Research Article

Expression Profile of Long Noncoding RNAs in Human Earlobe Keloids: A Microarray Analysis

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Background. Long noncoding RNAs (lncRNAs) play key roles in a wide range of biological processes and their deregulation results in human disease, including keloids. Earlobe keloid is a type of pathological skin scar, and the molecular pathogenesis of this disease remains largely unknown. Methods. In this study, microarray analysis was used to determine the expression profiles of lncRNAs and mRNAs between 3 pairs of earlobe keloid and normal specimens. Gene Ontology (GO) categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed to identify the main functions of the differentially expressed genes and earlobe keloid-related pathways. Results. A total of 2068 lncRNAs and 1511 mRNAs were differentially expressed between earlobe keloid and normal tissues. Among them, 1290 lncRNAs and 1092 mRNAs were upregulated, and 778 lncRNAs and 419 mRNAs were downregulated. Pathway analysis revealed that 24 pathways were correlated to the upregulated transcripts, while 11 pathways were associated with the downregulated transcripts. Conclusion. We characterized the expression profiles of lncRNA and mRNA in earlobe keloids and suggest that lncRNAs may serve as diagnostic biomarkers for the therapy of earlobe keloid.

1. Introduction

Keloids are defined as pathologically formed scars that exceed the boundary of the original wound [1]. They are also deemed as benign dermal tumors that are unique to humans. Etiologically, keloids may occur because of minor skin injury, such as body piercing and insect bites. In addition, it is widely agreed that the incidence rate of keloid is significantly higher in populations with darker skin, such as Africans and Asians. The external ear is one of the most common sites for keloid formation [2]. Many different treatment modalities such as surgical excision, intralesional corticosteroids, radiotherapy, and pressure earrings have been used for keloids [3, 4]. Although it has unclear etiology, the development of keloid could be considered as a process of abnormal wound healing, during which redundant extracellular collagen fibers as well as proteoglycans are deposited [5]. It is known that various molecular factors contribute to this process, for example, growth factors [6, 7], cytokines [8], and related gene pathways [9]. Some among them may be the key points that could stop or reverse this pathologic process. For example, transforming growth factor-β (TGF-β) receptor was recently reported to be a potential target in treating keloid [10]. However, deeper understanding of the molecular mechanism of keloid formation is still required for detecting critical biological factors and for the further development of effective therapies.

It is known that 90% of the human genome is transcribed to RNAs that do not code proteins (noncoding RNAs). A lot of evidence suggests that long noncoding RNA (lncRNA; >200 nucleotides) regulates protein-coding genes at the transcriptional and posttranscriptional levels, as well as transcription control [11, 12]. It is known that lncRNAs play important roles in cellular differentiation, development, and disease [11, 12]. However, for earlobe keloids, the expression or function of lncRNAs has not been studied to date.

It the present study, global expression profiles of the lncRNAs and the mRNAs from 3 pairs of earlobe keloid specimens and normal skin tissues were detected using a microarray technique, from which significantly dysregulated lncRNAs and mRNAs were screened. These results indicated
that the aberrant expression levels of lncRNAs may have important roles in the development of earlobe keloid and that knowing the differently expressed lncRNAs might provide useful biomarkers for earlobe keloid therapy and diagnosis.

2. Materials and Methods

2.1. Patients and Specimens. The study procedures were approved by the Ethics Review Board of Wuhan General Hospital of Guangzhou Military Command of the People's Liberation Army and it was carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association. Keloid was diagnosed by the overgrowth of a scar that obviously exceeded the boundary of the original wound. Demographic and clinical characteristics of the patients were extracted from their medical records. Earlobe keloid specimens were obtained from the resected keloid at our outpatient clinic. The normal skin specimens were obtained from the ear of the same patient. All patients were fully informed of the aim and protocol of the study and gave written informed consent to participate in the study.

2.2. RNA Isolation, Quantification, and Quality Control. Total RNA was extracted with the mirVana miRNA Isolation Kit (Applied Biosystems) and then eluted with 100 μL of nuclease-free water. Total RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific) and the integrity of RNA was determined using an Agilent 2100 bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies).

2.3. RNA Labeling and Array Hybridization. RNA sample preparation and microarray hybridization were performed according to Agilent One-Color Microarray-Based Gene Expression Analysis Protocol (Agilent Technologies, Santa Clara, CA, USA) with minor modifications. RNA was purified from 100 μg total RNA after removal of rRNA using RNeasy Mini Kit (Qiagen). After that, specimens were amplified and transcribed into cRNA, and cyanine-3-CTP was applied to label the cRNA (Quick Amp Labeling Kit: Agilent Technologies). Labeled cRNA was once again purified with the RNeasy Mini Kit (Qiagen) and quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific).

The cRNA was fragmented and hybridized using an Agilent Gene Expression Hybridization Kit (Agilent): 0.6 μg labeled cRNA was fragmented by adding 5.0 μL 10x blocking agent and 1.0 μL 25x fragmentation buffer, and then the mixture was heated at 60°C for 30 minutes. After that, 25 μL 2x GEx Hybridization Buffer was added to stop the fragmentation reaction. Finally, 50 μL hybridization solution was dispensed into the gasket slide and assembled to the lncRNA expression microarray slide. The slides were incubated for 17 hours at 65°C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed, and scanned with using the Agilent DNA Microarray Scanner (part number, G2505C).

2.4. Data Analysis. Data were extracted with Agilient Feature Extraction software 11.7.1.1. GeneSpring GX 12.5 (Agilent Technologies) was used to normalize the quantiles of the raw data. The lncRNAs are carefully constructed using the quality-controlled, public transcriptome databases (RefSeq, UCSC Known Genes, IncRNAWiki, LNCipedia, NONCODE v4, fRNAdb v3.4, Broad lncRNA, GENCODE, etc.), as well as landmark publications. After that, lncRNAs and mRNAs with significant differential expression between the two groups were identified, and the volcano plot was drawn. Hierarchical clustering was performed using MeV 4.9.0 (http://www.tm4.org/mev.html), and heat maps were obtained by this analysis. Gene Ontology (GO) analysis was performed based on Gene Ontology (www.geneontology.org), which provides three structured networks of defined terms that describe gene product functions. Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) database was used for pathway analysis of the differentially expressed genes.

2.5. Quantitative Real-Time PCR (qRT-PCR). The total RNA was isolated using mirVana miRNA Isolation Kit (Applied Biosystems) and then reverse-transcribed using Prime-Script RT reagent kit with gDNA Eraser (Perfect Real Time; TaKaRa). The expression of five upregulated lncRNAs and five downregulated lncRNAs was measured by qRT-PCR using SYBR Green assays (TaKaRa), and GAPDH was used as an internal control. The expression level of each lncRNA was represented as a fold change using \(2^{- \Delta \Delta C_t}\) methods. The expression levels of lncRNAs differentially expressed between earlobe keloid specimens and normal skin specimens were analyzed using Student's \(t\)-test with SPSS version 17.0 [13].

2.6. Statistics. Statistical analysis was performed with SPSS version 19.0. The differences in expression levels of tested lncRNAs and mRNAs between earlobe keloid and normal skin tissues were assessed using Student's \(t\)-test, and fold change \(\geq 2.0\) and \(P < 0.05\) were considered significant. Fisher's exact test was used for GO analysis and KEGG pathway analysis. \(P < 0.05\) was considered significant.

3. Results

3.1. Differentially Expressed lncRNAs. The baseline data for the 3 patients (3 pairs of specimens) included in the study are shown in Table 1. In order to compare the distributions

Table 1: Baseline data of included patients.

| Case | Age (years) | Gender | Reason of skin injury | Size of keloid (cm × cm × cm) | History of keloid (months) |
|------|-------------|--------|-----------------------|--------------------------------|----------------------------|
| 1    | 21          | Female | Earlobe piercing      | 2.0 × 1.3 × 0.8               | 10                         |
| 2    | 34          | Female | Earlobe piercing      | 1.8 × 1.5 × 1.2               | 14                         |
| 3    | 24          | Female | Earlobe piercing      | 2.7 × 2.0 × 1.5               | 17                         |
of intensities from all samples, we used a box plot to visualize the distributions of a dataset. Box-whisker plotting suggested similar distribution of the data from six RNA gene chips (Figure 1(a)). The expression profiles of 2068 IncRNAs indicated that they were differentially expressed (fold change ≥ 2.0 and \( P < 0.05 \)) between earlobe keloid specimens and normal skin specimens (shown in the IncRNA profiling). Variations in IncRNA expression among specimens were shown by volcano plotting and scatter plotting (Figures 1(b) and 1(c)). Among these IncRNAs, 1290 were upregulated more than twofold in the earlobe keloid specimens compared to the normal skin specimens, while 778 IncRNAs were downregulated more than twofold. IncRNA expression data are deposited at Gene Expression Omnibus under accession number GSE83286. The top 20 differentially expressed IncRNAs are listed in Tables 2 and 3. Finally, to infer the relationships among specimens, hierarchical clustering was performed to show distinguishable IncRNA expression patterns among samples (Figure 1(d)).

3.2. Differentially Expressed mRNAs. A total of 1511 mRNAs were differentially expressed between the two tissues (fold change ≥ 2.0 and \( P < 0.05 \)). A total of 1092 of 1511 mRNAs were expressed significantly higher in earlobe keloid
### Table 2: The top 20 upregulated lncRNAs.

| Seq. name | Source | Foldchange | Chrom. Strad. txStrat txEnd | Associated gene name |
|-----------|--------|------------|----------------------------|----------------------|
| NONHSAT120157 | NONCODEv4 | 302.566 | chr7 37946864 37949441 | SFRP4 |
| NONHSAT062994 | NONCODEv4 | 198.76 | chr19 18896928 18897844 | COMP |
| ENST00000424523 | Ensembl | 187.8763 | chr7 + 92484223 92546465 |
| NONHSAT016934 | NONCODEv4 | 121.1942 | chr10 127823937 127843874 | ADAM12 |
| NONHSAT016933 | NONCODEv4 | 66.81899 | chr10 127779305 127798357 | ADAM12 |
| NONHSAT033754 | NONCODEv4 | 59.65379 | chr13 + 50191636 50192101 |
| NONHSAT102388 | NONCODEv4 | 57.30576 | chr5 + 79377827 79379011 | THBS4 |
| NONHSAT016928 | NONCODEv4 | 56.89644 | chr10 127700956 127703336 | ADAM12 |
| NONHSAT076769 | NONCODEv4 | 55.34357 | chr2 + 216476099 216669548 | LINC00607 |
| NONHSAT033252 | NONCODEv4 | 45.90168 | chr13 − 38137358 38144948 | POSTN |
| NONHSAG013256 | NONCODEv4 | 45.0158 | chr13 − 38136835 38145672 | POSTN |
| ENST00000557618 | Ensembl | 40.71083 | chr14 + 60981837 61021634 |
| ENST00000597626 | Ensembl | 37.49051 | chr21 + 35287852 35341659 |
| NONHSAG030448 | NONCODEv4 | 36.29881 | chr2 − 216232403 216237205 | FN1 |
| NONHSAT056875 | NONCODEv4 | 34.17572 | chr18 + 907552 909671 | ADCYAP1 |
| NONHSAT125631 | NONCODEv4 | 32.53344 | chr9 + 34084331 34096676 | DCAF12 |
| NONHSAT100815 | NONCODEv4 | 31.91256 | chr5 + 28524293 28602803 |
| NONHSAT077639 | NONCODEv4 | 30.05823 | chr2 − 238241611 238243429 | COL6A3 |

### Table 3: The top 20 downregulated lncRNAs.

| Seq. name | Source | Foldchange | Chrom. Strad. txStrat txEnd | Associated gene name |
|-----------|--------|------------|----------------------------|----------------------|
| NONHSAT053431 | NONCODEv4 | 22.78803 | chr17 + 37395854 37400623 | FBXL20 |
| NONHSAT030286 | NONCODEv4 | 17.52123 | chr12 + 101988749 102021958 | MYBPC1 |
| FR244962 | fRNAdbv3.4 | 16.8388 | chr7 + 31551811 31552010 |
| ENST00000601148 | Ensembl | 15.31924 | chr19 + 51843949 51847370 |
| TCONS.00026076 | Broad lincRNA | 13.31326 | chr7 + 80804833 80828289 |
| FR193036 | fRNAdbv3.4 | 12.53563 | chr19 − 56526608 56527152 |
| NONHSAT125631 | NONCODEv4 | 12.31555 | chr8 + 25398695 25408293 |
| TCONS.00016248 | Broad lincRNA | 12.02637 | chr20 + 37230676 37256614 |
| NONHSAT030224 | NONCODEv4 | 12.02533 | chr12 − 100560001 100562998 | GOLGA2P5 |
| NONHSAT076673 | NONCODEv4 | 11.6689 | chr2 − 211074313 211081443 | ACADL |
| NONHSAT137402 | NONCODEv4 | 11.66708 | chrX + 69454505 69457167 | AWAT1 |
| ENST00000580420 | Ensembl | 11.61363 | chr18 + 29522538 29524119 |
| FR174595 | fRNAdbv3.4 | 11.10483 | chr6 + 118888744 118889041 |
| NONHSAT077942 | NONCODEv4 | 11.03293 | chr2 − 242455829 242457154 |
| NONHSAT060814 | NONCODEv4 | 10.87916 | chr19 + 7410710 7411049 |
| NONHSAT097800 | NONCODEv4 | 10.56074 | chr4 + 110897243 110898692 | EGF |
| NONHSAT070090 | NONCODEv4 | 10.32572 | chr2 + 36991437 36993016 | VIT |
| TCONS.00022478 | Broad lincRNA | 10.21703 | chr14 + 38205181 38208450 |
| ENST00000556024 | Ensembl | 9.802968 | chr14 − 38025363 38036300 |
| NONHSAT102735 | NONCODEv4 | 9.773005 | chr5 + 90142207 90144638 | GPR98 |

3.3. GO Analysis. The GO project is a collaborative effort in constructing gene and protein ontologies to functionally describe and classify all known genes. Variations in mRNA expression among specimens were shown by volcano plotting and scatter plotting (Figures 2(a) and 2(b)). Hierarchical clustering showed mRNA expression modes among samples were distinguishable (Figure 2(c)).

Specimens and mRNA were expressed significantly lower compared to normal skin specimens (shown in the mRNA profiling). mRNA expression data are deposited at Gene Expression Omnibus under accession number GSE83286.
meaningful annotation of genes and their products in a wide variety of organisms [14]. We performed GO analysis for lncRNAs to determine molecular function, biological processes, and cellular components. For molecular function (Figure 3(a)), calcium ion binding (GO:0005509) had the highest transcriptional domain coverage (TDC, 17.2%) in upregulated transcripts, while oxidoreductase activity (GO:0016491; TDC, 16.4%) was highest in downregulated transcripts. In biological processes (Figure 3(b)), it was found that upregulated genes were enriched most in the process of cell adhesion (GO:0007155; TDC, 18.8%). In contrast, downregulated genes were enriched most in the process of transmembrane transport (GO:0007155; TDC, 16.9%).

In the cellular components (Figure 3(c)), it was detected that integral to membrane (GO:0016021) had the highest enrichment of upregulated genes (TDC, 39.5%), and mitochondria had the highest enrichment of downregulated genes (GO:0005886; TDC, 27.7%).

3.4. KEGG Analysis. KEGG pathway enrichment analysis was used for differentially expressed genes to identify pathways represented among the lncRNAs identified in the earlobe keloid gene expression signature. KEGG analysis suggested that 24 pathways were significantly correlated with upregulated gene expression. The focal adhesion pathway had the highest enrichment of increased transcription (TDC,
27.8%) and comprised 35 targets genes. Pathway analysis also revealed that 11 pathways corresponded to downregulated transcripts and that the most enriched network was metabolic pathways (TDC, 49.2%), which comprised 30 target genes (Figure 3(d)). Many of these pathways are reported to be involved in keloid, including the gene category focal adhesion (Figure 3(d)).

3.5. QRT-PCR Validation. To verify the microarray data, five upregulated lncRNAs (NONHSAT021057, NONHSAT062994, NONHSAT016933, NR_024360.1, and FR39263) and five downregulated lncRNAs (NONHSAT053431, FR244962, ENST00000600148, TCONS_00022478, and XR_244388.1) were randomly selected from the differentially expressed lncRNAs. We detected the expression levels of these lncRNAs in 10 earlobe keloids tissues and normal skin samples (Supplemental Table 3) using qRT-PCR. As shown in Figure 4, the qRT-PCR results and microarray data are consistent.

4. Discussion
Emerging evidence shows that a set of noncoding RNAs (for example, miRNA) is involved in the mechanism of keloid formation. Our study is the first to verify the microarray data using qRT-PCR for multiple lncRNAs. These results support the involvement of lncRNAs in keloid pathogenesis, and further studies are needed to elucidate their specific roles.
Keloid formation has been associated with the deregulation of lncRNAs and mRNAs. The authors used high-throughput microarray techniques to analyze lncRNA and mRNA expression in earlobe keloid and normal skin tissues. They identified that 1290 lncRNAs and 1092 mRNAs were upregulated, and 778 lncRNAs and 419 mRNAs were downregulated in all 3 earlobe keloid and normal skin tissues (fold change ≥ 2.0, P < 0.05). GO and KEGG pathway analysis was used to explore the possible biological functions and potential mechanisms of lncRNAs and mRNAs in earlobe keloids. In fact, Liang and colleagues have previously identified differential expression of lncRNAs and mRNAs between 3 pairs of keloid and normal skin tissues by microarray. Compared with their results, our study has several differences. First, tissues used here were earlobe keloid and normal skin specimens, and the expression profiles of lncRNAs were significantly different from the previous results. Second, to verify the microarray data, the expression levels of five upregulated lncRNAs and five downregulated lncRNAs were detected in 10 earlobe keloids tissues and normal skin samples using qRT-PCR, and the results were consistent with microarray data. An integrative method including pathway was developed to identify possible functional relationships between the different RNA molecules. Based on the differentially expressed mRNAs, pathway analysis revealed which biological functions and mechanisms were involved in earlobe keloid formation. Our results suggest that different biological processes, such as cell-cell adhesion, cell migration, cell death, cell junction formation, epithelial to mesenchymal transition (EMT), TGF-β, and MAPK, are among the significantly enriched mRNAs. Most of these pathways are involved in the process of tissue fibrosis. For example, studies in a wide range of experimental models have revealed that TGF-β is a central mediator of keloid fibrogenesis. It is reported that Loureirin B attenuated the contraction of fibroblasts which was induced by TGF-β in hypertrophic scar formation (33). Yan et al. have reported that EMT plays crucial roles in keloid formation (29). Gobin et al. have shown that emodin-loaded liposomes decrease survival rate of keloids in keloid formation (33). Yan et al. have reported that EMT plays crucial roles in keloid formation. However, the profile and mechanism of earlobe keloid formation, which is not tightly associated signaling, may play an important role in the process of tissue fibrosis. For example, studies in a wide range of experimental models have revealed that TGF-β is a central mediator of keloid fibrogenesis. It is reported that Loureirin B attenuated the contraction of fibroblasts which was induced by TGF-β in hypertrophic scar formation (33). Yan et al. have reported that EMT plays crucial roles in keloid formation. 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cell tight, and adhesion related signaling series mRNAs may interact with IncRNAs. Previous reports demonstrated that ADAM proteins are involved in cell adhesion, cell fusion, cell signaling, and proteolysis. ADAM33 is a member of ADAM family that is associated with keloid scars in the northeastern Chinese population (36). ADAM12 are reluctant to adhere to fibronectin, a key ECM protein in keloids (37). Patients suffering from collagen VI related myopathies caused by mutations in COL6A1, COL6A2, and COL6A3 often also display skin abnormalities, like formation of keloids or “cigarette paper” scars, dry skin, striae rubrae, and keratosis pilaris (follicular keratosis) (38). Keloid fibroblasts were propagated in culture and their proliferative behaviour and response to the epidermal growth factor (EGF) were studied (39). Our results found that NONHSAT016934, NONHSAT016933, NONHSAT016928, and NONHSAT077639 expressions were increased, whereas NONHSAT097800 expression was decreased in earlobe keloid and normal tissues. These IncRNAs were associated with the related genes of keloid (ADAM12, COL6A3, and EGF). We will carry out further studies of these differentially expressed IncRNAs to establish their functions in earlobe keloid formation.

In conclusion, we studied the differential expression profile of IncRNAs and mRNAs in earlobe keloid and normal skin tissues. Our microarray analysis indicated that IncRNAs are involved in the pathological process of earlobe keloid formation. Therefore, subgroup analysis of IncRNAs should be performed to explore this relationship in the future. In addition, we will select numbers of samples to deepen the research into the IncRNA molecular mechanism and biochemical function in order to provide a novel accurate method for therapy of earlobe keloid.

Competing Interests

All the authors declare that they have no competing interests to disclose.

Authors’ Contributions

Liang Guo and Kai Xu contributed equally to this study.

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