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Tetrandrine induces microRNA differential expression in human hypertrophic scar fibroblasts *in vitro*

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ABSTRACT. MicroRNAs (miRNAs) have recently been shown to play a role in normal wound healing process. miRNAs may be linked to pathologic wound healing and closely related to the formation of hypertrophic scars. This study aimed to explore the effects of tetrandrine on the miRNA expression profile in human hypertrophic scar fibroblasts (HSFs) *in vitro*. HSFs were randomly divided into two groups: the tetrandrine treatment group and the control group. The experimental and control groups were collected and analyzed by miRNA array after a 48-h culture. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to confirm the array results. The targets of differentially expressed miRNA were functionally annotated using bioinformatic approaches. miRNA microarray analysis identified 193 differentially expressed miRNAs and the expression of 186 miRNAs in the experimental group decreased while that of 7 miRNAs increased compared to the control group. The most significantly

downregulated miRNA was hsa-miR-1246, and hsa-miR-27b had the highest expression level. Significant differentially expressed miRNAs were predicted to be related to several important signaling pathways related to scar wound healing. The differential miRNA expression identified in this study provides the experimental basis for further understanding the anti-fibrosis effect of tetrandrine.

Key words: Fibroblasts; Hypertrophic scars; MicroRNA; Expression profile; Tetrandrine

INTRODUCTION

Hypertrophic scars result from an abnormal fibrous wound healing process and represent a connective tissue response to trauma, inflammation, surgery, or burns. It is believed that proliferation of fibroblasts plays an important role in hypertrophic scar formation and is involved in reepithelialization, extracellular matrix (ECM) deposition, neovascularization, and ECM remodeling (van der Veer et al., 2009). Both experimental and clinical researchers have shown that tetrandrine, a bisbenzylisoquinoline alkaloid isolated from the root of *Stephania tetrandra*, exerted anti-inflammatory properties, attenuated ECM deposition, and exhibited antifibrogenic activity against fibroblasts (Reist et al., 1993; Huang and Hong, 1998; Oh and Lee, 2003). Our previous studies have provided evidence that tetrandrine significantly inhibits proliferation of hypertrophic scar fibroblasts (HSFs) and decreases the expression of DNA (Liu et al., 2001). However, the underlying mechanism remains unclear.

MicroRNAs (miRNAs) are a new class of regulatory noncoding single-stranded RNAs (19-22 nucleotides), which can suppress the expression of protein-coding genes by targeting the 3'-untranslated region (UTR) of messenger RNAs (mRNAs) and play a key role in cellular growth and differentiation, as well as in disease development (Bartel, 2004; Kloosterman and Plasterk, 2006; Zhao and Srivastava, 2007). It is predicted that up to 30% of human genes are regulated by miRNAs. In addition, each miRNA can target several hundred mRNA 3'-UTRs, making miRNAs a large family of 'regulatory' molecules (Aberdam et al., 2008). However, the anti-fibrosis metabolism mechanism of tetrandrine and whether it is related to miRNAs remains unclear. In this study, we tested the effects of tetrandrine on mRNA expression in HSFs and analyzed putative targets of differentially expressed miRNAs using bioinformatic approaches.

MATERIAL AND METHODS

Fibroblast isolation and cell culture

HSFs were established as a primary cell line from hypertrophic scar tissue obtained from severe burn patients who underwent orthopedic surgery at the Department of Plastic and Reconstructive Surgery of the First Affiliated Hospital of Nanchang University, China. Written informed consent was obtained according to the rules and regulations set by the Ethics Committee of the First Affiliated Hospital of Nanchang University.

Hypertrophic scar tissue was cut into 0.5- to 1-mm^3 pieces using a pair of scissors, and the epidermis and dermis were isolated by digestion with 0.25% Trypsin + EDTA (Gibco, USA) at 4°C

for 10-12 h. The pieces were then placed in 25-cm^2 cell culture flasks (Corning, USA), and 5 mL culture medium containing Dulbecco's modified Eagle's medium (DMEM) with 100 U/mL penicillin and 100 U/mL streptomycin (Solarbio) and 10% fetal bovine serum (HyClone, USA) was added. The flasks were maintained at 37°C in air containing 5% CO_2. The culture medium was changed (5 mL) every 5 days. HSFs grew to fusion for 14 days and were then subcultured into 25-mm^2 culture flasks. Experiments were performed with early passage cells (4-6) (Russell and Witt, 1976).

Drug treatment and morphological observation of cultured fibroblasts

HSFs (1 x 10^5) seeded on 6-well plates were subjected to different treatments: a control group consisting of fibroblasts cultured with DMEM only and a test group where 5 mg/mL tetrandrine (molecular formula $C_{38}H_{42}N_2O_6$, Yingtao, China) was added to the culture medium based on our previous results indicating that the inhibition ratio of HSFs was 50.72% at 5 mg/mL tetrandrine (Zunwen et al., 2012). After adding the tetrandrine, morphological changes in the HSFs were observed with an inverted microscope, and photos were taken every 24 h.

Sample harvest

After 72 h, the test and control group cells were digested and made into single-cell suspensions. Then, the cells were centrifuged at 1000 rpm for 5 min and the supernatant removed.

Total RNA extraction and purification

RNA was isolated using TRIZOL and then quantitated using a spectrophotometer. The total RNA quality was examined using formaldehyde denaturing gel. Total RNA from the cells was isolated using the mirVana™ miRNA Isolation Kit (Applied Biosystems) following the manufacturer protocol.

miRNA microarray analysis

We used 100 ng miRNA to perform the *in situ* oligonucleotide microarray. Fluorescent miRNA was labeled using the miRNA Complete Labeling and Hyb Kit (Agilent, USA) according to the manufacturer protocol. The hybridization was carried out for 20 h at 55°C in a rotating hybridization oven according to the instructions. After hybridization, slides were washed and then scanned by AgilentHD_miRNA. The Feature Extraction (v10.7) software was required for analysis of the images and extraction of scan data. Then, the GeneSpring software was used for data normalization, and the criterium using for differentially expressed miRNAs analysis was an absolute fold-change of at least more than 1-fold.

RT-PCR for miRNA expression analysis

To validate the miRNA microarray data, the significantly upregulated and downregulated miRNAs were selected and analyzed by RT-PCR assays, which were carried out using a 7900 HT Fast RealTime PCR system (Applied Biosystems). The miRNA-specific primers for hsa-miR-125b, hsa-miR-27b, and internal control U6 were purchased from Invitrogen (USA). Expression levels of each mature miRNA were evaluated using the comparative threshold cycle (Ct) method and

normalized to those of U6 small nuclear RNA for each condition (calculated using the comparative Ct method and analyzed by $2^{-\Delta\Delta Ct}$; Schmittgen and Livak, 2008).

Prediction of mRNA target

Three commonly used databases including miR, (http://www.microrna.org/microrna/home.do), PicTar (http://www.pictar.org/), and TargetScan (http://www.targetscan.org/) were used to obtain predicted gene targets for significantly differentially expressed miRNAs. The genes identified using at least two of the methods were considered potential target genes regulated by a given miRNA. Predicted target genes in combination with miRNA and whole-genome microarray data were used to visualize possible biological miRNA/mRNA processes correlating to HSF growth and/or differentiation. Then, the predicted target genes underwent enrichment analysis of cell signaling pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Hua et al., 2009).

RESULTS

Morphological changes in HSFs

HSFs cultured for 48 h exhibited a long spindle-shaped structure with larger cell bodies. The cytoplasms of the fibroblasts were rich and showed multiple-angle shapes, which grew two or three different synaptic lengths (Figure 1A). Compared to the control cells, the number of HSFs treated with tetrandrine decreased, the shape of cells became smaller and round, and spindles became shorter or disappeared (Figure 1B).

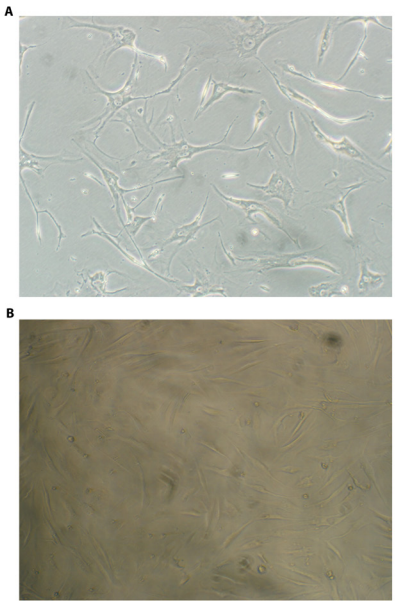

Figure 1. Morphological changes of hypertrophic scar fibroblasts (HSFs) observed under inverted microscope (magnification 200T). HSFs were subjected to different treatment: control group (**A**), tetrandrine group (**B**). It represents the morphological changes of HSFs for 48 h.

Table 1. Downregulated miRNAs in the test group relative to the control group.

miRNA-ID	Raw experimental signal values (test group)	Raw experimental signal values (control group)	Absolute fold-change (test *vs* control)
hsa-miR-1246	14.8059	295.8396	19.282063
hsa-miR-10b	7.13783	71.5005	9.666621
hsa-miR-760	2.24873	22.43954	9.629608
hsa-miR-150*	12.08515	117.3476	9.370315
hsa-miR-1268	15.42086	128.8487	8.063123
hsa-miR-939	22.99794	163.953	6.8795867
hsa-miR-378	7.06811	45.4653	6.2073846
hsa-miR-483-5p	11.66605	72.784	6.020657
hsa-miR-1290	13.611179	83.731895	5.9364524
hsa-miR-1275	41.9974	240.9115	5.53563
hsa-miR-503	4.55466	26.109589	5.5319195
hsa-miR-30d	4.70458	24.4761	5.020572
hsa-miR-1202	175.239	901.888	4.96654
hsa-miR-181b	4.28493	20.67487	4.656192
hsa-miR-224	8.87527	41.6338	4.526854
hsa-miR-542-5p	7.32674	34.3547	4.5248823
hsa-miR-1225-5p	99.1514	446.079	4.341553
hsa-miR-542-3p	4.43398	19.74572	4.297453
hsa-miR-630	37.1656	164.6234	4.27447
hsa-miR-188-5p	24.547901	98.9035	3.8880258
hsa-miR-99a	95.2809	374.3466	3.7914007
hsa-miR-1207-5p	249.101	921.96497	3.5716655
hsa-miR-1226*	7.88485	29.01783	3.5514302
hsa-miR-125a-3p	23.97645	86.0822	3.4646585
hsa-miR-299-3p	4.8526	17.40824	3.4618816
hsa-miR-1305	235.558	821.61597	3.3659143
hsa-miR-500a	27.36765	93.7181	3.3045917
hsa-miR-1977_v14.0	7.07214	23.9922	3.2737923
hsa-miR-1914*	150.7833	511.42902	3.2731354
hsa-miR-362-5p	9.51801	30.55838	3.0982468
hsa-miR-1295	12.04883	38.6351	3.0943468
hsa-miR-134	39.161	123.9933	3.055458
hsa-miR-532-5p	12.5388	39.5899	3.0469153
hsa-miR-30c	5.37916	16.6203	2.981648
hsa-miR-424	135.9667	419.182	2.9751027
hsa-miR-101	11.2223	33.744667	2.9017177
hsa-miR-1308_v15.0	434.11072	1303.179	2.896913
hsa-miR-30e	13.2306	38.25769	2.7904298
hsa-miR-575	87.0917	250.1585	2.7718544
hsa-miR-494	77.8722	223.0759	2.7644079
hsa-miR-886-3p_v15.0	199.30449	568.25476	2.7514274
hsa-miR-892b	57.324	163.1691	2.7468398
hsa-miR-218	40.8108	114.9884	2.719009
hsa-miR-762	273.9874	768.546	2.706893
hsa-miR-26a	112.953896	312.659	2.6711693
hsa-miR-23a*	16.78239	45.90035	2.6393325
hsa-miR-31	72.4642	196.56601	2.6176813
hsa-miR-320c	101.8523	275.8135	2.6132224
hsa-miR-638	218.59781	590.577	2.607129
hsa-miR-1288	109.0787	292.881	2.591094
hsa-miR-662	5.20603	13.94821	2.585495
hsa-miR-155	55.2021	147.10541	2.5716083
hsa-miR-152	19.8854	52.8124	2.5629108
hsa-miR-92a	12.6899	33.34922	2.5360584
hsa-miR-373*	4.28093	11.2446	2.5347652
hsa-miR-195	34.71508	90.1275	2.505365
hsa-miR-202	96.22861	246.725	2.474235
hsa-miR-342-3p	10.5277	26.3865	2.4186897
hsa-miR-497	7.44844	18.4124	2.3854864
hsa-miR-151-3p	13.4891	32.79506	2.3461585
hsa-miR-99b	22.57304	54.7146	2.3390799
hsa-let-7i	832.487	2007.053	2.3265529
hsa-miR-423-5p	5.87581	14.131	2.3207967
hsa-miR-1915	144.2714	345.15	2.308656
hsa-miR-320a	69.0542	163.539	2.2854054
hsa-miR-1260	77.47149	183.04642	2.2800863
hsa-miR-381	13.2529	31.1877	2.2709332
hsa-miR-128	4.76358	11.1013	2.2489104
hsa-miR-501-5p	27.15319	63.034397	2.240209
hsa-miR-137	52.1592	119.809494	2.2166247
hsa-miR-374b	12.55825	28.3029	2.1748722
hsa-miR-199b-5p	351.8671	789.69196	2.1657622
hsa-miR-125b	2194.671	4902.39	2.1556098
hsa-miR-16	599.572	1313.446	2.1139898
hsa-miR-26b	86.1997	188.4135	2.109299
hsa-miR-1224-5p	35.1823	76.369	2.0947142
hsa-miR-98	28.9707	62.66643	2.087411
hsa-miR-660	9.15352	19.6795	2.0747123
hsa-miR-654-3p	16.0017	34.3716	2.072839
hsa-miR-196a	276.8318	594.26404	2.0715487
hsa-miR-572	9.68648	20.7287	2.0650852
hsa-miR-31*	56.4231	119.8061	2.049055
hsa-miR-222	36.456	76.92737	2.0363092
hsa-let-7e	375.427	789.65796	2.0297637
hsa-miR-125a-5p	75.8074	158.9961	2.0239823
hsa-miR-324-3p	29.401241	61.6354	2.0230024
hsa-miR-625	5.16288	10.8205	2.0224943
hsa-miR-151-5p	99.916306	209.0674	2.0192115
hsa-miR-214	69.86972	145.8224	2.0140345
hsa-miR-199a-3p	2086.856	4347.41	2.0103414

Table 2. Upregulated miRNAs in the test group relative to the control group.

miRNA-ID	Raw experimental signal values (test group)	Raw experimental signal values (control group)	Absolute fold-change (test *vs* control)
hsa-miR-27b	246.7309	171.95459	1.4868867
hsa-miR-29b-1*	45.064598	32.8065	1.4234551
hsa-miR-193a-3p	35.012	28.9008	1.2553804
hsa-miR-493*	18.6228	16.9013	1.1418079
hsa-miR-100	1330.14	1253.593	1.0995347
hsa-miR-27a	1042.83	1010.114	1.0698209
hsa-miR-29b	1373.474	1334.3251	1.0666622

Differential miRNA expression between the test group and the control group

To distinguish the differentially expressed miRNAs, we examined the expression of miRNAs in the test and control groups using an miRNA microarray. Only miRNAs with changes of at least 1-fold were included. By this criterion, 193 miRNAs were identified, among which 186 were downregulated (Table 1 shows miRNAs whose expression exceeded 2-fold) and 7 were upregulated (Table 2) in the test group compared to that in the control group.

Validation of the microarray data by RT-PCR

To validate the results from the miRNA microarray, we further employed RT-PCR to measure the abundance of the miRNA, including downregulated hsa-miRNA-125b and upregulated hsa-miRNA-27b. The $2^{-\Delta\Delta Ct}$ values showed that hsa-miR-27b (1.92>1.0) was upregulated, while hsa-miR-125b (0.74<1.0) was downregulated. The RT-PCR data indicated that the transcriptional level of hsa-miRNA-125b and hsa-miRNA-27b coincided perfectly with the microarray results.

Putative targets of miRNAs and functional analysis by bioinformatics

All differentially expressed miRNAs were subjected to target gene prediction. Each miRNA potentially regulates many targets. To decrease the total number of false-positive targets, targets predicted by both methods were considered putative candidates. After carefully analyzing the putative targets, we identified targets for each miRNA. To elucidate the target pathways of miRNAs, KEGG pathway analysis was employed to clarify the biological significance of these potential target pathways, helping us to further understand the biological processes and corresponding metabolic networks regulated by potential miRNAs. We found that many targets played significant roles in several signaling pathways that play important roles in wound repair including vascular endothelial growth factor (VEGF), apoptosis, and the cell cycle. Table 3 shows part of the putative targets of hsa-miR-125b and hsa-miR-27b.

Table 3. Putative targets of hsa-miR-125b and hsa-miR-27b.

miRNA-ID	Targets	Gene name
hsa-miR-125b	*STARD13*	Star-related lipid transfer (START) domain containing 13
	COL4A3	Collagen, type IV, alpha 3
	FGFR2	Fibroblast growth factor receptor 2
	BCL2L12	BCL2-like 12
	TNFSF4	Tumor necrosis factor (ligand) superfamily, member 4
	ATP10D	Atpase, Class V, type 10D
hsa-miR-27b	*VEGFC*	Vascular endothelial growth factor C
	APAF1	Apoptotic protease activating factor
	COL19A1	Collagen, type XIX, alpha 1
	GDF8	Growth differentiation factor 8
	EGFR	Epidermal growth factor receptor

DISCUSSION

Hypertrophic scar formation occurs when the equilibrium between positive and negative cytokines stimulated by inflammation and the synthesis and metabolism of ECM is broken (van der Veer et al., 2009). The abnormal biological behavior of fibroblasts plays a critical role in the scar formation process. Tetrandrine is a bisbenzylisoquinoline alkaloid derived from *S. tetrandra*. Previous pharmacological and clinical studies have shown that tetrandrine possesses anti-inflammatory, anti-proliferative, immunosuppressive, and antitumor activities (Dong et al., 1997; Lai et al., 1998; Xie et al., 2002; Yoo et al., 2002; Lee et al., 2002; Kuo and Lin, 2003; Wang et al., 2004). In recent years, it was discovered that tetrandrine can inhibit proliferation of human Tenon's capsule fibroblasts and differentiation of osteoclasts (Takahashi et al., 2012; Li et al., 2012). In our previous studies, we showed that tetrandrine inhibited proliferation of HSFs and synthesis of collagen and DNA (Liu et al., 2001). However, it is still unclear how tetrandrine inhibits HSFs. MicroRNAs are known to play critical roles in development, cell proliferation, and other fundamental cellular processes (Bartel, 2004; Zhao and Srivastava, 2007). Our previous studies have confirmed distinct differences in miRNA expression between human hyperplastic scar tissue and normal skin, which may be closely correlated to the formation, development, and evolution of hyperplastic scarring (Ning et al., 2012). Furthermore, Wilmink et al. (2010) have shown that dermal fibroblasts differentially express 123 miRNAs when exposed to hyperthermia using an miRNA microarray. In this study, we identified 193 potential miRNAs that were differentially expressed between the tetrandrine test group and the control group with 186 miRNAs downregulated and 7 upregulated.

Some miRNAs identified in this study have been shown to play important roles in some cellular mechanisms. Gutierrez et al. (2011) demonstrated that thyrotroph embryonic factor is downregulated by miR-125b through activation of p53 and this novel regulation pathway helps determine the actin distribution and the shape of fibroblasts. Moreover hsa-miR-125b was observed to regulate cell proliferation and differentiation in both breast cancer and ovarian cancer cell lines and plays an important role in osteoblastic differentiation (Iorio et al., 2005; Mizuno et al., 2008; Guan et al., 2011). Hsa-miR-125a-3p and hsa-miR-125a-5p were found to affect the migration and invasion of lung cancer cells, while hsa-miR-99a was found to affect the differentiation of keratinocytes (Jiang et al., 2010; Lerman et al., 2011). Recent evidence has shown that hsa-miR-155 may promote cell proliferation by regulating its target genes and can regulate the expression of the angiotensin II type 1 receptor in primary human lung fibroblasts (Kong et al., 2010; Martin et al., 2013). While upregulated in the test group, hsa-miR-29b has been found to inhibit the expression of collagen 1 protein in skin fibroblasts by the way of classic mRNA transcriptional regulation *in vitro* (Wang et al., 2011). The results of the study by Crist et al. (2009) showed that overexpression of an miR-27b transgene in Pax3-positive cells in the embryo leads to downregulation of Pax3, resulting in interference with progenitor cell migration and in premature differentiation. Furthermore, miR-27b inhibitors were transfected into cultures of adult muscle satellite cells that normally express miR-27b at the onset of differentiation, which resulted in continuing Pax3 expression leading to more proliferation and a delay in the onset of differentiation.

To obtain a better understanding of the functional significance of miRNA, it was important to identify and validate the miRNA targets. In this study, hundreds of target genes were predicted, including several key mediators of cellular signaling pathways and some ECM proteins. Among them, we analyzed the putative targets of hsa-miR-125b and hsa-miR-27b, which were validated by RT-PCR. According to the results, these targets were involved in several signaling pathways

such as VEGF, the cell cycle, and apoptosis. The BCL2 protein played a key role in apoptosis, cell migration, proliferation, and differentiation (Cory and Adams, 2002). COL4 was closely related with collagen formation, and fibroblast growth factor was important for fibroblast growth (van der Veer et al., 2009). VEGF, the target gene of hsa-miR-27b, was not only an important growth factor for promoting endothelial cell proliferation but also played an important role in protein synthesis and the formation of granulation tissue in the inflammatory and wound repair proliferative phases (Ferrara et al., 2003). Moreover, the target genes of hsa-miR-27b including *COL19A1*, *GDF8*, and *EGFR* were also related to collagen formation, cell differentiation, and epidermal growth. GDF8 has been proven to be a pro-fibrogenic factor that promotes fibroblast proliferation and ECM synthesis in tendons and ligaments (van der Veer et al., 2009; Fulzele et al., 2010). miRNAs might contribute to the anti-fibrosis properties of tetrandrine by regulating its targets and influencing multiple-signaling pathways.

As a traditional Chinese medicine, tetrandrine has been used for the treatment of arthritis, arrhythmia, hypertension, inflammation, and silicosis for years in the clinic and as a therapeutic (Fang and Fang, 1996; Pang and Hoult, 1997; Shen et al., 2001). However, tetrandrine has not been utilized as a clinical treatment for hypertrophic scarring because its mechanism is unknown. This study has identified the differentially expressed miRNAs in HSFs treated with tetrandrine through genomic profiling, and the putative targets of these altered miRNAs are most likely involved in multiple signaling pathways that have previously been shown to be implicated in cellular growth. Hence, certain miRNAs might contribute to the anti-fibrosis of tetrandrine in HSFs through the signaling pathways of these targets.

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