Original Article

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Children with *GJB2* gene mutations have various audiological phenotypes

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Summary **The current study retrospectively investigated variations in audiological phenotypes in children with *GJB2* gene mutations. Subjects were 128 infants and young children who were seen as outpatients by Otology at Beijing Tongren Hospital from 2012 to 2018. Of the 128 subjects, 99 had biallelic truncating (T/T) mutations and 29 had truncating/nontruncating (T/NT) mutations. Genotypes, results of universal newborn hearing screening (UNHS), and the degree and symmetry of hearing loss were examined in the two groups. Twenty-two subjects (20.37%, 22/128) passed UNHS, including 13 children with T/T mutations and 9 with T/NT mutations. Of the 128 subjects, 22 had normal hearing, 2 had unilateral hearing loss, and 115 had bilateral hearing loss. Severe-to-profound hearing loss was the most prevalent phenotype in children with T/T mutations (73.23%), while normal hearing was prevalent in children with T/NT mutations (41.38%). Symmetrical hearing loss was the main phenotype in both groups, and the number of subjects with symmetrical hearing loss did not differ significantly between the two groups. Therefore, children with *GJB2* gene mutations have phenotypic variability in terms of their results of UNHS and their degree and symmetry of hearing loss. Subjects with T/NT mutations of the *GJB2* gene were more likely to pass UNHS and had milder hearing loss compared to those with T/T mutations. Symmetrical hearing loss was the main phenotype in the two groups, but 36.53% of children had bilateral asymmetric hearing loss. Parents of all subjects with sensorineural hearing loss were informed that their children may have a *GJB2* mutation.**

Keywords: *GJB2* gene, screening, Genotype

1. Introduction

Hearing loss is the most common congenital sensory disorder, with studies describing an incidence of neonatal congenital deafness of approximately 0.1-0.3%, 50% of which is caused by genetic factors (*1*). Hereditary deafness is divided into nonsyndromic hearing loss (NSHL) and syndromic hearing loss (SHL).

The *GJB2* gene that encodes connexin26 (Cx26) is the gene that most often causes NSHL (*2*). There are ethnic differences in *GJB2* gene mutations. In Caucasians in Europe and the US, c.35delG is the most common mutation (*3*), while c.235delC is most commonly found in Chinese with an allele frequency of approximately 11.90%, followed by c.299delAT with a frequency of approximately 2.22%, c.176del16 with a frequency of approximately0.65%, and c.35delG with a frequency of approximately 0.27% (*4*). A recent study been found that the p.V37I mutation is also highly prevalent in Chinese, with a carrier frequency of 6.2% (*5*).

GJB2-associated hearing loss can result in congenital severe-to-profound bilateral hearing loss, and the severity of hearing loss guides the clinical management

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of a mutation (*6*). There has been a growing recognition of phenotypic diversity associated with various *GJB2* genotypes. The degree of *GJB2*-associated hearing loss depends on the degree of damage to the coding protein connexin26. Truncating mutations, which create a premature stop codon and result in the absence of any functional connexin 26 protein, constitute nonsense mutations. The functions of nontruncating mutations, which result in altered proteins that may or may not be impaired, constitute missense mutations. Most *GJB2* gene mutations can be classified as truncating mutations (T) and nontruncating mutations (NT). Snoeckx *et al*. studied patients ages 0-26 with *GJB2* biallelic alleles; the most common mutation was c.35delC and the degree of hearing loss was related to the genotype (*7*). In those patients, the degree of hearing loss was most severe with a T/T mutation, followed by a T/NT mutation and an NT/NT mutation. In a previous study of *GJB2* mutations among Chinese ages 0-68, Dai *et al*. found that the degree of hearing loss was mild-to-profound and proposed detection of the *GJB2* gene for all patients with sensorineural hearing loss (*8*).

As a result of the promotion of "newborn screening for deafness genes," many patients with *GJB2*-associated hearing loss have been identified at a young age. Therefore, one aim of the current study was to classify children with *GJB2*-associated hearing loss depending on mutations in that gene. A second aim of this study was to examine genotypes, the results of universal newborn hearing screening (UNHS), and the degree of hearing loss in order to provide a basis for clinical genetic counseling.

2. Materials and Methods

Written informed consent was obtained from parents of all subjects. This study was in accordance with the principles of the Declaration of Helsinki and it was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University.

2.1. *Subjects*

Subjects were 128 children with *GJB2*-associated hearing loss who were seen by Otolaryngology and Head and Neck Surgery at Tongren Hospital (Beijing, China) between 2012 and 2018. All subjects were screened for nine loci in four genes, including *GJB2* c.235delC, c.299delAT, c.176dell6, and c.35delG; *GJB3* c.538C>T; SLC26A4 c.IVS7-2A>G and c.2168A>G; and mitochondrial *12S rRNA* m.1555A>G and m.1494C>T. Subjects with *GJB2* heterozygous mutations were screened for all exons and flanking splice sites of the *GJB2* gene. Exclusion criteria were as follows: carriers of GJB3 c.538C>T, SLC26A4 c.IVS7-2A>G, or c.2168A>G, or mitochondrial *12S rRNA* m.1555A>G or m.1494C>T mutations; familial segregation of hearing loss in an autosomal dominant, maternally transmitted, or X-linked manner; and individuals with syndromic hearing loss, conductive deafness, or secretory otitis media. Based on *GJB2* gene mutations, the subjects were divided into two groups, those with biallelic truncating (T/T) mutations and those with compound heterozygous truncating/nontruncating (T/NT) mutations.

2.2. *Clinical evaluation*

The following demographic information was collected for each subject: sex, date of birth and birth history, date of initial otolaryngological consultation, and major comorbidities.

2.3. *DNA analysis*

Genomic DNA was extracted from 2 mL of whole blood from each subject using the Blood DNA kit (Tiangen Biotech, Beijing, China). All exons and flanking splice sites of the *GJB2* genes were screened for mutations through amplification with PCR and bidirectional sequencing.

2.4. *Auditory evaluation*

Subjects underwent universal newborn hearing screening and a physical examination, including an otoscopic examination, with special attention to hearing. Comprehensive audiological evaluation included auditory brainstem response (ABR), 40-Hz auditory event-related potential, distortion product otoacoustic emission, auditory steady-state response (ASSR), acoustic immittance, and pediatric behavioral audiometry. The hearing threshold was calculated as the average hearing level at 0.5, 1.0, 2.0, and 4.0 kHz according to the 1997 standards of the World Health Organization. The severity of hearing impairment was defined as mild (26-40 dB), moderate (41-60 dB), severe (61-80 dB), or profound (> 80 dB). Owing to the subjects' young age, the ABR threshold and/or ASSR were recorded, and mean thresholds at frequencies in the 0.5-4 kHz range were averaged to obtain an approximation for directional conditioned reflex. For children lacking behavioral thresholds and ASSR results, the ABR threshold was considered the high-frequency auditory threshold (*9,10*). Subjects with normal hearing and unilateral hearing loss were excluded, and asymmetrical hearing loss was defined as a difference in HL > 10 dB between the ears for at least two frequencies (*11*).

2.5. *Statistical analysis*

The statistical software SPSS21.0 was used to analyze data with the chi-squared (χ^2) test.

3. Results

3.1. *Demographics*

Of the 128 children, 59.37% were male and 40.63% were female. Subjects ranged in age from 4 to 112 months (mean, 44.13 ± 23.59 months). Ninety-one percent of subjects ranged in age from 4-72 months. The age at first visit ranged from 2 to 83 months (mean, 10.52 ± 14.59 months). Table 1 shows the clinical characteristics of the two groups. All subjects were Chinese.

3.2. *Genetic testing*

The 128 subjects had a total of 9 mutations that were grouped into 14 genotypes. Of the 9 mutations, 5 were truncating mutations and 4 were nontruncating mutations. The most common mutation locus was c.235delC at a frequency of 63.67%. c.299delAT was found at a frequency of 19.92% and c.109G>A was found at a frequency of 8.99%. Five infrequent mutations were identified, including c.230G>A, c.257C>G, c.427C>T, c.583A>G, and c.9G>A. Of the 14 genotypes, 7 were found in children with T/

Table 1. Comparison of basic parameters of the two groups (*n* = 128 newborns)

group	Number	Sex: male/female	Age (months): Range (Mean)	Age at first visit (months): Range (Mean)
T/T	99	55/44	4-112 (45.44 ± 23.15)	2-77 (9.85 ± 12.81)
T/NT	29	21/8	9-112 (39.62 ± 24.92)	2-83 (10.66 ±16.07)
Total	128	76/52	4-112 (44.13 ± 23.59)	2-83 (10.52 ± 14.59)

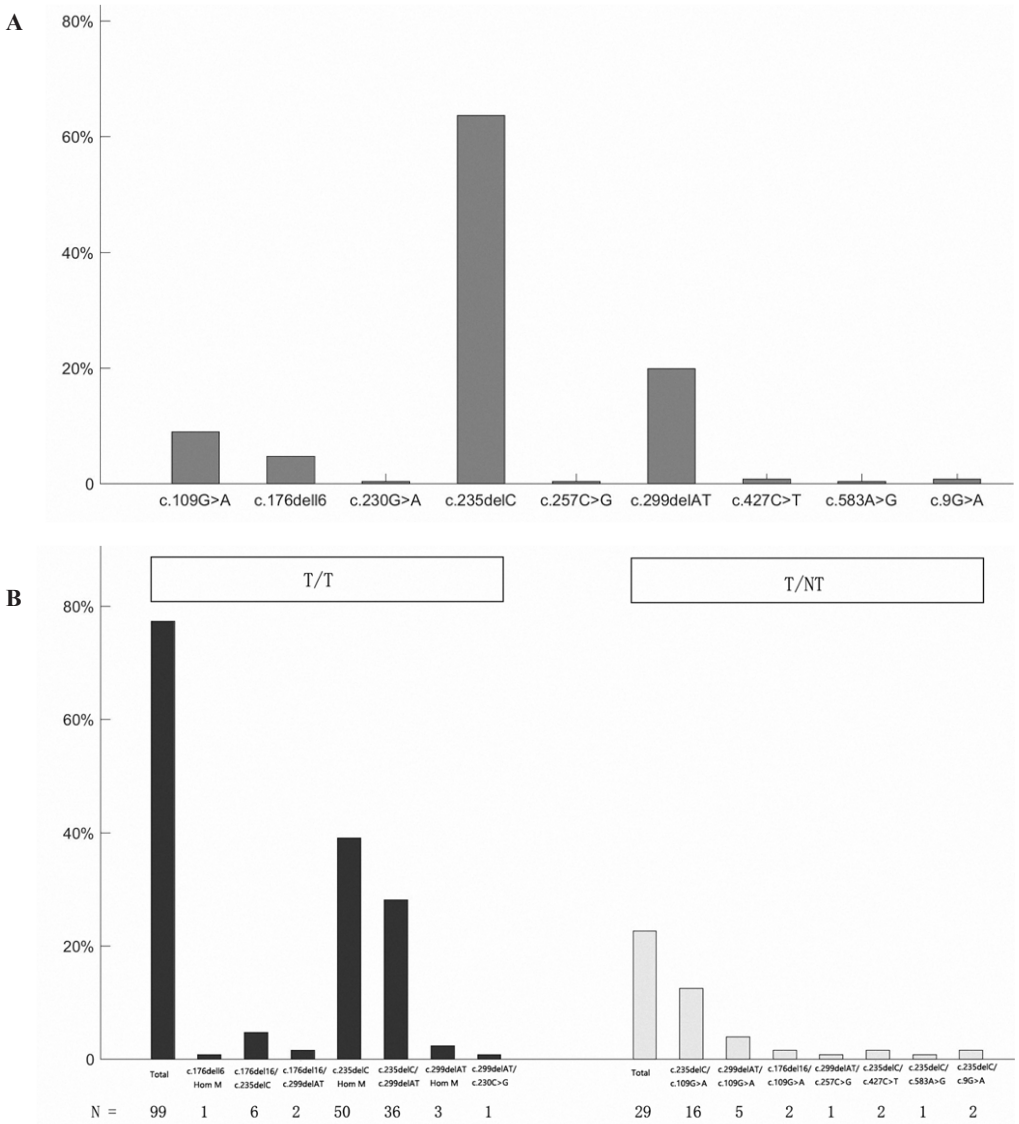

Figure 1. Results of genetic testing of subjects. (A) In total, 9 mutations were found in subjects. **(B)** Subjects had 14 genotypes, including 7 in children with T/T mutations and 7 in children with T/NT mutations. The number of subjects is shown under each subgroup. Hom M: homozygous mutation; CHM: compound heterozygous mutations.

T mutations and 7 were found in children with T/NT mutations. c.235delC Hom M was the most common genotype in children with T/T mutations (39.06%). In children with T/NT mutations, c.109G>A /c.235delC CHM was the most common genotype with a frequency of 12.50% (Figure 1).

3.3. *Phenotype and UNHS*

Table 2 shows the comparison of the results of UNHS for the two groups. In UNHS, 106 subjects had normal hearing, including 96 with bilateral normal hearing and 10 with unilateral passing results, whereas 22 passed UNHS. In UNHS, most subjects in both groups had bilateral normal hearing; this was true for 81.82% of children with T/T mutations and 51.72% of children with T/NT mutations (Table 2). Pair-wise comparisons of the two groups revealed significant differences (χ^2 = 26.05, P = 0.0038). In addition, 22 subjects passed UNHS with at least one ear. Bilateral hearing loss was examined in those subjects, revealing 18 children with T/T mutations and 4 with T/NT mutations. In addition, 3 children with T/NT mutations were diagnosed with normal hearing in at least one ear.

3.4. *Phenotype and diagnosis of audiology*

3.4.1. *Phenotype and the symmetrical/asymmetrical hearing loss.*

Of the 128 subjects, 11 had normal hearing, 2 had unilateral hearing loss, and 115 had bilateral hearing loss. Of subjects with bilateral hearing loss, 73 had symmetrical hearing loss and 42 had asymmetrical hearing loss. Symmetrical hearing loss was highly prevalent in children with T/T mutations (62.63%) or T/NT mutations (68.75%) (Table 3). Pair-wise comparisons of the two groups revealed no differences (χ^2 = 0.22, P = 0.7824)

3.4.2. *Phenotype and the degree of hearing loss*

Of 258 ears, 24 were normal and 234 ears had sensorineural hearing loss. Normal ears were only noted in children with T/NT mutations (41.83%, 24/58), and genotypes in subjects with normal hearing were c.109A>G compound heterozygous mutations. Of the 234 ears with hearing loss, 96 had profound hearing loss, 61 had severe hearing loss, 56 had moderate hearing loss, and 19 had mild hearing loss. Profound hearing loss was the most common phenotype in children with T/T mutations with a frequency of 46.46%, following by severe hearing loss with a frequency of 36.77%. However, mild and moderate hearing loss were more often detected in children with T/NT mutations (with a respective frequency of 24.14% and 13.79%) (Table 4). Pair-wise comparisons of the two groups revealed significant differences (χ^2 = 131.87, P < 0.0001). Table 5 shows the average hearing threshold for 14 genotypes in two groups. Subjects with T/T mutations had more severe hearing impairment, with an average hearing threshold of 75.40 ± 22.33

Table 2. Comparison of the results of UNHS in the two groups (*n* = 128 cases)

Group	Subjects undergoing UNHS (%)			χ^2	P
	Bilateral pass	Unilateral pass	Bilateral normal hearing		
T/T	13 (13.13)	5 (5.05)	81 (81.82)		
T/NT	9 (31.04)	5 (17.24)	15 (51.72)	26.05	0.0038
Total	22 (17.19)	10 (7.81)	96 (75.00)		

Table 3. Comparison of symmetrical/asymmetrical hearing loss in the two groups (*n* = 115 cases)

Group	Incidence of hearing loss (%)		χ^2	P
	symmetrical	asymmetrical		
T/T	62 (62.63)	37 (37.37)		
T/NT	11 (68.75)	5 (31.25)	0.22	0.7824
Total	73 (63.47)	42 (36.53)		

Table 4. Comparison of the degree of hearing loss in the two groups (*n* = 256 ears)

Group	The degree of hearing loss (%)					χ^2	P
	normal	moderate	mild	severe	profound		
T/T	0 (0)	11 (5.56)	42 (21.21)	53 (26.77)	92 (46.46)		
T/NT	24 (41.38)	8 (13.79)	14 (24.14)	8 (13.79)	4 (6.90)	26.05	0.0038
Total	24 (9.38)	19 (7.42)	56 (21.88)	61 (23.83)	96 (37.50)		

Table 5 Average hearing threshold for 14 genotypes (*n* = 256 ears)

Group	Genotype	Ear	Average hearing threshold (dB)	Average hearing threshold for the group (dB)
T/T	c.176dell6 Hom M	2	100.00 ± 0	75.40 ± 22.33
	c.176del16 / c.235delC CHM	6	92.30 ± 11.20	
	c.176del16 / c.299delAT CHM	4	88.25 ± 11.61	
	c.235delC Hom M	100	77.99 ± 21.63	
	c.235delC / c.299delAT CHM	72	68.87 ± 22.42	
	c.299delAT Hom M	6	77.50 ± 26.91	
	c.299delAT / c.230C>G CHM	2	37.00 ± 1.14	
T/NT	c.235delC / c.109G>A CHM	32	31.20 ± 19.87	39.69 ± 25.92
	c.299delAT / c.109G>ACHM	10	38.30 ± 15.75	
	c.176del16/ c.109G>A CHM	4	38.00 ± 30.99	
	c.299delAT/ c.257C>G CHM	2	56.50 ± 0.71	
	c.235delC/ c.427C>T CHM	4	100.00 ± 0	
	c.235delC/ c.583A>G CHM	2	52.50 ± 16.26	
	c.235delC/ c.9G>A CHM	4	40.50 ± 24.02	

dB, and subjects with T/NT mutations a mean hearing threshold of 39.69 ± 25.92 dB.

4. Discussion

The *GJB2* gene is the main cause of hereditary nonsyndromic deafness, and its normal expression in the inner ear is required for normal development and signal transduction between inner ear sensory cells and supporting cells. Over 50% of cases of congenital autosomal recessive nonsyndromic deafness hearing loss (ARNSHL) are caused by mutations of the *GJB2* gene (*12*). The rates of *GJB2* mutation vary by country, and 21% of mutations have been found in Chinese patients (*13*). Therefore, *GJB2* mutations are a critical component of audiological screening and genetic counseling.

GJB2 gene mutations vary widely among ethnic groups. c.35delG is the most common variant in Caucasian populations (*14*). However, in Chinese populations, the c.235delC mutation has the highest prevalence (11.90%), followed by c.299delAT (2.22%), c.176del16 (0.65%), and c.35delG (0.27%) (*4*). Recent studies have indicated that the rate of the c.109G>A mutation is high in the Chinese population as well. Jiang *et al*. examined 155 patients with NSHL and reported that the incidence of c.109G>A was 9.03% (28/310) (*15*). Dai *et al*. performed genetic testing on 2,063 patients with NSHL and found that in the Chinese population the most common genotype of a *GJB2* gene mutation was c.235delC/c.235delC, followed by c.235delC/c.299delAT (*16*). A total of nine variants of the *GJB2* gene were detected in the current study, including c.235delC (63.67%), followed by c.299delAT (19.92%) and c.109G>A (8.99%). The most common genotype was c.235delC/c.235delC, and the c.35delG mutation was not detected. This finding is consistent with the results of previous studies. Five pathogenic variants (c.230G>A, c.257C>G, c.427C>T, c.583A>G, and c.9G>A) that had previously been reported were detected in the current study at lower rates.

Early studies suggested that hearing loss caused by *GJB2* gene mutations was mostly congenital and penetrant at birth and that the hearing phenotype was mainly bilateral, symmetrical, and moderate-to-profound SNHL. As research progressed, some researchers found that approximately 3.8-6.9% of children with *GJB2* gene mutations were able to pass UNHS, with delayed-onset hearing loss (*17,18*). This confirms that those patients had non-penetrance at birth to some extent. In the current study, 22 children had passing results on UNHS for both ears while 10 had passing results for one ear. Children with T/NT mutations had a significantly higher passing rate on UNHS than did children with T/T mutations. Wu *et al*. conducted hearing screening in combination with screening for deafness genes in 5,173 neonates, and they detected c.109G>A/c.109G>A mutations in 62 subjects, c.109G>A/c.235delC mutations in 16, and m.1555A>G mutations in 4. In that study, 46 subjects (56.1%) passed UNHS (*19*). In the current study, 109G>A compound heterozygous mutations were predominant in children with T/NT mutations, indicating that children with the c.109G>A mutation were more likely to pass UNHS. Subjects in both groups with bilateral SNHL had at least one ear that passed UNHS.

There are several possible reasons for this: (*i*) delayed-onset hearing loss, according to the study by Wu *et al*. The incidence of the c.109G>A mutation was 20% in children of Han nationality with delayed-onset hearing loss, and that rate was much higher than that in the normal hearing group, suggesting that this variant is associated with delayed-onset hearing loss. (*ii*) The results of UNHS were unreliable, to some extent, due to poor quality control during screening. A study indicated that approximately 11% to 31% of children with hearing loss who passed UNHS would eventually develop permanent hearing loss (*20*).

Three children (17.1%, 7/41) with T/NT mutations were referred for UNHS, and at least one ear was diagnosed with normal hearing in the current study. Li

et al. conducted genetic tests on 173 children who had been referred for UNHS and diagnosed with normal hearing (5). They detected the p.V37I mutation at a rate of 5.8% (10/173), which was much higher than the rate in children who passed UNHS (0.14%). Children with p.V37I compound heterozygous mutations may have subclinical or borderline slight hearing loss at birth. In addition to the reasons mentioned above, other causes could be that cochlear hair cells are relatively immature in some newborns or that middle ear effusion was present during UNHS, thus affecting the results of otoacoustic emissions. According, the sample size needs to be increased and an in-depth study needs to be conducted in the future.

A study found that hearing loss caused by *GJB2* gene mutations could be mild to profound and that patients with T/T mutations had a greater degree of hearing loss than patients with NT/T mutations (*7*). In the current study, children with T/T mutations mainly had severe-to-profound hearing loss (73.23%), while children with NT/T mutations mainly had normal hearing and mild-to-moderate hearing loss (79.31%). The severity of hearing loss differed significantly between the two groups. This finding was consistent with the results of previous studies. Chan *et al*. examined 52 children with *GJB2* gene mutations and found that the average hearing threshold was 100.3 dB in those with T/T mutations and 53.9 dB in those with NT/T mutations (*6*). In the current study, the average hearing threshold was 75.40 ± 22.33 dB in children with T/T mutations and 39.69 ± 25.92 dB in children with NT/T mutations. Both numbers were both lower than those reported by Chan *et al*. This may be due to differences in research methodologies, ethnicity, or age distribution. The subjects in the study by Chan *et al*. included Caucasians, Latinos, and Asians with an average age of 5.6 years, and the c.35delG mutation was common. However, the subjects in the current study were Chinese Han children with an average age of 3.7 years, and the c.235delC mutation was common. In the current study, the average age of enrollment was lower than that used by Chan *et al*. Therefore, patients with *GJB2* gene mutations could have progressive hearing loss (*21,22*), and the average hearing thresholds of the two groups in the current study were lower than those cited in the previous study by Chan *et al*. Dai *et al*. found that the *GJB2* mutation was detected at a higher rate in patients with bilateral SNHL and at a lower rate in patients with unilateral hearing loss.

Of the 117 subjects with hearing loss in this study, 2 had unilateral hearing loss and 115 had bilateral hearing loss, indicating that these subjects mainly had bilateral SNHL. Although symmetric hearing loss was predominant, 36.53% of children had asymmetric hearing loss. The number of subjects with symmetric hearing loss did noti differ significantly between the two groups. This suggested that children with *GJB2* mutations had varied levels of hearing loss, indicating a high degree of genetic heterogeneity.

5. Conclusion

Children with *GJB2* gene mutations have phenotypic variability in terms of their results on UNHS and their degree and symmetry of hearing loss. Hearing phenotypes were evident as normal hearing or mild-to-profound hearing loss. Subjects with T/NT mutations of the *GJB2* gene were more likely to pass UNHS and had milder hearing loss compared to those with T/T mutations. Symmetrical hearing loss was the main phenotype of the two groups, but 36.53% of children had bilateral asymmetric hearing loss. Therefore, parents of all subjects with sensorineural hearing loss were informed of the possibility that their child may had a *GJB2* mutation. In addition, a small number of children (17.18%) passed UNHS despite having bilateral hearing loss, indicating that delayed-onset hearing loss may occur later on.

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