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MicroRNA expression profiling and target genes study in congenital microtia

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ABSTRACT

Objective: Microtia is a complicated congenital anomaly with a genetic and environmental predisposition, and the molecular events underlying this disease are not fully understood. MicroRNAs (miRNAs) are a class of 20–22 nucleotide non-coding RNAs that function to control post-transcriptional gene expression. We want to find the miRNA expression profiling of microtia by using Affymetrix GeneChip® miRNA 2.0 Arrays.

Methods: We selected 9 microtia cartilages and 3 normal controls for GeneChip® miRNA 2.0 Arrays analysis. The altered miRNA were analyzed by poly (A) RT-PCR from 58 microtia samples and 16 normal controls. We predicted the target genes of miRNAs by bioinformatics and RT-PCT was used to confirm the target genes.

Results: We found 11 miRNAs with significantly altered expression in the microtic group compared to the normal controls, which included 6 up-regulated miRNAs and 5 down-regulated miRNAs. These miRNAs were further examined using poly (A) RT-PCR analysis, we found that *miR-451* and *miR-486-5p* were significantly up-regulated and *miR-200c* was significantly down-regulated in the microtic group compared to the normal controls ($p < 0.05$). Several complementary target messenger RNAs (mRNAs) had been predicted. *OSR1*, the target gene of *miR-451* and *miR-200c*, was significantly up-regulated ($p < 0.01$); *TRPS1*, the target gene of *miR-200c*, was significantly down-regulated in the microtic group compared to the controls ($p < 0.0001$).

Conclusion: The reduction in *miR-200c* expression and the accretion of *miR-451* and *miR-486-5p* expression in microtic samples could be possible causes of the abnormal development of the external ear. *OSR1* and *TRPS1*, as the complementary target mRNAs, may play important roles during the development of the external ear. Further studies are still needed to identify the miRNA target genes and to determine their function in the pathogenesis of microtia. This is the first report of a relationship between miRNAs and microtia.

1. Introduction

Microtia is a rare congenital malformation of the external ear with an overall prevalence of 2.06 per 10,000 births [1]. Phenotypes range from minor deformities, such as pre-auricular tags, to anotia, the complete absence of the external ear [2]. Microtia is often related to other conditions such as deafness, ear canal atresia and ossicular malformation.

In literature reports, there are more than 18 different microtia-associated syndromes for which single-gene defects or chromosomal aberrations have been reported. Alasti et al. found a missense variant in HOXA1 co-segregating with autosomal-recessive bilateral microtia and hearing loss in a onsanguineous family. A dominant mutation of the murine Hox-2.2 gene, the ortholog for HOXB6, has been shown to cause open eyes at birth, cleft palate and microtia [3–8]. Mouse models have shown that mutations in specific genes like PACT and Hoxa2 were necessary for ear development result in microtia [9–12]. However, few studies have focused on possible genetic causes of isolated

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congenital microtia, and no direct gene has been linked to microtia [13–15]. A number of studies have found that in utero exposure to toxins, influenza virus or drugs during the early stages of pregnancy may lead to microtia occurrence [16–18]. Environmental changes may cause epigenetic changes [19], and some studies have focused on possible epigenetic causes of congenital microtia [20,21].

MicroRNAs are a class of 20–22 nucleotide non-coding RNAs that function to control post-transcriptional gene expression. These small RNAs have been shown to regulate cell growth, differentiation and apoptosis. After the identification of hundreds of miRNAs, the challenge is now to understand their specific biological function [22,23]. MicroRNA arrays are a newly developed high-throughput screening technology that can be used to detect different expression levels of miRNAs in human tissues. They have been applied to a number of study areas, such as human cancer, systemic scleroderma and leukaemia [24–26]. We propose that miRNAs may play a role in the pathogenesis of microtia, which has not been previously reported. This study attempts to reveal the involvement of miRNAs in microtia pathogenesis by screening miRNAs in microtic auricular tissue and normal controls by miRNA expression profiling.

2. Materials and methods

2.1. Sample collection: patients and normal controls

This study was reviewed by the Ethics Committee of the Eye & ENT Hospital of Fudan University. Informed consent was obtained from all participating subjects. Auricular cartilage and soft tissue samples were obtained from 58 patients with microtia after reconstructive ear surgeries and 14 normal controls after other otology surgeries. An additional 4 normal controls were included from individuals that died in traffic accidents within 4 h of death and were provided by the Department of Forensic Medicine. All patients with microtia were diagnosed by a group of ENT doctors using Marx classification [27]. Microtic cartilages and soft tissues were collected during ear reconstruction surgery, quickly cut into small pieces and placed in RNAlater (Ambion, USA). A total of 14 of the 18 normal auricular cartilages were obtained from other otology surgeries, such as wall-up tympanoplasty, in which part of the auricular cartilages were discarded, and these samples were immediately placed in RNAlater. All the samples were stored in RNAlater for 12 h in room temperature and then stored at −80 °C.

2.2. Affymetrix GeneChip® miRNA 2.0 Array and data analysis

The GeneChip® miRNA 2.0 Array probes were synthesised according to the Sanger miRBase V15. The arrays included 15,644 probe sets and targeting 1105 human miRNAs. We selected 9 microtia cartilages and 3 normal controls for miRNA array analysis. Microarray experiments were conducted according to the manufacturer's instructions. Briefly, 1 μg of total RNA was labelled using the Flash Tag Biotin Labelling Kit (Affymetrix, USA). The labelling reaction was hybridised with the miRNA Array in an Affymetrix Hybridization Oven 640 (Affymetrix, USA) at 48 °C rotating at 60 rpm for 16 h. The arrays were stained in the Fluidics Station 450 using fluidics script FS450_0003 (Affymetrix, USA) and then scanned on the GeneChip® Scanner 3000 (Affymetrix, USA).

MicroRNA probe outliers were defined per the manufacturer's instructions (Affymetrix, USA), and further analysis included data summarisation, normalisation and quality control using the web-based miRNA QC Tool software (www.affymetrix.com). To identify miRNAs that had significantly different expression between microtic cartilages and the normal controls, we filtered samples for fold changes greater than 2.0. The filtered data were analysed using GenePattern, BRB-Array Tools and Microarray Software Suite 4. Finally, we performed tree visualisation using Cluster 3.0 and Treeview 1.0.4.ext.

2.3. Poly (A) RT-PCR analysis of miRNAs

Analysis of mature miRNA expression by poly (A) RT-PCR was carried out by the method described previously [28]. Total RNA was isolated using an RNAprep pure kit (Tiangen, China) from 58 microtia samples and 16 normal controls. Poly (A) tails were then added to the 3′ end of the RNAs by *E. coli* poly (A) polymerase (New England BioLabs, USA). Reverse transcription of tailed total RNAs with anchor RT primer (composed of three parts: 5′stem-loop, oligo-dT and 3′anchor base) was performed using M-MLV reverse transcriptase (Promega, USA). We then detected altered miRNAs using PCR with miRNA-specific forward primers and a universal reverse primer (Table 4).

The 18S poly (A) was selected as the endogenous reference. RT-PCR was performed for each sample in triplicate and a no-template experimental control based on the protocol for SYBR Green (TaKaRa, Japan). Expression levels of the miRNAs were calculated using the comparative Ct method followed by statistical analysis with SPSS13.0.

2.4. RT-PCR analysis of target genes

Analysis of mRNA expression by RT-PCR was carried out by the method described previously [29]. Total RNA was isolated using an RNAprep pure kit (Tiangen, China) from 46 microtia samples and 18 normal controls. Reverse transcription of total RNAs was performed using PrimeScript® RT reagent Kit (TaKaRa, Japan). We then detected target mRNAs using PCR with specific primers (Table 5).

The β-actin was selected as the endogenous reference. RT-PCR was performed for each sample in triplicate and a no-template experimental control based on the protocol for SYBR Green (TaKaRa, Japan). Expression levels of the target mRNAs were calculated using the comparative Ct method followed by statistical analysis with SPSS13.0.

3. Results

3.1. MicroRNA expression array

Using Affymetrix GeneChip® miRNA 2.0 Arrays (Bioassay Laboratory of CapitalBio Corporation, Beijing, China), we evaluated miRNA expression profiles of auricular tissues from 9 congenital microtia tissues and 3 normal controls (Table 1). 6 miRNAs were up-regulated greater than two fold (*miR-486-5p*, *miR-451*, *miR-185*, *miR-16*, *miR-140-3p* and *miR-126*) in congenital microtia tissues compared with normal controls. 5 miRNAs were down-regulated greater than two fold (*miR-708*, *miR-1308*, *miR-203*, *miR-200c* and *miR-205*). Cluster analysis including the above differentially expressed miRNAs was used to generate a tree that showed clear distinction between microtia and normal auricular tissues (Fig. 1, Table 2).

3.2. Poly (A) RT-PCR

Poly (A) RT-PCR analysis for mature miRNAs was used to validate the 11 miRNAs differentially expressed between microtic tissues and normal controls using Affymetrix GeneChip® miRNA 2.0 Arrays. A total of 58 microtic auricular tissues and 16 normal auricular tissues were used for poly (A) RT-PCR (Table 3). The results showed that the expression of *miR-486-5p* and *miR-451* was significantly up-regulated and that *miR-200c* was significantly