International Journal of Pediatric Otorhinolaryngology 93 (2017) 78–82

Contents lists available at ScienceDirect

International Journal of Pediatric Otorhinolaryngology

journal homepage: http://www.ijporlonline.com/

Mutational analysis of *GSC*, *HOXA2* and *PRKRA* in 106 Chinese patients with microtia

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ARTICLE INFO

Article history:
Received 27 July 2016
Received in revised form
21 December 2016
Accepted 21 December 2016
Available online 26 December 2016

Keywords:
Microtia
GSC gene
HOXA2 gene
PRKRA gene
Chinese

ABSTRACT

Objective: Microtia is defined as a developmental malformation characterized by a small, abnormal shaped auricle, with atresia or stenosis of the auditory canal. Genes responsible for nonsyndromic microtia have remained elusive. We therefore report a mutational analysis of *GSC*, *HOXA2* and *PRKRA* in 106 congenital microtia patients without any combined malformation to explore the relationship between *GSC*, *HOXA2*, *PRKRA* and nonsyndromic microtia.

Methods: A total of 106 patients with a clinical diagnosis of congenital microtia and a control group (100 unaffected controls) were recruited through the Eye and ENT Hospital of Fudan University in China. Genomic DNA was extracted following a standard protocol. DNA sequencing analysis was performed in all exons and the exon-intron borders of *GSC*, *HOXA2* and *PRKRA*.

Results: We identified 5 genomic variants in *GSC*, *HOXA2* and *PRKRA*. As to the *GSC*, we obtained a reported variant g.994C > T in exon 2, which resulted in no change of protein. Our results revealed that g.994C > T was also detected in 10 control cases. We also detected 2 novel variants, g.90G > A and g.114A > C, in the 5′UTR of *HOXA2*. No class 5 or 4 genomic variant of *PRKRA* was identified in our microtia patients. Additionally, two previously reported SNVs in *GSC* and *PRKRA* were also presented.

Conclusions: We suggest that g.994C > T is a new SNV, which is different from the previous report. Further study is needed to prove the function of 2 novel variants in the 5′UTR of *HOXA2*, and to explore the possible mechanism of these variants in the occurrence of microtia.

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1. Introduction

Microtia is defined as a developmental malformation characterized by a small, abnormal shaped auricle, with atresia or stenosis of the auditory canal. Marx classification is widely used to describe the degree of the microtia, including four grades. Grade I microtia has all of the normal outer ear structures with a small pinna. Grade II has an abnormal, but definable, auricle and pinna. Grade III displays only soft tissue rudiments, with no definable structures of the auricle. Grade IV microtia simply referred to as anotia [1].

The prevalence of microtia was reported in the range of 0.76–4.34 per 10,000 births [2–4], and it has been reported to be 5.18 in China [5]. Most cases of microtia are sporadic, with a right-sided bias and a slight male excess [3]. More than 80% of microtia cases are unilateral [2,4,6], and approximate 90% of microtia patients have conductive hearing loss in the dysplastic ears due to the malformation of the external acoustic canal and middle ear [4]. In addition to the deformed external ear, microtia also may occur with other abnormalities, including hemifacial microsomia, renal anomalies, vertebral abnormalities, cardiac defects and so on. Although many cases of microtia display no apparent cause, several risk factors have been reported to be associated with microtia, including maternal age [7], infant sex [3,8–10], birth weight [2,11], low maternal education [8], isotretinoin treatment during pregnancy [12], acute maternal illness [13], and even seasonal variation in birth date [14].

The auricle and external acoustic cannal develops from the

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http://dx.doi.org/10.1016/j.ijporl.2016.12.026
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groove between the first and the second branchial arch, and complicated tissue interactions are needed to form the ear during embryonic period. Many genes are expressed in the first and the second branchial arch to manipulate the morphogenesis of the external ear, and genetic factors can be one of the important causes in the occurrence of microtia. A host of genes have been reported to cause syndromic microtia, including *GSC* [15], *FGF* [16], *TCOF1* [17], *TBX1* [18], *EYA1* [19], *SIX5* [20], *HMX1* [21], *HOXA2* [22] and *HOXA1* [22]. However, genes responsible for nonsyndromic microtia have remained elusive.

GSC is a homeodomain transcription factor which plays an essential role in early embryonic development [23]. Mice with a homozygous disruption of *Gsc* had the similar phenotype as the patient of microtia [24]. Another candidate gene implicated in genetic microtia is *HOXA2*. HOX genes are essential for the development of hindbrain and branchial arches [25]. Missense variants in *HOXA2* have been reported in an Iranian family of autosomal recessive microtia with cleft palate [22]. Animal model studies can also provide another possible method to confirm candidate gene for mutational analysis. Prkra is a doublestranded RNA-activated protein kinase [26]. Rowe et al. [27] established the *Prkra*−/− mouse model which had small malformed pinnas, stenosis or atresia of the external ear canals, malformed ossicles and associated conductive hearing loss. These results suggested the association between *PRKRA* and microtia.

We therefore report a mutational analysis of *GSC*, *HOXA2* and *PRKRA* in 106 congenital microtia patients without any combined malformations. The aim of our study was to explore the relationship between *GSC*, *HOXA2*, *PRKRA* and nonsyndromic microtia. DNA sequence analysis was performed to analyze the gene variant in the patients and control group.

2. Patients and methods

2.1. Patients

A total of 106 patients with a clinical diagnosis of congenital microtia and a control group (100 unaffected controls) were recruited through the Eye and ENT Hospital of Fudan University in China. Among these patients, 73 patients were male and 33 were female. The ages ranged from 6 months to 14 years. All patients underwent a cautious physical examination to exclude defined syndromic microtia, and none of the patients had family history. The study protocols were previously approved by the Ethics Committee of the hospital, and blood samples were collected after informed consent was obtained from patients or their legal guardians.

3. Methods

Peripheral blood samples were obtained from patients and controls, then stored immediately at −80 °C until use. Genomic DNA was extracted following a standard protocol. All exons and the exon-intron borders of *GSC*(ENSG00000133937), *HOXA2*(ENSG00000105996) and *PRKRA*(ENSG00000180228) were amplified by PCR under optimal conditions using specific primers constructed by Invitrogen Biotechnology Company (Table 1).

A mixture with a total volume of 25 μl was prepared for each reaction including 10 × PCR buffer, 2.0 mM Mg^{2+}, 0.2 mM dNTP, 0.2 μM of each primer, 0.2 μl Taq polymerase (Takara) and 1 μl template DNA. The cycling program was 94 °C for 15 min; 35 cycles of denaturation at 95 °C, annealing temperature as indicated in Table 1, and elongation at 72 °C for 40–50 s; and 72 °C for 5 min. The PCR products were purified using SAP and ExoI. The final products were then analyzed using ABI Prism 3730 Genetic Analyzer (Applied Biosystems, USA). Sequencing data was analyzed against human gene sequence of *GSC*, *HOXA2* and *PRKRA* obtained from Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/) and compared with the non-affected controls using the Mutation Surveyor software. Identified polymorphisms were compared to SNV information tracks on Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/) and UCSC (http://genome.ucsc.edu/) to determine if they were reported normal variants.

4. Results

We identified 5 genomic variants in *GSC*, *HOXA2* and *PRKRA*. As to the *GSC*, we obtained a reported variant g.994C > T in the exon 2, which resulted in no change of protein. However, the variant g.994C > T in the exon 2 of *GSC* was also detected in 10 normal control cases, so we suggested that g.994C > T in the exon 2 of *GSC* was a new SNV. We also detected 2 novel variants, g.90G > A and g.114A > C, in the 5′UTR of *HOXA2*. No class 5 or 4 genomic variant in *PRKRA* was identified in our microtia patients. Additionally, two previously documented SNVs were presented in *GSC* and *PRKRA*. Total 32 patients were confirmed with 5 variants, and 25 of the patients exhibited Grade III microtia in unilateral or bilateral ear. There were 11 patients who heterozygous for both g.994C > T and g.1243G > T of *GSC* with various phenotype. A summary of the clinical findings in all subjects with identified *GSC*, *HOXA2* and *PRKRA* variants are described in Tables 2 and 3. All of the variants found within our patients are showed in Fig. 1.

5. Discussion

The structures of vertebrate animals all result from the development of six pharyngeal arches (PA) during embryonic period. The development of outer and middle ear derives from the mesenchyme through complex cell interactions between PA I, PA II and migrating neural crest cells (NCCs) [28]. The external ear begins to develop in the sixth week of gestation at the dorsal end of the first branchial cleft, and completes by the third month's gestation. The auricle derives from the fusion of six hillocks in PA I and PA II, and the external auditory meatus originates from the first branchial cleft between the mandibular and hyoid arches. The auricles are initially at the base of the neck, however they migrate to the normal position as the mandible develops. The development of middle ear requires sequential interactions between the epithelia and the underlying mesenchyme. The formation of normal ear is a complicated process of cell interactions controlled by various genes. Gene-inactivation experiments and animal models have identified several genes which are responsible for formation of the ear, such as *GSC*, *HOXA2* and *PRKRA* which are all likely involved in the ear patterning and morphogenetic processes.

Human Goosecoid (*GSC*) locating in 14q32 [15] is a homeodomain transcription factor which plays an essential role in early embryonic development [23]. The expression of *GSC* has time phase property and plays an important role in the migration of NCCs [29]. Mice with a homozygous disruption of *Gsc* had the phenotype of lower mandible, deformed inner ear and dysplastic external auditory meatus [24]. The results suggest that the hypofunction of Gsc has a profound influence in the process of bone morphogenesis, resulting in the dysplasias of external and inner ear. Kelberman et al. [15] analyzed the coding regions of *GSC* in 120 sporadic cases of hemifacial microsomia (HFM) and two HFM families, however no pathogenic genomic variant was detected. Zhang et al. [30] found six patients had synonymous variant c.197C > T in exon 2, and a missense variant c.125A > G in exon 3 occurred in two cases. As to the *GSC* gene in our study, we obtained an variant g.994C > T in exon 2 and a reported SNV in exon 3. We analyzed the base

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