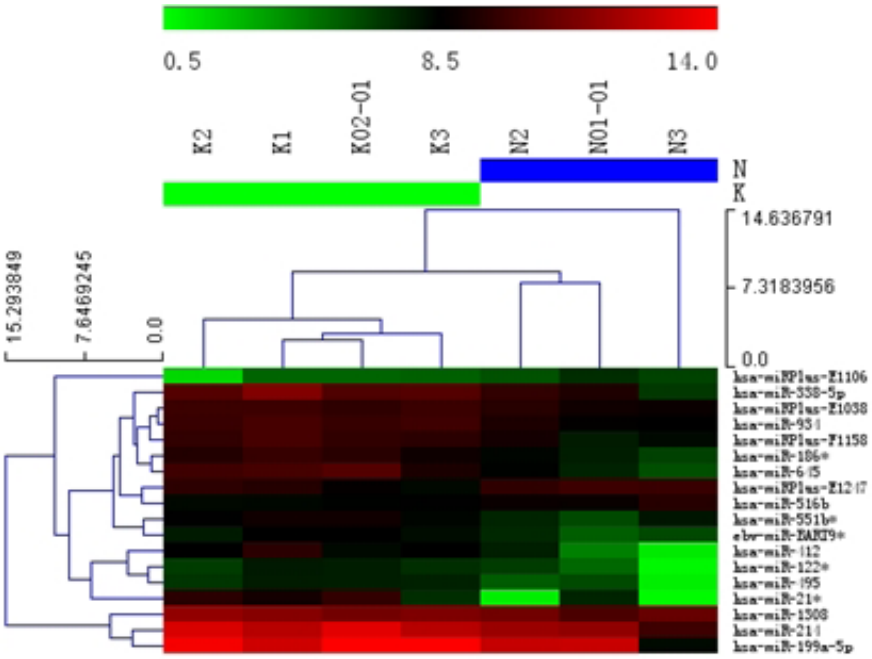

Figure 2. Cluster analysis diagram between keloid and normal skin tissue.

Table 1. Differential expression of microRNA in keloid and normal skin tissue.

ID	Name	Fold-change	P	Regulation
11151	hsa-miRPlus-E1106	2.9471054	0.0323476	Up
46312	hsa-miR-516b	1.5488775	0.0403448	Up
46445	hsa-miRPlus-E1247	1.635632	0.044607	Up
11014	hsa-miR-214	0.3348936	0.0281083	Down
17613	hsa-miR-645	0.2015858	0.0108087	Down
17825	hsa-miR-338-5p	0.3025472	0.0447802	Down
17863	hsa-miR-934	0.4737911	0.0017724	Down
29562	hsa-miR-199a-5p	0.3420021	0.0348098	Down
42524	hsa-miR-21*	0.1067548	0.0445511	Down
42551	hsa-miR-122*	0.2905647	0.0350182	Down
42614	hsa-miR-186*	0.3116197	0.0087563	Down
42676	hsa-miR-495	0.2417453	0.0056144	Down
42764	hsa-miR-412	0.165942	0.0308919	Down
42781	hsa-miR-551b*	0.3840699	0.0086062	Down
46474	hsa-miRPlus-F1158	0.4040254	0.0103133	Down
46537	hsa-miRPlus-E1038	0.6078815	0.0060700	Down
46739	hsa-miR-1308	0.6151837	0.0444737	Down

qRT-PCR detection

The amplification curve and melting curve for qRT-PCR of miR-199a-5p showed good amplification efficiency and specificity, respectively (Figure 3). miRNA levels were normalized to U6 snRNA levels using the formula ΔCt = [Ct(miRNA)] - [Ct(U6 snRNA)]. The mean ΔCt ± SD is reported. The results showed that, compared to normal skin, the expression of miR-199a-5p in keloid tissue was significantly downregulated. This is consistent with the microarray results (Table 2).

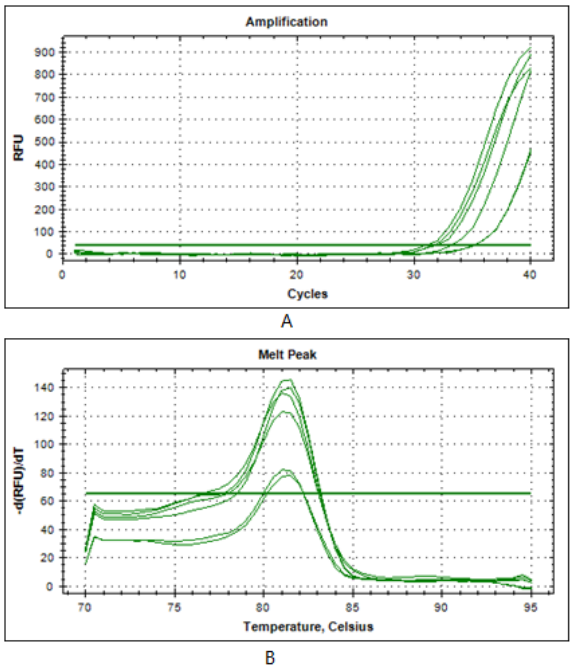

Figure 3. A. MiR-199a-5p fluorescence amplification curve; **B.** MiR-199a-5p melting curve.

Table 2. Comparison of miR-199a-5p expression levels in keloid and normal skin (N = 8).

	miR-199a-5p
Keloid ΔCT (means ± SD)	9.76 ± 0.79
Normal skin ΔCT (means ± SD)	5.67 ± 0.86
t	17.239
P	<0.001

EdU detection

miR-199a-5p was transiently overexpressed in KF cells.after 46 Hours, the cellular proliferation rate was determined using EdU assay. All nuclei were stained blue using Hoechst stain. Only the nuclei in replicating cells were stained red by the EdU dye. When the images of Hoechst-stained and EdU-stained cells are overlapped, proliferating cells can be identified as purple nuclei (Figure 4). Compared with the mock-transfected group, the number of EdU positive cells (i.e., proliferating cells) was significantly lower in the miR-199a-5p-transfected cells (Table 3). There was also a difference in the cell cycle of miR-199a-5p-transfected and mock-transfected cells. miR-199a-5p-transfected cells showed significantly longer S and G2/M phases (Table 4).

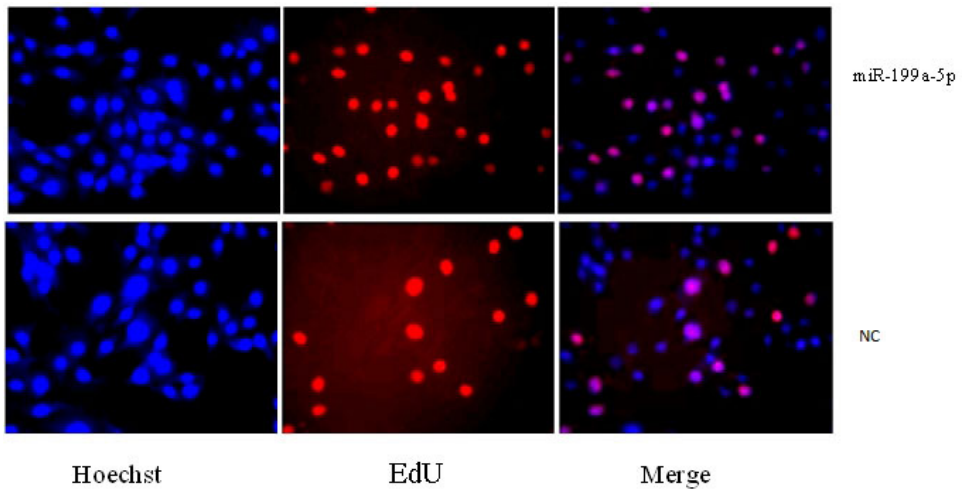

Figure 4. EdU staining detection about the influence of miR-199a-5p and negative control (NC) group on the proliferation of keloid fibroblast.

Table 3. EdU positive cell rate in experimental group and the negative control (NC) group.

	EdU positive cell rate (%)
MiR-199a-5p mimics	20.72 ± 2.50
NC	27.68 ± 4.92
t	2.183
P	0.047

Table 4. Experimental group and the negative control (NC) group of cell cycle changes.

	G1 (%)	S (%)	G2/M (%)
MiR-199a-5p mimics	54.91 ± 2.07	33.93 ± 1.30	10.87 ± 0.80
NC	59.09 ± 0.91	31.39 ± 0.79	9.27 ± 0.46
t	-3.199	2.889	3.013
P	0.016	0.022	0.020

DISCUSSION

miRNAs are a recently discovered class of 20-22 nucleotide long RNAs that are present in a wide variety of eukaryotic organisms. Although they do not encode proteins, through the RISC complex, they can regulate mRNA expression by binding to the 3'UTR of target genes and causing mRNA degradation or translational inhibition. The mature miRNA is derived by the processing of a long hairpin pre-miRNA transcript by the Dicer endonuclease. miRNA genes are present in the intergenic regions of genomes (they may have independent promoters regulating their expression) or in the introns of genes (these miRNAs may have their own independent promoters or may be transcribed from the promoter of the host gene). It is speculated that there may be more than 1000 miRNA genes in the human genome (Bentwich et al., 2005). A single miRNA can regulate more than 200 target genes and miRNAs are thought to regulate about 1/3 of the genes involved in cell proliferation, differentiation, development, metabolism, apoptosis, and other physiological activities (Carthew, 2006). Therefore, the abnormal expression of miRNAs may be closely related to the occurrence, development, and prognosis of certain diseases.

Much progress has been made in studying the genetic aspects of keloid formation. Some tumor-related genes have been confirmed to participate in the formation and development of keloids. Mutations in *FAS*, one of the main genes mediating apoptosis, may cause the abnormal apoptosis of KF cells and lead to the formation of keloids. In fact, transfecting normal copies of the *FAS* gene into KFs using a recombinant adenovirus vector caused an increase in FAS expression and induced normal apoptosis and prevented the onset of keloids (Lu and Gao, 2005). As early as 1998, scientists identified and confirmed the presence of a mutation in the tumor-suppressor gene *P53* in keloid tissue that was absent in normal skin tissue (Saed et al., 1998). In addition, BCL-2 and the proteins of the caspase family have been shown to have abnormal expression in keloid tissue (Teofoli et al., 1999; Akasaka et al., 2005). miRNAs have been shown to regulate disease-causing genes. Huang et al. discovered that the expression of *Fas* and miR-20a were negatively correlated in 8 osteosarcoma cell lines. Experiments *in vitro* and *in vivo* showed that, in osteosarcoma, Fas expression was downregulated by miR-20a from the miR-17-92 cluster (Huang et al., 2012). miR-15 and miR-16 were found to induce apoptosis in chronic lymphocytic leukemia cells by regulating bcl-2 expression (Cimmino et al., 2005). Fang et al. showed that miR-378 can inhibit the expression of its target gene caspase-3, thereby reducing ischemic injury in myocardial cells (Fang et al., 2012).

Since miRNAs have been shown to play a role in the onset and progression of more than 70 different diseases, we hypothesized that miRNAs may play an important role in the formation of keloids (Lu et al., 2008). Therefore, we used the miRNA chip miRCURYTM LNA Array (v14.0) to identify, in a high-throughput manner, miRNAs that are differentially expressed in keloid *vs* normal skin. Indeed, we established the miRNA expression profile of keloid tissue and validated the results from the microarray analysis by qRT-PCR.

The gene encoding miR-199a is in the 16th intron of the *DNM2* gene. The majority of pre-miRNAs are processed in the cytoplasm by Dicer into siRNA-like double-stranded miRNAs that consist of a duplex of 2 miRNA fragments- miRNA and miRNA*- which are also called -5p/-3p, respectively. According to the literature, miR-199a-5p is closely associated with the onset and development of various diseases, including organ fibrosis, malignancy, etc. In 2006, Murakami et al. reported that, compared to normal tissue, the expression of miR-199a-5p and miR-199a-3p was downregulated in hepatocellular carcinoma cells. Their expression levels were negatively correlated with the degree of differentiation of the hepatocellular carcinoma (Murakami et al., 2006). Shen et al. (2010) used qRT-PCR analysis to discover that miR-199a-5p expression was significantly decreased in 65.2% hepatocellular carcinoma tissues and in 4/5 cell lines studied. Reduced expression of miR-199a-5p was found to promote the metastasis of hepatocellular carcinoma by inhibiting the activity of the miR-199a-5p target gene *DDR1* (Shen et al., 2010). Its expression was correlated with the degree of malignancy in testicular cancer, and it was found to inhibit tumor migration and invasion and cell proliferation. One of the target genes of miR-199a-5p, *PODXL*, showed overexpression in testicular cancer (Cheung et al., 2011). Intracellular loss of *PODXL* has been shown to inhibit the invasion of cancer (Mota et al., 2001). The relationship between *PODXL* and miR-199a-5p expression suggests that PODXL might be a downstream effector of miR-199a-5p function (Cheung et al., 2011). miR-199a expression was found to be significantly higher in gastric cancer tissues than in normal gastric tissue and non-metastatic gastric cancer tissues. This miRNA was found to regulate the expression of mitogen-activated protein kinase kinase kinase 11(MAP3K11). MAP3K11 inhibits the growth of tumor cells, and decreased the MAP3K11 protein level from gastric cancer cells causes a marked reduction in the metastasis of these cells (Song et al., 2010). miR-199a-5p expression is also upregulated in a mouse model of cardiac hypertrophy, while appearing to be significantly downregulated at early stages of heart failure (Rane et al., 2010). Recently, Yoshiki et al. found that 4 miRNAs- miR-199a, miR-199a*, miR-200a, and miR-200b, were upregulated in human and mouse liver fibrosis. The expression level of miR-199a-5p was related to the degree of hepatic fibrosis. Furthermore, in hepatic stellate cells, the expression of the genes related to fibrosis, for example *TIMP1*, increased with the overexpression of these 4 miRNAs, which suggested that these 4 miRNAs might participate in the process of hepatic fibrosis (Murakami et al., 2011).

The results of the EdU staining experiment showed that the proliferation rate of keloid fibroblasts transfected with miR-199a-5p was remarkably lower than of mock-transfected cells. There were also significant changes in the cell cycles of the miR199a-5p-transfected and mock-transfected cells. The proportion of cells in the G1 phase decreased significantly, while the proportion of cells in the S phase and the G2/M phase increased significantly upon miR-199a-5p transfection. The miR-199a-5p-transfected cells likely become arrested in the in S phase or the G2/M phase of their cell cycle. Taken together, this data suggests that miR-199a-5p may inhibit the proliferation of KFs. KFs are a good model for keloid formation and growth. KFs grow much faster *in vitro*, show disordered structure, and show increased collagen synthesis as compared to normal skin cells (Abergel et al., 1985). Hence, factors that cause excessive the proliferation or functioning of KFs may also lead to the formation of keloids. Therefore, we inferred that the overexpression of miR-199a-5p can inhibit the proliferation of keloid cells. Downregulation of miR-199a-5p may inhibit apoptosis and promote the formation of keloids. The mechanism of action of miR-199a-5p may be mediated through

changes in the cell cycle. This study provides a novel basis for studying the mechanism underlying the etiology of keloids.

Based on our knowledge of miRNA function, it is likely that miR-199a-5p may inhibit the expression of genes involved in the proliferation of KFs. However, further studies are required to determine the exact mechanism of miR-199a-5p action in the etiology of keloids.

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