Original Article

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Analysis of p.V37I compound heterozygous mutations in the *GJB2* gene in Chinese infants and young children

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Summary **The p.V37I (c.109G>A) mutation in the *GJB2* gene is the common frequent cause of congenital deafness; however, its pathogenicity is debated. The present study investigated the prevalence of p.V37I in Chinese infants and young children and associated clinical characteristics. The subjects of the present study were screened for mutations in *GJB2* (235delC, 299delAT, 176dell6, 35delG), *SLC26A4* (IVS7-2A>G, 2168A>G), *GJB3* (538C>T), and in the mitochondrial *12S rRNA* gene (1555A>G, 1494C>T). Subjects with p.V37I underwent an audiological evaluation. *GJB2* exon sequencing revealed that 20 subjects had p.V37I compound heterozygous mutations, one of whom had a family history; the mutations included c.235delC/p.V37I (*n* = 12), c.299AT/p.V37I (*n* = 7), and c.176del16/p.V37I (*n* = 1). Of the 20 subjects, 12 were referred for Universal Newborn Hearing Screening (UNHS). Nine of the 20 subjects had mild hearing loss in the better ear and 5 had moderate hearing loss in the better ear while 4 had normal hearing. Among subjects with the c.235delC/p.V37I mutation, 5 had mild hearing loss and 2 had moderate hearing loss while 3 had normal hearing. Among subjects with the c.299AT/p.V37I mutation, 3 had mld hearing loss and 3 had moderate hearing loss while 1 had normal hearing. One subject with the c.176del16/p.V37I mutation had mild hearing loss. Few studies have reported on the clinical characteristics of Chinese infants with p.V37I compound heterozygous mutations identified *via* screening for deafness genes and *GJB2* sequencing. The c.235delC/p.V37I mutation was the most prevalent mutation found in subjects. The degree of hearing loss associated with p.V37I compound heterozygous mutations was mainly mild to moderate.**

Keywords: Infant, children, gene, mutation, audiological evaluation, hearing loss

1. Introduction

Deafness, which refers to varying degrees of hearing loss, is one of the most common sensory disorders. Although there are many causes of deafness, genetic factors account for approximately 50-60% of cases (*1*). The *GJB2* gene is the most frequently mutated gene in cases of hereditary hearing loss, but the mutation spectrum varies among ethnic groups. For example, among Caucasians the most common *GJB2* mutation is c.35delG, with a carrier frequency of 2-4% (*2*). However, c.235delC is most frequently observed among Asians, with an allele frequency in the hearing impaired ranging from 5-22% (*3-5*). Notably, p.V37I (c.109G>A) also has a high incidence in Asia, with a frequency of 8.5% in Thailand (*6*), 1.75% in Japan (*7*), and 6.2% in China (*8*) in patients with hearing loss.

The p.V37I mutation was first identified as a polymorphism because it was present on one chromosome in normal individuals but not in the deaf (*9*). However, compound heterozygous mutations (*i.e.*, p.V37I and R143W heterozygous mutations) were later observed in a Japanese family that exhibited deafness (*10*). R143W was later confirmed to cause recessive nonsyndromic

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Table 1. Comparison of previous studies investigating the relationship between the *GJB2* p.V37I mutation and hearing status

Study	PT	Subjects	GT	MT	Conclusion
Snoeckx *et al*. (*19*)	2005	Persons with congenital HL. Patients were ages 0–70 years, with a median age of 8.	Not described	Hom M	The p.V37I homozygous mutation is associated with mild to moderate hearing loss.
Dai *et al*. (*22*)	2009	Patients with HL and an average age of 13.7 ± 4.5.	*GJB2* sequencing	Hom M	Revealed a unique *GJB2* mutation spectrum in Chinese patients with HL. The p.V37I mutation may be pathogenic, with an allele frequency of 6.7%.
Kim *et al*. (*23*)	2013	Children under the age of 15 with HL (n = 103). Five carried the p.V37I variant of *GJB2*.	Sanger sequencing of *GJB2* and targeted capture of exons and flanking sequences of 82 deafness genes	Het M	The p.V37I variant of *GJB2* contributes to the pathogenesis of mild HL and may justify sequencing of *GJB2* in Korean patients with mild to moderate hearing loss.
Chai Y *et al*. (*24*)	2015	945 subjects with HL and a mean age over 6. Twenty-five subjects were identified as p.V37I homozygous.	*GJB2* sequencing	Hom M	The homozygous p.V37I variant of *GJB2* is associated with diverse hearing phenotypes
Huang Y *et al*. (*25*)	2015	Of a total of 300 infants ages 0-3 months diagnosed with HL, 26 exhibited the p.V37I mutation of *GJB2*.	*GJB2* sequencing	Hom M, Het M	The p.V37I mutation of *GJB2* is also strongly correlated with SNHL in infants, whose hearing pheno-types ranged from mild to profound. In addition, 69.6% of p.V37I carriers exhibit mild to moderate HL, indicating that even patients with mild or moderate HL must be tested for *GJB2*.
Bakhchane A *et al*. (*26*)	2016	A large cohort of 152 Moroccan families with HL.	*GJB2* sequencing	Hom M, CH M	*GJB2* mutations may play a role in 43.42% of the Moroccan patients diagnosed with HL. p.V37I had an allele frequency of 3.29%. This mutation was homozygous and compound heterozygous in patients with moderate HI.

PT: time of publication; GT: Genetic testing; MT: mutation type; HL: hearing loss; Hom M: homozygous mutation; Het M: heterozygous mutation; CH M: compound heterozygous mutation; SNHL:sensorineural hearing loss; HI:hearing impaired.

sensorineural deafness (*11*). A study of the p.V37I genotype of *GJB2*, a genetic risk factor for permanent childhood hearing impairment, revealed that the mutation was closely related to late-onset hearing loss in Chinese children (*8*). Many studies (*12-14*) have also found that the mutation is more prevalent in patients than in control subjects; it has therefore been classified as a recessive missense mutation.

GJB2 has two modes of inheritance, *i.e.*, dominant and recessive, that often manifest as syndromic and nonsyndromic deafness, respectively. Most individuals with *GJB2*-associated deafness are nonsyndromic and exhibit autosomal recessive inheritance (*15*); *GJB2*-associated deafness was initially described as bilateral, severe-to-profound, autosomal-recessive, nonprogressive, sensorineural hearing loss (*16,17*). However, there is growing recognition of a wide range of phenotypes associated with *GJB2* mutations, ranging in severity from mild to profound hearing loss with varying degrees of progression (*18,19*). Given that p.V37I is associated with mild and progressive hearing loss, clinical identification of affected individuals is vital (*20,21*).

Recent studies on p.V37I have focused on the incidence of p.V37I (*6-8*) and the hearing status of individuals with a p.V37I homozygous mutation or heterozygous mutation (Table 1). Subjects in those studies generally had a mean age over 6 except those in the study by Huang *et al*. (*25*), where subjects had a mean age of 0-3 months. Nonetheless, the principle was the same: subjects were initially the hearing impaired; *GJB2* sequencing was performed as part of genetic testing, and results suggested that p.V37I-associated hearing loss was mild to moderate or diverse hearing loss (*19,22-26*). In China, newborn screening for deafness genes was first instituted in Beijing in 2012, and newborns are screened for 9 loci in 4 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. Numerous newborns have been referred for screening for deafness genes. In the process of genetic counseling and clinical diagnosis, a large number of infants with a single locus mutation in *GJB2* have been identified. In a preliminary study, the current authors screened 915 newborns for 4 *GJB2* mutant alleles, *i.e.*, c.235delC, c.299delAT, c.176del16, c.35delG, and *GJB2* gene sequencing revealed that

p.V37I had a frequency of 4.04%. However, no study has examined the clinical significance of the p.V37I compound heterozygous mutation in deafness (*27*). This was addressed in the present study by examining the clinical characteristics of children with this mutation.

2. Materials and Methods

Parents provided written, informed consent for subjects to participate in this study. The protocol was in accordance with the Declaration of Helsinki principles and was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University.

2.1. *Subject recruitment*

From 2012 to 2016, 1,348 Chinese newborns who underwent screening for deafness genes were recruited from among patients seeking genetic testing and counseling at the Department of Otolaryngology, Head and Neck Surgery, Tongren Hospital (Beijing, China). Newborns were screened for 9 loci in 4 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. After sequencing of *GJB2*, 20 children with compound heterozygous mutations in *GJB2*, *i.e.*, c.235delC, c.299delAT, c.176del16, or c.35delG, and p.V37I, were included in the study.

Inclusion criteria were as follows: 1 of the 4 mutations in *GJB2* identified in screening for deafness genes and the presence of the p.V37I mutation according to sequencing. 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; *GJB2* homozygous or compound heterozygous 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.

2.2. *Clinical evaluation*

The following demographic information was collected for each patient: sex, date of birth and birth history, history of maternal pregnancy, family history, date of initial otolaryngological consultation, and major comorbidities. All subjects were Chinese.

2.3. *DNA analysis*

Genomic DNA was extracted from 2 mL of whole blood from each patient, using the Blood DNA kit (Tiangen Biotech, Beijing, China). All exons and flanking splice sites of *GJB2* were screened for mutations using PCR amplification and bidirectional sequencing (*22*).

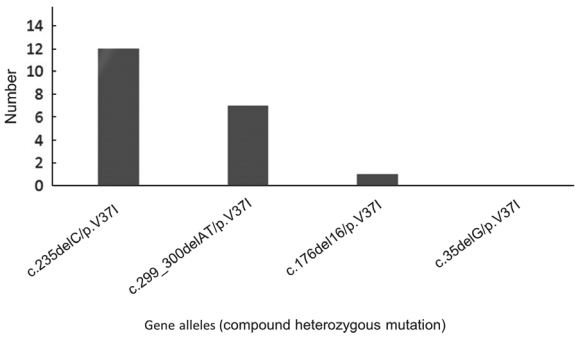

Figure 1. Distribution of *GJB2* genotypes among subjects.

2.4. *Auditory evaluation*

Subjects carrying known compound heterozygous mutations in *GJB2* (c.235delC, c.299delAT, c.176del16, or c.35delG and p.V37I) underwent a physical examination, including an otoscopic examination, with special attention to hearing. Comprehensive audiological evaluation included tympanometry, distortion product otoacoustic emission test, auditory brainstem response, auditory steady-state response (ASSR), and conditioned orientation reflex audiometry. The hearing threshold was calculated as the average hearing level at 0.5, 1.0, 2.0, and 4.0 kHz for the better ear according to the standards of the World Health Organization (1997). The severity of hearing impairment was defined as mild (26-40 dB), moderate (41-60 dB), severe (61-80 dB), or profound (> 80 dB) hearing loss. Given the subjects' young age, the auditory brainstem response threshold and/or ASSR were recorded, and mean thresholds at frequencies in the 0.5- to 4-kHz range were averaged to obtain an approximation for the conditioned orientation reflex (*23*). Subjects were considered as lost to follow-up if hearing results could not be obtained.

3. Results

3.1. *Demographic data*

DNA sequencing identified 20 unrelated children with a p.V37I compound heterozygous mutation, with the other locus being either c.235delC, c.299delAT, c.176del16, or c.35delG. Subjects included 14 males and 6 females with a mean age of 24.2 ± 14.7 months. One subject had a family history of hearing loss. None had an abnormal birth history or abnormal history of maternal pregnancy. The age at first visit was 3-6 months for 12 subjects, younger than 3 months for 3 subjects, and 6-12 months for 5 subjects.

3.2. *Genetic testing*

Three different sequence variants were identified in the coding region of *GJB2* (Figure 1). The most common mutation was the c.235delC/p.V37I compound

Table 2. Demographic information and audiological diagnoses of subjects (*n* = 20)

No.	Sex	Age (m)	Fvma (m)	Gene (p.V37I/X)	UNHS (L)	UNHS (R)	DP	AI	DHL (L)	DHL (R)
01	M	42	4	c.235delC	1	1	1	A	mild	mild
02	M	22	3	c.235delC	1	1	1	A	moderate	moderate
03	M	56	3	c.235delC	1	1	1	A	moderate	moderate
04	M	19	12	c.235delC	0	1	1	A	0	0
05	M	15	3	c.235delC	1	1	down	A	mild	mild
06	M	39	2	c.299delAT	1	1	down	A	mild	mild
07	M	26	9	c.299delAT	1	1	1	A	moderate	moderate
08	M	25	6	c.176del16	1	1	1	A	mild	mild
09	M	38	2	c.235delC	1	1	down	A	mild	mild
10	M	10	2	c.235delC	1	1	down	A	mild	mild
11	M	13	5	c.235delC	0	0	1	A	0	0
12	M	9	4	c.299delAT	0	1	down	A	moderate	moderate
13	M	6	4	c.299delAT	0	0	down	A	0	0
14	F	31	5	c.299delAT	1	1	1	A	moderate	moderate
15	F	8	3	c.235delC	0	0	down	A	mild	mild
16	M	11	3	c.235delC	0	0	down	A	0	0
17	F	7	3	c.299delAT	0	0	down	A	mild	mild
18	F	14	4	c.299delAT	0	0	down	A	mild	mild
19	F	44	8	c.235delC	0	0	/	/	/	/
20	F	40	12	c.235delC	0	0	/	/	/	/

Fvma: age at first visit (months); DP: distortion product otoacoustic emission test; AI: tympanometry; DHL: degree of hearing loss; M: male; F: female; m: months; L: left; R: right; 0: normal; 1: abnormal; /: not determined; A: normal tympanometry type.

Table 3. Patient characteristics

Fvma (m)	Sex		Alleles (with p.V37I)			UNHS		DHL (*n* = 18)		
	M	F	c.235delC	c.299delAT	c.176del16	Pass	Refer	Mild	Moderate	Normal
0-3	3	0	2	1	0	0	3	3	0	0
3-6	8	4	7	5	0	6	6	4	4	3
6-12	3	2	3	1	1	2	3	2	1	1
Total	14	6	12	7	1	8	12	9	5	4

Fvma: age at first visit; m: months; F: female; M: male; DHL: degree of hearing loss.

heterozygous mutation, which was detected in 12 of 20 subjects. Seven had the c.299AT/p.V37I compound heterozygous mutation and 1 had the c.176del16/p.V37I compound heterozygous mutation. No subjects had a c.35delG mutation.

3.3. *Audiological evaluation*

Clinical examination and medical history ruled out the involvement of environmental factors in the hearing loss of the 20 subjects and did not reveal clinical evidence of syndromic features. No pre- or postnatal risk factors were identified. Twelve subjects were referred for hearing screening, including two subjects who were referred for unilateral screening (right ear). Eight subjects passed the screening. Tympanometry revealed normal tympanic membranes in 18 subjects. Audiological results were obtained for 18 subjects; 2 subjects were lost to follow-up. Of the 18 subjects, 9 had mild hearing loss and 5 had moderate hearing loss while 4 had normal hearing (in the better ear). The 2 subjects who were lost to follow-up passed the hearing screening and were female (Table 2).

3.4. *Relationship between mutations and patient characteristics*

Table 4. Results of hearing screening by *GJB2* alleles (*n* = 20)

Alleles (with p.V37I)	UNHS Pass	UNHS Refer
c.235delC	5	7
c.299delAT	3	4
c.176del16	0	1

The age at first visit, sex, mutation, hearing screening, and degree of hearing loss were compared among subjects (Table 3). Most patients had their first visit at the age of 3-6 months (7 of those had the c.235delC/p.V37I compound heterozygous mutation and 5 had the c.299delAT/p.V37I compound heterozygous mutation). However, only 1 subject had the c.176del16/p.V37I mutation, and the subject's first visit was after the age of 6 months. The age at first visit was most often 3-6 months for subjects who passed the hearing screening ($n = 6$). A point worth noting is that 3 subjects who were referred for the hearing screening test were under the age of 3 months at first visit. The degree of hearing loss was determined for 18 subjects; mild hearing loss occurred irrespective of age at first visit.

Hearing screening test results were examined with respect to *GJB2* mutations (Table 4). Among subjects

Table 5. Degree of hearing loss by compound heterozygous GJB2 mutations (*n* = 18)

Degree of hearing loss	genetic loci		
	c.235delC/p.V37I	c.299delAT/p.V37I	c.176del16/p.V37I
Mild	5	3	1
Moderate	2	3	0
Severe	0	0	0
Profound	0	0	0
Normal	3	1	0
Total	10	7	1

with the c.235delC/p.V37I compound heterozygous mutation, 7 were referred for the hearing screening test and 5 passed. Among subjects with the c.299delAT/p.V37I mutation, 4 were referred for testing and 3 passed. One subject with the c.176del16/p.V37I mutation was referred for testing.

The degree of hearing loss in subjects was examined with respect to *GJB2* compound heterozygous mutations (Table 5). Among subjects with the c.235delC/p.V37I compound heterozygous mutation, 5 had mild hearing loss and 2 had moderate hearing loss while 3 had normal hearing. Among subjects with the c.299delAT/p.V37I mutation, 3 had mild hearing loss and 3 had moderate hearing loss while 1 had normal hearing. One subject with the c.176del16/p.V37I mutation exhibited mild hearing loss.

4. Discussion

Hearing impairment is a common disorder with a major impact on public health. Many factors can cause deafness, but genetics account for approximately 50-60% of cases (*1*). *GJB2* is the gene that is most frequently implicated in hearing impairment, and its normal expression in the inner ear is required for normal development and signal transduction between inner ear sensory cells and supporting cells (*28*). The *GJB2* gene mutation spectrum varies according to ethnicity. For example, the most common *GJB2* mutation among Caucasians is c.35delG (*2*), whereas c.235delC is the most prevalent mutation among Asians (*3-5*). The spectrum of *GJB2* gene mutations associated with autosomal recessive nonsyndromic hearing loss is continually expanding (*29*).

The p.V37I (c.109G>A) mutation is highly prevalent in Asia (*6-8,27*), but its pathogenicity is debated. Up to 6% of Han Chinese are carriers of p.V37I, suggesting that there are over 4 million people with p.V37I homozygous mutations in China (*24*). A previous study reported the occurrence of a compound heterozygous mutation (p.V37I and R143W) in a Japanese family with hearing impairment (*10*). R143W has been confirmed to cause recessive non-syndromic sensorineural deafness (*11*). However, p.V37I is also thought to be linked to hearing impairment, as it is found more often in patients than in normal controls (*10,12-14,30*). Therefore, p.V37I is classified as a recessive missense gene.

First, studies on p.V37I have focused on the incidence of p.V37I (*6-8*). Recent studies have increasingly revealed the hearing status of individuals with p.V37I homozygous mutation or heterozygous mutation (Table 1). In most of those studies, subjects generally had a mean age over 6 except those in the study by Huang *et al*. (*25*), where subjects had a mean age of 0-3 months. Nonetheless, the principle was the same: subjects were initially the hearing impaired, *GJB2* sequencing was performed as part of genetic testing, and results suggested that p.V37I-associated hearing loss was mild to moderate or diverse hearing loss (*19,22-26*). In China, newborn screening for deafness genes was first instituted in Beijing, in 2012, and newborns are screened for 9 loci in 4 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. Numerous newborns have been referred for screening for deafness genes. In the process of genetic counseling and clinical diagnosis, a large number of infants with a single locus mutation in *GJB2* have been identified. In a preliminary study, the current authors screened 915 newborns for 4 *GJB2* mutant alleles, *i.e*., c.235delC, c.299delAT, c.176del16, c.35delG, and *GJB2* sequencing revealed that p.V37I had a frequency of 4.04%. However, no study has examined the clinical significance of the p.V37I compound heterozygous mutation in deafness (*27*). This was addressed in the present study by examining the clinical characteristics of children with this mutation.

The present study examined the rate of *GJB2* compound mutations, *i.e*., p.V37I concurrent with either c.235delC, c.299delAT, c.176del16, or c.35delG, in Chinese children under the age of 5. Results revealed that more males than females were compound heterozygotes. The most common compound heterozygous mutation was c.235delC/p.V37I, which was detected in 12 of 20 subjects, while no subjects had the c.35delG mutation. This is consistent with previous findings that c.235delC is common among Chinese whereas c.35delG is rare (*2-5*).

More subjects were referred for the hearing screening test than subjects who passed. Of those who

underwent screening, only 1 had normal hearing; the others exhibited mild to moderate hearing loss. Of those that passed the hearing screening test and were followed up, half had mild hearing loss. A study found that biallelic mutations in *GJB2* are a rare occurrence among newborns that pass the screening test if p.V37I is screened for (*31*). The inability to identify an infant who is most likely deaf based on genetic testing may be attributable to technical limitations or to the late onset of hearing loss. This would contradict the contention that the hearing loss caused by *GJB2* mutations is usually congenital, moderate to profound, and nonprogressive (*16,17*).

The p.V37I mutation is associated with mild to moderate hearing loss (*30-32*), which was corroborated by the current findings. Biallelic truncating mutations (*i.e.*, frameshift and nonsense mutations such as c.35delG, c.235delC, and c.167delT) are detected more frequently in individuals with severe to profound hearing loss, whereas biallelic amino acid substitutions (missense mutations; *e.g.*, p.V37I) occur more frequently in individuals with mild to moderate hearing loss. Mutations in the *GJB6* gene should be considered when infants present with hearing impairment since these mutations are significantly correlated with *GJB2* mutations in individuals with nonsyndromic sensorineural hearing loss (*33*).

5. Conclusion

The *GJB2* c.235delC/p.V37I compound heterozygous mutation was the mutation that was detected most often in the Chinese children in this study, and that mutation was mainly associated with mild to moderate hearing loss. Therefore, children need to undergo hearing screening or screening for the *GJB2* p.V37I mutation. Although the current findings need to be verified in a larger sample, they nonetheless provide a reference for clinical diagnosis and treatment of hearing loss in children.

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