SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 10: 379-386, 2014

Prevalence and range of GJB2 and SLC26A4 mutations in patients with autosomal recessive non-syndromic hearing loss

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Received September 11, 2013; Accepted March 13, 2014

DOI: 10.3892/mmr.2014.2148

Abstract. The frequency and distribution of genetic mutations that cause deafness differ significantly according to ethnic group and region. Zhejiang is a province in the southeast of China, with an exceptional racial composition of the population caused by mass migration in ancient China. The purpose of the present study was to investigate the prevalence and spectrum of gap junction-β2 (GJB2), solute carrier family 26 (anion exchanger) member 4 (SLC26A4) and GJB3 mutations in patients with autosomal recessive non-syndromic hearing loss (ARNHL) in this area. A total of 176 unrelated pediatric patients with ARNHL were enrolled in the study. A genomic DNA sample was extracted from the peripheral blood. Polymerase chain reaction was employed, and the products were sequenced to screen for mutations in GJB2. In addition, a SNaPshot sequencing method was utilized to detect four hotspot mutations in SLC26A4 (IVS7-2A>G and c.2168A>G) and GJB3 (c.538C>T and c.547G>A). All patients were subjected to a temporal bone computed tomography scan to identify enlarged vestibular aqueducts (EVA). In total, 14 different mutations, including two new mutations (p.W44L and p.D66N) of GJB2, were detected. The most common pathogenic mutation of GJB2 was c.235delC (15.1%), followed by c.176_191del16 (1.7%), c.299_300delAT (1.7%), c.508_511dup (0.85%) and c.35delG (0.28%) of the total alleles. Mutation analysis of SLC26A4 demonstrated that 13.6% (24/176) of patients carried at least one mutant allele. The patients with EVA (84.2%) had SLC26A4 mutations, and 31% had homozygous mutations. Only one patient carried a heterozygous mutation of GJB3 (c.538C>T). Compared with the other regions of China, in the present population cohort, the prevalence and spectrum of mutations in GJB2 was unique, and in patients with EVA the frequency of a homozygous mutation in SLC26A4 was significantly lower. These findings may be of benefit in genetic counseling and risk assessment for families from this area of China.

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Key words: GJB2, SLC26A4, autosomal recessive non-syndromic hearing loss

Introduction

Sensorineural hearing loss is one of the most common birth disorders, with an incidence of ~1 in 1,000 newborns worldwide (1). In China, there are ~30,000 newborns with congenital sensorineural hearing loss every year, and the prevalence is ~1.5 per 1,000 at birth (2,3). Among those with congenital sensorineural hearing impairment, half have a genetic cause and 70% of these cases with genetic hearing loss are non-syndromal (4). The gap junction-β2 (GJB2) gene is the most prevalent gene associated with autosomal recessive non-syndromic hearing loss (ARNHL) and is responsible for almost 50% of genetic cases in numerous populations, including that of China (5,6). The GJB2 gene is located on chromosome 13q12 at the DFNB1 locus, encoding the connexin 26 protein, which is a transmembrane protein forming gap junction channels in cochlear cells (7). The function of the connexins is considered to be the recycling of potassium ions to the endolymph of the cochlear duct, serving auditory signal transduction (8-10). The malfunction of the connexins is considered to cause the accumulation of potassium ions in the endolymph and result in hair cell dysfunction (11). At present, >150 mutations, polymorphisms and unclassified variants have been reported in GJB2 (12). The c.35delG, c.167delT and c.235delC mutant alleles are the most frequent mutations (13-15). GJB3, which encodes connexin 31, has been demonstrated to interact with GJB2 (16). In the Chinese population, mutations in GJB3 have been identified to cause autosomal recessive and autosomal dominant non-syndromic hearing loss (17-18). Additionally, the association between mutations of GJB3 and non-syndromic hearing impairment has been detected in other populations (19).

Following GJB2, solute carrier family 26 (anion exchanger) member 4 (SLC26A4) is the second most common gene contributing to genetic deafness. The SLC26A4 gene encodes an anion transporter transmembrane protein, pendrin, which is expressed in the thyroid, kidney and cochlea (20). In the inner ear, pendrin is responsible for the exchange of anions, including Cl^- and HCO_3^- to maintain the anion composition

of endolymph (21). The malfunction of pendrin causes an increase in endolymph volume, which leads to the degeneration of inner ear sensory cells (22). SLC26A4 mutations lead to Pendred syndrome and ARNHL (DFNB4) with enlargement of the vestibular aqueduct (EVA) (6,23). It has been documented that mutations in SLC26A4 contribute to ~5% of all cases of congenital recessive hearing loss in East Asia (24). However, in China, the incidence of SLC26A4 mutations in non-syndromic hearing impairment is 14-17% (25-27).

It has been demonstrated that the frequency and distribution of genetic mutations causing deafness differ significantly according to ethnic group and region (14,24,28-31). In China, GJB2 and SLC26A4 are the two most common genes responsible for genetic hearing loss (6). However, mutations in these two genes have been identified to show a significant difference in various regions in China (15,25). Zhejiang is a southeastern province of China. Due to several mass migrations in ancient China, the racial composition of the population in this area may be exceptional. The aim of the present study was to investigate the frequency and spectrum of GJB2 mutations by polymerase chain reaction (PCR) sequencing, and the four hotspot mutations of SLC26A4 (IVS7-2A>G and c.2168A>G) and GJB3 (c.538C>T and c.547G>A) by SNaPshot sequencing in unrelated patients with ARNHL in Zhejiang province.

Materials and methods

Patients and samples. A total of 176 unrelated pediatric patients with ARNHL were enrolled in the present study. The patients consisted of 82 males (46.6%) and 94 females (53.4%) aged between 9 months and 18 years old, with a mean age of 5±2.7 years. The patients originated from various regions of Zhejiang province. Each patient received careful physical examinations and a detailed clinical history was recorded. The patients with a family history of deafness, a history of aminoglycoside exposure and syndromic hearing loss were excluded from the study. All subjects underwent audiometric testing, including auditory brain-stem response tests, otoacoustic emission tests and tympanometry. The results demonstrated that all the subjects suffered from severe (71-90 dB) to profound (>90 dB) bilateral sensorineural hearing impairment. Furthermore, a computed tomography (CT) scan of the temporal bones was also performed. The diagnosis of EVA was based on the criterion that the diameter of the vestibular aqueduct is >1.5 mm at the midpoint between the common crus and the external aperture (32). Peripheral blood samples were obtained from the patients for genomic DNA isolation.

Informed consent was obtained from the parents of the subjects, and the present study was approved by the Ethics Committee of the Second Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, Zhejiang).

Mutation analysis

GJB2 mutation detection by PCR. Two exons of GJB2 were amplified from DNA samples by PCR. The primers are listed in Table I. The amplification reactions were performed in a 20 μl mixture. For exon 1 this contained 1X HotStarTaq buffer, 2.0 mM Mg^{2+}, 0.2 mM dNTPs, 0.2 μM of each primer, 1 unit HotStarTaq polymerase and 10 ng template DNA. For exon 2 the mixture contained 1X GC buffer I, 0.2 mM dNTPs, 0.2 μM of each primer, 1 unit HotStarTaq polymerase and 10 ng template DNA. The cycling programs were as follows: For exon 1, 95˚C for 15 min, 11 cycles of 94˚C for 15 sec, 62-0.5˚C per cycle for 40 sec and 72˚C for 1 min, and 24 cycles of 94˚C for 15 sec, 57˚C for 30 sec, 72˚C for 1 min and 72˚C for 2 min; for exon 2, 95˚C for 15 min, 9 cycles of 94˚C for 15 sec and 72-0.5˚C per cycle for 1 min, and 26 cycles of 94˚C for 15 sec, 68˚C for 1 min and 72˚C for 2 min. For the purification of the PCR products, 1 unit shrimp alkaline phosphatase (SAP; Promega Corporation, Madison, WI, USA) and 6 units exonuclease I (Epicentre, Madison, WI, USA) were added into 8 μl of the PCR products. The mixture was incubated at 37˚C for 60 min, followed by incubation at 70˚C for 10 min.

The purified PCR products were sequenced using the dideoxy chain termination method on an ABI 3730XL DNA sequencer with the ABI Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The primers were the same as those for the PCR amplification.

GJB3 and SLC26A4 mutation detection by SNaPshot sequencing methods. The final 20 μl of PCR reaction mixture contained 1X GC buffer I, 3.0 mM Mg^{2+}, 0.3 mM dNTPs, 1 unit HotStarTaq polymerase, 10 ng template DNA and 1 μM primer mixture, including four pairs of primers to amplify four mutation regions (Table I). The PCR conditions were as follows: 95˚C for 2 min, 11 cycles of 94˚C for 20 sec, 65-0.5˚C per cycle for 40 sec and 72˚C for 90 sec, and 24 cycles of 94˚C for 20 sec, 59˚C for 30 sec, 72˚C for 90 sec and 72˚C for 2 min. For the purification of the PCR products, 5 units SAP (Promega Corporation) and 2 units exonuclease I (Epicentre) were added to 15 μl of PCR products. The mixture was incubated at 37˚C for 60 min, followed by incubation at 75˚C for 15 min.

The single base extension (SBE) was performed in a final 10 μl of reaction mixture, containing 5 μl SNaPshot Multiplex kit (Applied Biosystems), 2 μl purified multiplex PCR product and 0.8 μM extension primer mixture (Table II). The reaction program was 96˚C for 1 min, 28 cycles of 96˚C for 10 sec, 52˚C for 5 sec, 60˚C for 30 sec and 4˚C for the rest of the program. The SBE products were then purified using SAP. For the sequence analysis, 0.5 μl purified multiplex SBE products were mixed with 0.5 μl 120 Liz Size Standard (Applied Biosystems) and 9 μl Hi-Di (Applied Biosystems), and denatured at 95˚C for 5 min. The products were then sequenced by the ABI 3730XL DNA sequencer. The data was analyzed by GeneMapper v4.1 software (Applied Biosystems).

Results

Mutation of GJB2. Among the 176 patients, 14 different mutations were identified (Tables III and IV). A total of 140 (79.5%) patients were identified to carry mutations. However, pathogenic mutation alleles were identified in only 69 (19.6%) out of the total 352 alleles and affected 40 (22.7%) patients. The most common pathogenic mutation was c.235delC (15.1%), followed by c.176_191del16 (1.7%), c.299_300delAT (1.7%), c.508_511dupAACG (0.85%) and c.35delG (0.28%) of the total alleles. In all 40 affected patients, 18 individuals carried homozygous mutations, 11 individuals were compound

Table 1. PCR primer sequences.

Gene	PCR primers	Predicted size, bp
GJB2		
Exon 1	F: 5'-TGGGGAACTCATGGGGGCTCAAAG-3' R: 5'-AGGTTCCTGGCCGGGCAGTCC-3'	425
Exon 2	F: 5'-TCAGAGAAGTCTCCCTGTTCTGTCC-3' R: 5'-TGAGGCCTACAGGGGTTTCAA-3'	916
SLC26A4		
IVS7-2A>G	F: 5'-GATCGGTTTAGACACAAAATCCCAGTC-3' R: 5'-GGCTCCATATGAAATGGCAGTAGC-3'	184
c.2168A>G	F: 5'-GCTGGGCATGGTAGGGTGTG-3' R: 5'-TGACCCTCTTGAGATTTCACTTGGT-3'	337
GJB3		
c.538C>T c.547G>A	F: 5'-CTGCCCCAACATCGTGGACT-3' R: 5'-CAGCGGCAGGTGGAAGCTC-3'	216

PCR, polymerase chain reaction; SLC26A4, solute carrier family 26 (anion exchanger) member 4; GJB, gap junction β.

Table II. Extension primer sequences.

Gene	Extension primers
SLC26A4	
IVS7-2A>G	R: 5'-TTTTTTTTTTTGAAATGGCAGTAGCAATTATCGTC-3'
c.2168A>G	R:5'-TTTTTTTTTTTTTACTTGGTTCTGTAGATAGAGTATAGCATCA-3'
GJB3	
c.538C>T	F: 5'-CGTGGACTGCTACATTGCC-3'
c.547G>A	R: 5'-CCATGAARTAGGTGAAGATTTTCTTCT-3'

SLC26A4, solute carrier family 26 (anion exchanger) member 4; GJB3, gap junction-β3.

heterozygotes and 11 patients carried one heterozygous mutation. Homozygous c.235delC was the most prevalent genotype (16/40; 40%), followed by compound heterozygous c.235delC/c.176_191del16 (4/40; 10%) and heterozygous c.235delC (4/40; 10%). The c.508_511dupAACG allele was detected in three (3/40; 7.5%) patients. Two of these patients had the compound heterozygous mutation c.299_300delAT/c.508_511dupAACG. The other was heterozygous for c.508_511dupAACG. The c.35delG allele was identified in only one patient with a compound heterozygote mutation c.35delG/c.235delC. In all 176 patients, the controversial p.V37I mutation was detected in 23 alleles (6.5%) and 22 patients (12.5%). Of these 22 patients, three patients had the p.V37I mutation compounded with c.235delC, one patient was homozygous for p.V37I and one patient had the p.V37I mutation compounded with a novel mutation c.131G>T (p.W44L). The other novel mutation that was detected in 176 patients was c.196G>A (p.D66N). The patients carried the p.D66N mutation without any other mutations in GJB2.

Mutation of GJB3 and SLC26A4. Four mutations, c.538C>T and c.547G>A of the GJB3 gene and IVS7-2A>G and c.2168A>G of the SLC26A4 gene, were detected by the SNaPshot sequencing method (Table V). The mutations of GJB3 were identified in only one patient who carried heterozygous c.538C>T and had no pathogenic mutation in SLC26A4 and GJB2. The mutations in SLC26A4 were detected in 24 (13.6%) patients and 32 (9.1%) alleles. The mutation IVS7-2A>G was more common than c.2168A>G in the cohort. Two patients carried compound heterozygous IVS7-2A>G/c.2168A>G mutations. Similarly, the patients who carried the mutation in SLC26A4 had no pathogenic mutation in GJB2 and GJB3.

CT scans. A total of 19 (19/176; 10.8%) patients with EVA were identified by temporal bone CT scans. Among them, three patients had EVA with Mondini dysplasia. The mutation in SLC26A4 was detected in 16 (16/19; 84.2%) patients, however, only six individuals had a biallelic mutation in SLC26A4 (five homozygous IVS7-2A>G and one homozygous c.2168A>G). In the other 10 patients with only one mutant allele, nine patients had one mutant IVS7-2A>G allele and one patient had one mutant c.2168A>G allele. Among the other three patients with EVA, one patient carried heterozygous c.235delC in GJB2. However, eight patients with the mutation in SLC26A4 had no EVA or inner ear malformation and two of them carried compound heterozygous IVS7-2A>G/c.2168A>G mutations.

Table III. Mutations identified in GJB2 in 176 unrelated pediatric ARNHL patients.

Genotypes	Consequence of amino acid change	Mutation type	Number of alleles
c.79G>A	p.V27I	Polymorphism	97
c.341A>G	p.E114G	Polymorphism	74
c.235delC	Frame shift	Pathogenic	53
c.109G>A	p.V37I	Controversy	23
c.608T>C	p.I203T	Polymorphism	15
c.176_191del16	Frame shift	Pathogenic	6
c.299_300delAT	Frame shift	Pathogenic	6
c.508_511dupAACG	Frame shift	Pathogenic	3
c.368C>A	p.T123N	Unknown	3
c.478G>A	p.G160S	Polymorphism	1
c.35delG	Frame shift	Pathogenic	1
c.131G>T	p.W44L	Novel	1
c.196G>A	p.D66N	Novel	1
c.499G>A	p.V167M	Unknown	1

ARNHL, autosomal recessive non-syndromic hearing loss; GJB2, gap junction-β2.

Figure 1. Zhejiang province lies in the southeast of China and consists of 11 regions.

Discussion

Zhejiang is a province in the southeast of China (Fig. 1) and is one of the most developed areas. The racial composition of the population in this area may be exceptional compared with other regions of China. In ancient China, there were several mass migrations (300-1300 AD). The south area of the Yangtse River, including Zhejiang province, was a common destination. Particularly in the Song Dynasty (~1300 AD), due to war, almost the whole population of the Dynasty migrated from the north of China to the area of Zhejiang province; this is recorded as the largest migration in Chinese history. However, in this exceptional population, the prevalence and spectrum of ARNHL-related gene mutations have not been well documented. In the present study, the patients were from 11 different areas of Zhejiang, covering all regions of the province. To identify the mutations in GJB2, the two exons were sequenced. The results demonstrated that 22.7% of patients carried a confirmed pathogenic mutation. c.235delC was the most common pathogenic mutation, accounting for 76.8% of all pathogenic mutant GJB2 alleles. The c.35delC mutation, which is the most common mutation in Caucasians, was only detected in one patient (13). In addition, c.167delT, the most common mutation in Ashkenazi Jews (14), was not identified in the present patient cohort. These results were similar to those previously reported (6,15,27). The c.299_300delAT and the c.176_191del16 mutant alleles are the second most common mutations in the Chinese population (6,15). However, the prevalence of the c.299_300delAT allele and the c.176_191del16 allele is markedly different according to the region. In a study by Dai *et al*, a total of 23 provinces were included to investigate the GJB2 mutation spectrum. The incidence of the c.299_300delAT allele in Guangxi and Hubei was zero, however, in Qinghai, it was 46.2% of the total mutant alleles (15). Similarly, the c.176_191del16 allele was not detected in approximately half of those provinces, and the highest prevalence was 14.5% of all mutant alleles in Jiangsu. In the present cohort, the incidence of c.299_300delAT and c.176_191del16 in all detected pathogenic alleles was 8.7% for each, respectively, which was also markedly different from other studies investigating GJB2 mutations in other provinces of China (6,33). Following these three common GJB2 mutations, the fourth most common GJB2 mutation in the present population cohort was c.508_511dupAACG. Although the c.508_511dupAACG mutation is not listed on the website of the GJB2 mutation database (12), it was first reported by Wu *et al* and was recognized as a pathogenic mutation (34). In the present study, the incidence of the c.508_511dupAACG

Table IV. Genotypes of patients with GJB2 mutations.

Category	Genotypes		Affected patients, n
	Allele 1	Allele 2	
Homozygous pathogenic mutations	c.235delC	c.235delC	16
	c.235delC	c.235delC, c.478G>A	1
	c.176_191del16	c.176_191del16	1
Compound heterozygous pathogenic mutations	c.235delC	c.176_191del16	4
	c.235delC	c.299_300delAT	3
	c.299_300delAT	c.508_511dupAACG	2
	c.235delC	c.299_300delAT, c.499G>A	1
	c.235delC	c.35delG	1
Heterozygous pathogenic mutations	c.235delC	No variant	4
	c.235delC	c.109G>A	3
	c.235delC	c.79G>A, c.341A>G	2
	c.235delC	c.79G>A	1
	c.508_511dupAACG	c.79G>A, c.341A>G	1
Unidentified mutations	c.109G>A	No variant	7
	c.79G>A, c.109G>A	c.341A>G	7
	c.109G>A	c.79G>A	2
	c.79G>A	c.368C>A	2
	c.79G>A, c.109G>A	c.368C>A	1
	c.109G>A	c.131G>T	1
	c.109G>A	c.109G>A	1
	c.196G>A	No variant	1
Polymorphism	c.79G>A	c.341A>G	35
	c.79G>A	No variant	9
	c.608T>C	No variant	9
	c.79G>A, c.341A>G	c.79G>A, c.341A>G	8
	c.79G>A	c.79G>A, c.341A>G	6
	c.79G>A	c.608T>C	4
	c.341A>G	No variant	3
	c.79G>A	c.608T>C, c.341A>G	2
	c.79G>A, c.341A>G	c.341A>G	1
	c.79G>A	c.79G>A	1

GJB2, gap junction-β2.

mutation was 4.3% of all pathogenic GJB2 mutant alleles. However, in other studies identifying GJB2 mutations in the Chinese population, this mutation was not detected, although the numbers of involved individuals were larger than in the present cohort (6,15,33). While certain studies detected the c.508_511dupAACG allele of GJB2 in the Chinese population, the incidence was <1% of all mutant alleles (35,36). Furthermore, 62 patients were found to carry the p.V27I and p.E114G alleles together, which was recorded as a pathogenic mutation in the GJB2 mutation database. Whereas another previous study demonstrated that this compound mutation is not pathogenic (37).

p.V37I is a controversial mutation of GJB2. It was first reported as a polymorphism of GJB2 by Kelley *et al* (38). However, Bruzzone *et al* (39) revealed that homozygous p.V37I can result in a simple loss of channel activity. Later, certain studies indicated that p.V37I was associated with mild and moderate hearing loss (35,40-42). In the study by Ma *et al* (35), 10.3% of the newborn infants with hearing loss (hearing threshold >35 dB nHL) were homozygous for p.V37I. By contrast, the allele frequency of p.V37I was ~4.5% in the Chinese Han population. Furthermore, 89% of the newborn infants with homozygous p.V37I had hearing thresholds of ≤65 dB nHL (35). In the present study, the prevalence of the p.V37I allele was 6.5%, which was near that

Table V. Genotypes of patients with SLC26A4 mutations or GJB3 mutations.

Gene	Affected patients, n			Total affected patients/alleles, n
	Homozygous	Heterozygous	Compound heterozygous	
SLC26A4				
IVS7-2A>G	5	13	2	24/32
c.2168A>G	1	3		
GJB3				
c.538C>T	0	1	0	1/1
c.547G>A	0	0		

SLC26A4, solute carrier family 26 (anion exchanger) member 4; GJB3, gap junction-β3.

of the controls reported by Ma *et al* (35). Furthermore, only one patient was homozygous for p.V37I. A possible explanation is that the individuals in the present cohort had severe to profound sensorineural hearing loss. The present results supported the findings reported in previous studies (35,40-42).

In the present study, 16.5% (29/176) of patients had two pathogenic mutant alleles in the GJB2 gene and 11 patients had only one pathogenic mutant allele. Although the percentage of patients with a single GJB2 mutation allele varies in different ethnic populations or in different regions of China, such patients occupy a significant proportion of the population (15,27,33,43,44). The patients with ARNHL in the present study carried a single pathogenic mutant allele of GJB2, which may be ascribed to the presence of another pathogenic mutation in other regions of GJB2 or other genes that were not investigated.

Two new mutations, c.131G>T (p.W44L) and c.196G>A (p.D66N), of GJB2 were detected in the present cohort. W44 and D66 lie in the highly-conserved amino acid residues, which indicate that these mutations may affect the function of GJB2. Further studies are required to elucidate whether they are pathogenic mutations.

SNaPshot sequencing, a technique for detecting several mutations of different genes simultaneously in a single reaction, has been used for phylogenetic analysis and detection of numerous pathogenic mutations of human hereditary disorders (45-47). This technique is more economic and effective than direct sequencing of the gene, and more recently it was used to screen hotspot mutations of several hearing loss-related genes (48). In the present study, this technique was utilized to identify the spectrum of four hotspot mutations in two genes in the cohort. This included IVS7-2A>G and c.2168A>G of SLC26A4, which are the two most common mutations in the Chinese population (25,26), and c.538C>T and c.547G>A of GJB3, which were first identified in the Chinese population as dominant mutations and recently also detected in patients with recessive non-syndromic hearing impairment (17,27).

Mutation analysis of SLC26A4 demonstrated that 13.6% (24/176) of patients carried at least one mutant allele, with IVS7-2A>G being the most common mutation, and 4.5% (8/176) of individuals had two mutant alleles. The prevalence was lower than that from typical areas in the north and south of China (6). However, the total variant detection rate neared that reported in a previous study involving patients from 27 different regions of China (25). In addition, the proportion of patients with two mutant alleles in all individuals with the SLC26A4 mutation was equal to that reported by Qu *et al* (27). However, the frequency of the SLC26A4 mutation also differed geographically in China, from 3.3% in Guangxi to 26.3% in Gansu (25). IVS7-2A>G is the most common mutation of SLC26A4 in the Chinese population, followed by c.2168A>G (6,24,25,47). The IVS7-2A>G and c.2168A>G mutations account for 73.2% of the mutant alleles (24). In Caucasians, IVS7-2A>G is also more prevalent than c.2168A>G (50), while in Japanese and Korean individuals, the most prevalent mutation is c.2168A>G, followed by IVS7-2A>G (51,52).

In the present patient cohort, 84.2% (16/19) of patients with EVA had a SLC26A4 mutation and 31% (6/19) had homozygous mutations. The high frequency of the SLC26A4 mutation in patients with EVA was also identified in previous studies of the Chinese population (6,25,49). However, in those studies, the proportion of patients with two mutant alleles was 75-92% and the majority of them were homozygous IVS7-2A>G or homozygous 2168A>G (6,25,49). The lower frequency of homozygous mutations in the patients with EVA in the present cohort is extraordinary, although certain studies have indicated that one mutation in SLC26A4 is common in patients with EVA (50,53). As other confirmed pathogenic mutations in SLC26A4 in Chinese individuals are relatively rare, additional screening of SLC26A4 in other regions may increase the proportion only mildly. By contrast, in Caucasians, only 40% of patients with EVA carry a SLC26A4 mutation and 60% of these have a biallelic mutation (54). In Brazilian patients with EVA, only 39% carry a SLC26A4 mutation and approximately half of these carry two mutant alleles (55). Complex factors, including other gene mutations and environmental factors, may possibly contribute to the etiology of EVA.

GJB3 mutations originally reported by Xia *et al* (17) demonstrated an association with autosomal dominant non-syndromic deafness in Chinese patients. Later, Liu *et al* (18) reported that GJB3 was also able to mediate ARNHL in the Chinese population. Previous studies have demonstrated that the expression of connexin 31 in the cochlea is partially overlapped with connexin 26 and that these two connexins are able to form heterotypic channels (6,16). Losing any two alleles of these heterotypic

connexins may result in hearing impairment (16). Certain studies have confirmed that the digenic form of GJB2 and GJB3 may cause ARNHL (6,16). In the present patient cohort, no individual carried both the GJB2 and GJB3 mutations. The reason may be that only hotspot mutations in GJB3 (c.538C>T and c.547G>A) were investigated. Although these two mutations were originally identified in patients with dominant non-syndromic hearing loss, previous studies also detected them in a patient with ARNHL (27). Additionally, in the present patient cohort, one patient carried the heterozygous c.538C>T mutation, although the parents had normal hearing. Blood samples from the parents were not, however, obtained. It is possible that the c.538C>T mutation occurred only in the patient and resulted in congenital hearing loss.

In conclusion, the present study identified the prevalence and spectrum of GJB2, SLC26A4 and GJB3 mutations in ARNHL patients from a southeastern province of China. Compared with other regions of China, this population cohort demonstrates a unique spectrum of mutations in the GJB2 gene and a different prevalence of SLC26A4 mutations in patients with EVA. The present findings may be of benefit in genetic counseling and risk assessment for families from the Zhejiang province of China. Further studies screening whole coding regions of SLC26A4, GJB3 and other genes are required in order to improve our understanding of the genotype in ARNHL patients from Zhejiang.

References

1. Morton NE: Genetic epidemiology of hearing impairment. Ann NY Acad Sci 630: 16-31, 1991.
2. Wang QJ, Zhao YL, Rao SQ, Guo YF, He Y, Lan L, *et al*: Newborn hearing concurrent gene screening can improve care for hearing loss: a study on 14,913 Chinese newborns. Int J Pediatr Otorhinolaryngol 75: 535-542, 2011.
3. Dai P, Liu X, Yu F, Zhu QW, Yuan YY, Yang SZ, *et al*: Molecular etiology of patients with nonsyndromic hearing loss from deaf-mute schools in 18 provinces of China. Chinese J Otol 1: 1-5, 2006.
4. Smith RJ, Bale JF Jr and White KR: Sensorineural hearing loss in children. Lancet 365: 879-890, 2005.
5. Kenneson A, Van Naarden Braun K and Boyle C: GJB2 (connexin 26) variants and nonsyndromic sensorineural hearing loss: a HuGE review. Genet Med 4: 258-274, 2002.
6. Yuan Y, You Y, Huang D, Cui J, Wang Y, Wang Q, *et al*: Comprehensive molecular etiology analysis of nonsyndromic hearing impairment from typical areas in China. J Transl Med 7: 79, 2009.
7. Goodenough DA, Goliger JA and Paul DL: Connexins, connexons, and intercellular communication. Annu Rev Biochem 65: 475-502, 1996.
8. Bruzzone R, White TW and Paul DL: Connections with connexins: the molecular basis of direct intercellular signaling. Eur J Biochem 238: 1-27, 1996.
9. Simon AM and Goodenough DA: Diverse functions of vertebrate gap junctions. Trends Cell Biol 8: 477-483, 1998.
10. Wangemann P: K^+ cycling and the endocochlear potential. Hear Res 165: 1-9, 2002.
11. Kikuchi T, Kimura RS, Paul DL, Takasaka T and Adams JC: Gap junction systems in the mammalian cochlea. Brain Res Brain Res Rev 32: 163-166, 2000.
12. The Connexin-Deafness Homepage. http://davinci.crg.es/deafness. Accessed August 20, 2013.
13. Gabriel H, Kupsch P, Sudendey J, Winterhager E, Jahnke K and Lautermann J: Mutations in the connexin26/GJB2 gene are the most common event in non-syndromic hearing loss among the German population. Hum Mutat 17: 521-522, 2001.
14. Morell RJ, Kim HJ, Hood LJ, Goforth L, Friderici K, Fisher R, *et al*: Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. N Engl J Med 339: 1500-1505, 1998.
15. Dai P, Yu F, Han B, Liu X, Wang G, Li Q, *et al*: GJB2 mutation spectrum in 2,063 Chinese patients with nonsyndromic hearing impairment. J Transl Med 7: 26, 2009.
16. Liu XZ, Yuan Y, Yan D, Ding EH, Ouyang XM, Fei Y, *et al*: Digenic inheritance of non-syndromic deafness caused by mutations at the gap junction proteins Cx26 and Cx31. Hum Genet 125: 53-62, 2009.
17. Xia JH, Liu CY, Tang BS, Pan Q, Huang L, Dai HP, *et al*: Mutations in the gene encoding gap junction protein beta-3 associated with autosomal dominant hearing impairment. Nat Genet 20: 370-373, 1998.
18. Liu XZ, Xia XJ, Xu LR, Pandya A, Liang CY, Blanton SH, *et al*: Mutations in connexin31 underlie recessive as well as dominant non-syndromic hearing loss. Hum Mol Genet 9: 63-67, 2000.
19. Alexandrino F, Oliveira CA, Reis FC, Maciel-Guerra AT and Sartorato EL: Screening for mutations in the GJB3 gene in Brazilian patients with nonsyndromic deafness. J Appl Genet 45: 249-254, 2004.
20. Everett LA, Morsli H, Wu DK and Green ED: Expression pattern of the mouse ortholog of the Pendred's syndrome gene (Pds) suggests a key role for pendrin in the inner ear. Proc Natl Acad Sci USA 96: 9727-9732, 1999.
21. Wangemann P, Nakaya K, Wu T, Maganti RJ, Itza EM, Sanneman JD, *et al*: Loss of cochlear HCO3- secretion causes deafness via endolymphatic acidification and inhibition of $Ca2^+$ reabsorption in a Pendred syndrome mouse model. Am J Physiol Renal Physiol 292: F1345-F1353, 2007.
22. Everett LA, Belyantseva IA, Noben-Trauth K, Cantos R, Chen A, Thakkar SI, *et al*: Targeted disruption of mouse Pds provides insight about the inner-ear defects encountered in Pendred syndrome. Hum Mol Genet 10: 153-161, 2001.
23. Campbell C, Cucci RA, Prasad S, Green GE, Edeal JB, Galer CE, *et al*: Pendred syndrome, DFNB4, and PDS/SLC26A4 identification of eight novel mutations and possible genotype-phenotype correlations. Hum Mutat 17: 403-411, 2001.
24. Park HJ, Shaukat S, Liu XZ, Hahn SH, Naz S, Ghosh M, *et al*: Origins and frequencies of SLC26A4 (PDS) mutations in east and south Asians: global implications for the epidemiology of deafness. J Med Genet 40: 242-248, 2003.
25. Yuan Y, Guo W, Tang J, Zhang G, Wang G, Han M, *et al*: Molecular epidemiology and functional assessment of novel allelic variants of SLC26A4 in non-syndromic hearing loss patients with enlarged vestibular aqueduct in China. PLoS One 7: e49984, 2012.
26. Hu X, Liang F, Zhao M, Gong A, Berry ER, Shi Y, *et al*: Mutational analysis of the SLC26A4 gene in Chinese sporadic nonsyndromic hearing-impaired children. Int J Pediatr Otorhinolaryngol 76: 1474-1480, 2012.
27. Qu C, Sun X, Shi Y, Gong A, Liang S, Zhao M, *et al*: Microarray-based mutation detection of pediatric sporadic nonsyndromic hearing loss in China. Int J Pediatr Otorhinolaryngol 76: 235-239, 2012.
28. Murgia A, Orzan E, Polli R, Martella M, Vinanzi C, Leonardi E, *et al*: Cx26 deafness: mutation analysis and clinical variability. J Med Genet 36: 829-832, 1999.
29. Liu Y, Ke X, Qi Y, Li W and Zhu P : Connexin26 gene (GJB2): prevalence of mutations in the Chinese population. J Hum Genet 47: 688-690, 2002.
30. Pryor SP, Madeo AC, Reynolds JC, Sarlis NJ, Arnos KS, Nance WE, *et al*: SLC26A4/PDS genotype-phenotype correlation in hearing loss with enlargement of the vestibular aqueduct (EVA): evidence that Pendred syndrome and non-syndromic EVA are distinct clinical and genetic entities. J Med Genet 42: 159-165, 2005.
31. Dai P, Yuan Y, Huang D, Zhu X, Yu F, Kang D, *et al*: Molecular etiology of hearing impairment in Inner Mongolia: mutations in SLC26A4 gene and relevant phenotype analysis. J Transl Med 6: 74, 2008.
32. Valvassori GE and Clemis JD: The large vestibular aqueduct syndrome. Laryngoscope 88: 723-728, 1978.
33. Chen G, He F, Fu S and Dong J: GJB2 and mitochondrial DNA 1555A>G mutations in students with hearing loss in the Hubei Province of China. Int J Pediatr Otorhinolaryngol 75: 1156-1159, 2011.
34. Wu BL, Lindeman N, Lip V, *et al*: Effectiveness of sequencing connexin 26 (GJB2) in cases of familial or sporadic childhood deafness referred for molecular diagnostic testing. Genet Med 4: 279-288, 2002.
35. Ma Y, Yang T, Li Y, *et al*: Genotype-phenotype correlation of two prevalent GJB2 mutations in Chinese newborn infants ascertained from the Universal Newborn Hearing Screening Program. Am J Med Genet A 152A: 2912-2915, 2010.

36. Ji YB, Han DY, Lan L, Wang DY, Zong L, Zhao FF, *et al*: Molecular epidemiological analysis of mitochondrial DNA12SrRNA A1555G, GJB2, and SLC26A4 mutations in sporadic outpatients with nonsyndromic sensorineural hearing loss in China. Acta Otolaryngol 131: 124-129, 2011.
37. Tekin M, Xia XJ, Erdenetungalag R, Cengiz FB, White TW, Radnaabazar J, *et al:* GJB2 mutations in Mongolia: complex alleles, low frequency, and reduced fitness of the deaf. Ann Hum Genet 74: 155-164, 2010.
38. Kelley PM, Harris DJ, Comer BC, Askew JW, Fowler T, Smith SD, *et al*: Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. Am J Hum Genet 62: 792-799, 1998.
39. Bruzzone R, Veronesi V, Gomès D, Bicego M, Duval N, Marlin S, *et al*: Loss-of-function and residual channel activity of connexin26 mutations associated with non-syndromic deafness. FEBS Lett 533: 79-88, 2003.
40. Snoeckx RL, Huygen PL, Feldmann D, Marlin S, Denoyelle F, Waligora J, *et al*: GJB2 mutations and degree of hearing loss: a multicenter study. Am J Hum Genet 77: 945-957, 2005.
41. Oguchi T, Ohtsuka A, Hashimoto S, Oshima A, Abe S, Kobayashi Y, *et al*: Clinical features of patients with GJB2 (connexin 26) mutations: severity of hearing loss is correlated with genotypes and protein expression patterns. J Hum Genet 50: 76-83, 2005.
42. Huculak C, Bruyere H, Nelson TN, Kozak FK and Langlois S: V37I connexin 26 allele in patients with sensorineural hearing loss: evidence of its pathogenicity. Am J Med Genet A 140: 2394-2400, 2006.
43. Hayashi C, Funayama M, Li Y, Kamiya K, Kawano A, Suzuki M, *et al*: Prevalence of GJB2 causing recessive profound non-syndromic deafness in Japanese children. Int J Pediatr Otorhinolaryngol 75: 211-214, 2011.
44. Liu XZ, Xia XJ, Ke XM, Ouyang XM, Du LL, Liu YH, *et al*: The prevalence of connexin 26 (GJB2) mutations in the Chinese population. Hum Genet 111: 394-397, 2002.
45. Paneto GG, Köhnemann S, Martins JA, Cicarelli RM and Pfeiffer H: A single multiplex PCR and SNaPshot minisequencing reaction of 42 SNPs to classify admixture populations into mitochondrial DNA haplogroups. Mitochondrion 11: 296-302, 2011.
46. Speiser PW and White PC: Congenital adrenal hyperplasia. N Engl J Med 349: 776-788, 2003.
47. Kirchhoff T, Gaudet MM, Antoniou AC, McGuffog L, Humphreys MK, Dunning AM, *et al*: Breast cancer risk and 6q22.33: combined results from Breast Cancer Association Consortium and Consortium of Investigators on Modifiers of BRCA1/2. PLoS One 7: e35706, 2012.
48. Sagong B, Baek JI, Oh SK, Na KJ, Bae JW, Choi SY, *et al*: A rapid method for simultaneous screening of multi-gene mutations associated with hearing loss in the Korean population. PLoS One 8: e57237, 2013.
49. Wang QJ, Zhao YL, Rao SQ, Guo YF, Yuan H, Zong L, *et al*: A distinct spectrum of SLC26A4 mutations in patients with enlarged vestibular aqueduct in China. Clin Genet 72: 245-254, 2007.
50. Choi BY, Stewart AK, Madeo AC, Pryor SP, Lenhard S, Kittles R, *et al*: Hypo-functional SLC26A4 variants associated with nonsyndromic hearing loss and enlargement of the vestibular aqueduct: genotype-phenotype correlation or coincidental polymorphisms? Hum Mutat 30: 599-608, 2009.
51. Tsukamoto K, Suzuki H, Harada D, Namba A, Abe S and Usami S: Distribution and frequencies of PDS (SLC26A4) mutations in Pendred syndrome and nonsyndromic hearing loss associated with enlarged vestibular aqueduct: a unique spectrum of mutations in Japanese. Eur J Hum Genet 11: 916-922, 2003.
52. Park HJ, Lee SJ, Jin HS, Lee JO, Go SH, Jang HS, *et al*: Genetic basis of hearing loss associated with enlarged vestibular aqueducts in Koreans. Clin Genet 67: 160-165, 2005.
53. Azaiez H, Yang T, Prasad S, Sorensen JL, Nishimura CJ, Kimberling WJ, *et al*: Genotype-phenotype correlations for SLC26A4-related deafness. Hum Genet 122: 451-457, 2007.
54. Albert S, Blons H, Jonard L, Feldmann D, Chauvin P, Loundon N, *et al*: SLC26A4 gene is frequently involved in nonsyndromic hearing impairment with enlarged vestibular aqueduct in Caucasian populations. Eur J Hum Genet 14: 773-779, 2006.
55. de Moraes VC, dos Santos NZ, Ramos PZ, Svidnicki MC, Castilho AM and Sartorato EL: Molecular analysis of SLC26A4 gene in patients with nonsyndromic hearing loss and EVA: identification of two novel mutations in Brazilian patients. Int J Pediatr Otorhinolaryngol 77: 410-413, 2013.