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Mitochondrial 12S rRNA variants in 1642 Han Chinese pediatric subjects with aminoglycoside-induced and nonsyndromic hearing loss

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Abstract

In this report, we investigated the frequency and spectrum of mitochondrial 12S rRNA variants in a large cohort of 1642 Han Chinese pediatric subjects with aminoglycoside-induced and nonsyndromic hearing loss. Mutational analysis of 12S rRNA gene in these subjects identified 68 (54 known and 14 novel) variants. The frequencies of known 1555A>G and 1494C>T mutations were 3.96% and 0.18%, respectively, in this cohort with nonsyndromic and aminoglycoside-induced hearing loss. Prevalence of other putative deafness-associated mutation at positions 1095 and 961 were 0.61% and 1.7% in this cohort, respectively. Furthermore, the 745A>G, 792C>T, 801A>G, 839A>G, 856A>G, 1027A>G, 1192C>T, 1192C>A, 1310C>T, 1331A>G, 1374A>G and 1452T>C variants conferred increased sensitivity to ototoxic drugs or nonsyndromic deafness as they were absent in 449 Chinese controls and localized at highly conserved nucleotides of this rRNA. However, other variants appeared to be polymorphisms. Moreover, 65 Chinese subjects carrying the 1555A>G mutation exhibited bilateral and sensorineural hearing loss. A wide range of severity, age-of-onset and audiometric configuration was observed among these subjects. In particular, the sloping and flat shaped patterns were the common audiograms in individuals carrying the 1555A>G mutation. The phenotypic variability in subjects carrying these 12S rRNA mutations indicated the involvement of nuclear modifier genes, mitochondrial haplotypes, epigenetic and environmental factors in the

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phenotypic manifestation of these mutations. Therefore, our data demonstrated that mitochondrial 12S rRNA is the hot spot for mutations associated with aminoglycoside ototoxicity.

Keywords

hearing loss; aminoglycosides; 12S rRNA; variants; mitochondrial; Chinese

1. INTRODUCTION

Hearing loss is one of the most common human health problems, affecting one in 700–1000 newborns (Morton, 1991; Mel et al. 2002; Morton, 2002). Hearing loss can be caused by the gene aberrations or environmental factors including ototoxic drugs such as aminoglycoside antibiotics (Morton, 2002). In familial cases of ototoxicity, aminoglycoside hypersensitivity is often maternally transmitted, suggesting that mutation(s) in mitochondrial DNA (mtDNA) is the molecular basis for this susceptibility (Fischel-Ghodsian, 2005; Guan, 2005). As mitochondrial ribosomes share more similarities to bacterial ribosomes than do cytosolic counterparts, the human mitochondrial 12S rRNA was proposed to be the primary targeting site for aminoglycosides (Fischel-Ghodsian, 2005; Guan, 2005). Recently, the 12S rRNA 1555A>G mutation has been found to be responsible for both aminoglycoside-induced and nonsyndromic hearing loss in many families worldwide (Fischel-Ghodsian et al., 1993; Prezant et al. 1993; Pandya et al., 1997; Estivill et al. 1998; del Castillo et al., 2003; Li et al. 2004a; 2004b; Jacobs et al., 2005; Young et al. 2005; 2006; Yuan et al., 2005; Zhao et al., 2005b; Tang et al. 2007; Chen et al. 2008; Wang et al., 2008; Lu et al., 2010). On the other hand, the 12S rRNA 1494C>T mutation has been associated with both aminoglycoside-induced and nonsyndromic hearing loss only in some Chinese and Spanish families (Zhao et al., 2004a; Rodriguez-Ballesteros et al., 2006; Wang et al., 2006; Han et al., 2007; Chen et al., 2007; Zhu et al. 2009).

The 1555A>G and 1494C>T mutations are located at the highly conserved A-site of mitochondrial 12S rRNA (Prezant et al., 1993; Zhao et al., 2004a; 2005a; Qian et al., 2009). The A1555 and C1494 (equivalent to positions 1491 and 1409 of *Escherichia coli* 16S rRNA, respectively) are in apposition to each other but do not form a base-pair. The 1555A>G or 1494C>T mutation creates a new G-C or A-U pair base-pair, thereby extending the adjacent stem by one nucleotide and making the secondary structure of mitochondrial 12S rRNA more closely resemble the corresponding region of *E. coli* 16S rRNA and altering binding properties of aminoglycosides such as paromomycin, neomycin, gentamicin, and kanamycin at the A-site of 12S rRNA (Hamasaki and Rando, 1997; Zhao et al., 2004a; Qian and Guan, 2009). Thus, the administration of aminoglycosides can induce or worsen hearing loss in these subjects carrying the 1555A>G or C1494T mutation. In the absence of aminoglycosides, matrilineal relatives within and among families carrying the 1555A>G or C1494T mutation exhibited a considerable phenotypic variation with respect to severity and age-of-onset and penetrance of hearing loss (Prezant et al. 1993; Matthijs et al. 1996; Estivill et al. 1998; Li et al. 2004a; 2004b; Zhao et al., 2004a; Young et al. 2005; 2006; Tang et al. 2007; Chen et al., 2007; Chen et al. 2008). Therefore, additional modifier factors such as aminoglycosides, nuclear and mitochondrial genetic modifiers contributed to the phenotypic variability of these mtDNA mutations (Guan et al. 1996; 2000; 2001; 2006; Zhao et al. 2004a; 2005a).

However, the incidences of the 1555A>G and 1494C>T mutations were only reported in the small cohorts of hearing-impaired subjects (Hutchin et al., 1993; Usami et al., 2000; Malik et al., 2003; Li et al., 2004b; 2005; Jacobs et al., 2005). As these mutations are only responsible for a portion of patients with hearing loss, it is anticipated that additional mutations causing hearing loss can be found in the same gene. In the present investigation, we carried out a

systematic and extended mutational screening of 12S rRNA gene in a large cohort of 1642 hearing-impaired Han Chinese pediatric subjects from Zhejiang Province, China. Mutational analysis of 12S rRNA gene in these subjects identified 68 nucleotide changes including the 1555A>G mutation in 65 subjects and the 1494C>T mutation in 3 subjects. These 12S rRNA variants have been further evaluated by phylogenetic analysis, structure-function relation and allelic frequency of these variants in the 449 Han Chinese controls from the same region. Furthermore, we performed the clinical characterization of 65 subjects carrying the 1555A>G mutation and 21 subjects carrying one of 12 putative deafness-associated 12S rRNA mutations. In addition, it was implied that *TRMU* and *GJB2* mutations modulated the phenotypic manifestation of hearing loss associated with the A1555G mutation (Guan et al., 2006; Abe et al., 2001). To further examine the role of the *TRMU* and *GJB2* genes in the phenotypic manifestation of the 1555A>G mutation and 12 putative 12S rRNA mutations, we performed a mutational analysis of the *TRMU* and *GJB2* genes in these hearing-impaired subjects carrying one of these mutations.

2. SUBJECTS AND METHODS

2.1. Subjects and audiological examinations

A total of 1642 unrelated hearing-impaired Chinese subjects, who were younger than 18 years old from Zhejiang Province, and some of their family members, were enrolled in this study under an institutional review board-approved protocol of informed consent at the Cincinnati Children's Hospital Medical Center Institutional Review Board and Ethics Committee of Wenzhou Medical College, China. As shown in Figure 1, these subjects consisted of 1441 students from 17 Schools of Deaf Children among 14 cities/counties of Zhejiang Province, China and 201 patients who were diagnosed with aminoglycoside-induced and nonsyndromic hearing impairment by the Otology Clinic of Wenzhou Medical College. A comprehensive history and physical examination for these participating subjects were performed to identify any syndromic findings, the history of the use of aminoglycosides, genetic factors related to the hearing impairment. An age-appropriate audiological examination was performed and this examination included pure-tone audiometry (PTA) and/or auditory brainstem response (ABR), immittance testing and Distortion product otoacoustic emissions (DPOAE). The PTA was calculated from the average of the audiometric thresholds at 500, 1000, 2000, 4000 and 8000 Hz. The severity of hearing impairment was classified into five grades: normal <26 Decibel (dB); mild =26–40 dB; moderate=41–70 dB; severe=71–90 dB; and profound >90 dB. The 449 control DNA used for screening for the presence of mtDNA variants were obtained from a panel of unaffected subjects from Han Chinese recruited from approximately 31 subjects per city/county among 14 cities/counties of Zhejiang Province.

2.2. Mutational analysis of mitochondrial 12S rRNA gene

Genomic DNA was isolated from whole blood of participants using Puregene DNA Isolation Kits (Gentra Systems, Minneapolis, Minnesota, USA). Subject's DNA fragments spanning the 12S rRNA gene were amplified by PCR using oligodeoxynucleotides corresponding to positions 618–635 and 1988–2007 (Rieder et al., 1998). Each fragment was purified and subsequently analyzed by direct sequencing in an ABI 3700 automated DNA sequencer using the Big Dye Terminator Cycle (Applied Biosystems, Foster City, California, USA) sequencing reaction kit. The resultant sequence data were compared with the updated consensus Cambridge sequence (GenBank accession number: AC_000021.2) (Andrews et al., 1999). The homoplasmy of the 1555A>G and 1494C>T mutations in these subjects were determined as detailed previously (Li et al., 2004a; 2005). The frequency of variants in the 12S rRNA gene in 449 Chinese control subjects was determined by direct sequencing of PCR products as described above.

2.3. Structural analysis

The published secondary structures for the 12S rRNA (Neefs et al., 1991; Springer and Douzery, 1996) were used to define the stem and loop structure. The secondary structure of human mitochondrial 12S rRNA was predicted by using the RnaViz program (De Rijk and De Wachter, 1997).

2.4. Phylogenetic analysis

A total of 14 primate mitochondrial 12S rRNA sequences were used in the interspecific analysis. These include *Homo sapiens*, *Gorilla gorilla*, *Pan paniscus*, *Pan troglodytes*, *Pongo pygmaeus*, *Pongo abelii*, *Hylobates lar*, *Macaca mulatta*, *Macaca sylvanus*, *Papio hamadryas*, *Cebus albifrons*, *Tarsius bancanus*, *Nycticebus coucang*, and *Lemur catta* (Genbank). The conservation index (CI) was calculated by comparing the human nucleotide variants with other 13 primates. The CI was then defined as the percentage of species from the list of 14 different primates that have the wild-type nucleotide at that position.

2.5. Mutational analysis of GJB2 gene

The DNA fragments spanning the entire coding region of *GJB2* gene were amplified by PCR using the following oligodeoxynucleotides: forward-5'TATGACACTCCCCAGCACAG3' and reverse-5'GGGCAATGCTTAACTGGC3'. PCR amplification and subsequent sequencing analysis were performed as detailed elsewhere (Li et al., 2004b). The results were compared with the wild type *GJB2* sequence (Version 1, GenBank accession number: M86849) to identify the mutations.

2.6. Genotyping analysis of TRMU gene

DNA fragments spanning exon 1 of *TRMU* gene were amplified by PCR using the following oligodeoxynucleotides: forward-5'-ACAGCGCAGAAGAAGAGCAGT-3' and reverse-5'-ACAACGCCACGACGGACG-3'. PCR amplification and subsequent sequencing analysis were performed as detailed elsewhere (Guan et al., 2006). The resultant sequence data were compared with the *TRMU* genomic sequence (Version 1, GenBank accession number AF448221) (Guan et al., 2006).

3. RESULTS

3.1. Study samples

The study samples consisted of 873 males and 769 females. The age of all participants ranged from 1 years old to 17 years old, with the median age of 9 years old. Patients from each location were shown in Figure 1. All participants were Han Chinese from 14 cities/counties of Zhejiang Province, Eastern China. Based on a clinician review of the medical record, 461 subjects (268 males and 193 females) had a history of exposure to aminoglycosides including gentamicin, streptomycin and kanamycin, accounting for 28% cases of hearing loss in this cohort. These subjects, due to infections or other illness, received a conventional daily dosage of aminoglycosides (3–5 mg/kg/dose every 8 h for gentamicin or 15–25 mg/kg/dose every 12 h for streptomycin, 15 mg/kg/dose every 8 h for kanamycin) at younger than 10 years old. Hearing impairment occurred from 3 days to three months after the administration of drugs. Audiological evaluation showed that 156 subjects had severe hearing loss and 305 individuals exhibited profound hearing loss. Furthermore, there was the wide range of severity of hearing loss in 1181 affected subjects who did not have a history of exposure to aminoglycosides: 738 subjects exhibited profound hearing loss, 387 subjects had severe hearing loss, 35 individuals suffered from moderate hearing loss and 21 subjects had mild hearing impairment. The onset of the hearing loss ranged from congenital to 10 years old.

3.2. Mutational spectrum of mitochondrial 12S rRNA gene

DNA fragments spanning 12S rRNA gene were PCR-amplified from genomic DNA of 1642 hearing-impaired Chinese subjects and each fragment was purified and subsequently analyzed by DNA sequencing. Comparison of the resultant sequence with the Cambridge consensus sequence identified 68 nucleotide changes in the 12S rRNA gene as shown in Table 1. All the nucleotide changes were verified by sequence analysis of both strands and appeared to be homoplasmy. Of these, 3 subjects with profound or severe hearing loss carried the 1494C>T mutation. Two of three subjects carrying the 1494C>T mutation had a history of exposure to aminoglycosides (Chen et al., 2007). A total of 65 hearing-impaired subjects harbored the 1555A>G mutation. The 1555A>G and 1494C>T mutations were indeed present in homoplasmy in these subjects (data not shown). These translate to a frequency of ~3.96 % and ~0.18% for the 1555A>G and 1494C>T mutations in this Chinese pediatric deafness population, respectively. Furthermore, 10 subjects harbored the known deafness-associated 1095T>C mutation (Tessa et al., 2002; Thyagrajan et al., 2002) and 29 subjects carried the putative deafness-associated mutations at position of 961 (961insC, 961delT and 961T>C) (Bacino et al., 1995; Casano et al., 1999; Li et al., 2004b; Li et al., 2005), respectively.

In addition to the mutations mentioned above, there were 48 known and 14 novel variants in the 12S rRNA gene (Brandon et al., 2005). These variants were first evaluated by examining the allelic frequency in 449 Han Chinese control population. Thirty-six out of 68 variants were absent in this Chinese control population. Of other 32 variants, the frequencies of 15 variants were <1% in 449 Chinese controls, while the allelic frequency of other 17 variants was >1% in this control population. Furthermore, we used the secondary structure of 12S rRNA (Neefs et al., 1991; Ruiz-Pesini and Wallace, 2006) to localize each variant with either a stem or a loop and to analyze if the base changes within stems alter classic Watson-Crick (WC) base pair (De Rijk and De Wachter, 1997; Ruiz-Pesini and Wallace, 2006). As shown in Figure 2, 44 variants were located at the loops, while 24 variants occurred in the stems of this rRNA. As shown in Table 1 and Figure 2, 7 variants 710T>C, 921T>C, 922C>T, 1393G>A, 1494C>T, 1503G>A and 1555A>G created a putative base-pairing(s), while 17 variants 663A>G, 681T>C, 709G>A, 745A>G, 792C>T, 839A>G, 856A>G, 951G>A, 980T>C, 1007G>A, 1095T>C, 1310C>T, 1382A>C, 1438A>G, 1452T>C, 1520T>C, and 1535T>C abolished a putative base pairing(s). This suggested that the nucleotide variants were more frequent in loops than in stems. In addition, phylogenetic analysis was performed by comparing the human 12S rRNA nucleotide variants with other 13 primates. As shown in Table 1, conservation index (CI) among the variants ranged from 7.1% (750A>G variant) to 100% (792C>T and 801A>G variants). In particular, CI of 28 variants including 1555A>G and 1494C>T mutations were >78%, CI of other 19 variants was between 78% and 50% and CI for the remaining variants was <50%. In addition to the 1555A>G and 1494C>T mutations, the 745A>G, 792C>T, 801A>G, 839A>G, 856A>G, 1027A>G, 1192T>C, 1192C>A, 1310C>T, 1331A>G, 1374A>G and 1452T>C variants, which are absent in the 449 Chinese controls and whose CIs were >78%, were the putative deafness-associated variants. On the other hand, other 14 variants such as 663A>G, 681T>C, 752C>T, 735A>G, 827A>G, 1107T>C, 1382A>C and 1438A>G, whose CIs were >78%, which were present in the controls, appeared to be the polymorphisms.

3.3. Clinical and genetic characterization of 21 hearing-impaired Chinese subjects carrying one of 12 putative 12S rRNA mutations

Comprehensive medical histories of 21 probands carrying one of 12 putative 12S rRNA mutations and other members in these families showed no other clinical abnormalities, including diabetes, muscular diseases, visual loss and neurological disorders. As shown in Table 2, 19 subjects received a regular dose of aminoglycosides including gentamicin, streptomycin and kanamycin for various illnesses at the age of 6 months to 3 years old, while other two subjects did not have a history of exposure to aminoglycosides. This was no evidence

that these subjects had any known cause to account for hearing loss. Audiological examination indicated that two subjects suffered from severe hearing loss and 19 subjects exhibited profound hearing loss. Variable patterns of audiometric configurations were detected in these subjects: 6 subjects with slope-shaped pattern and 15 individuals with flat-shaped pattern. Of these, 20 Chinese subjects carrying one of these putative 12S rRNA mutations, as shown in Figure 3, did not have a family history of hearing loss. However, three of 61 members in the pedigree QZ062, as shown in Figure 3, suffered from hearing loss. Of these, the proband IV-3 and subject III-14, who did not have a history of exposure of aminoglycosides, experienced the loss of hearing at the age of younger than 4, while the subject IV-1 received a regular dose of gentamicin for high fever at the age of 2 years old.

3.4. Clinical evaluation of 65 Chinese subjects carrying the 1555A>G mutation

As shown in Table 3, 65 Chinese individuals carrying the 1555A>G mutation consisted of 24 females and 41 males. Comprehensive medical histories of those probands showed no other clinical abnormalities, including diabetes, muscular diseases, visual dysfunction, and neurological disorders. Of these, 48 subjects received a regular dose of aminoglycosides including gentamicin, streptomycin and kanamycin for various illnesses at the age of 3 months to 4 years old. This was no evidence that these subjects had any known cause to be responsible for hearing loss. As shown in Table 3, audiological assessments showed that 33 and 15 subjects with the aminoglycoside treatments exhibited profound and severe hearing loss, respectively. All subjects were tested for their ability to hear various sound frequencies by audiometer. All these individuals showed the loss of hearing at high frequencies. Both of their ears exhibited similar degrees of hearing deficit (symmetric). Furthermore, 17 individuals, who did not have a history of exposure to aminoglycosides, exhibited a variety of severity and age-of-onset of hearing impairment. Audiometric studies showed that 8 individuals suffered from profound hearing impairment, 5 subjects exhibited severe hearing impairment, 3 probands had moderate hearing impairment and 1 subjects exhibited mild hearing impairment. The age-of-onset of hearing loss in these subjects ranged from infant to 10 years old. All affected individuals showed the loss of high frequencies. Hearing impairment in 16 affected subjects was symmetric. In one individual (SX018-V7) with asymmetric hearing impairment, the severe dysfunction in the left ear was due to sudden hearing loss at the age of 4 years old. We then studied the audiograms of 65 patients carrying the 1555A>G mutation, and characterized the audiometric configuration of their hearing loss. These subjects exhibited the variable patterns of audiometric configurations: 47 subjects with Slope-shaped pattern (72.3%), 11 individuals with Flat-shaped pattern (16.9), 5 subjects with U-shaped pattern (7.7%) and 2 individuals with Rising-shaped pattern (3.1%).

3.5. Mutational analysis of GJB2 gene

To examine if the *GJB2* gene contributed to a deafness phenotype, we performed the mutational screening of *GJB2* gene in 65 subjects carrying the 1555A>G mutation and 21 subjects carrying one of 12 putative 12S rRNA mutations. The homozygous 235delC mutation in *GJB2* gene was detected in the subjects YJ042-IV-7 and YQ052-IV-5 carrying the 1555A>G mutation and in the subject YQ062-IV-3 with the 1027A>G mutation. Further analysis of *GJB2* gene in the members of YQ062 family showed that her parents and her sister IV-1 carried the heterozygous 235delC mutation and her brother IV-2 lacked the 235delC mutation. However, none of other mutations in *GJB2* gene was found in other affected subjects. Indeed, the absence of mutation in the *GJB2* gene in those subjects with hearing impairment indicated that the *GJB2* gene did not contribute to the deafness phenotype in those subjects.

3.6. Mutational analysis of TRMU gene

Our previous study showed that the *TRMU* 10A>S mutation modulated the phenotypic manifestation of the 1555A>G mutation in the Israeli/European pedigrees (Guan et al., 2006). To assess if the *TRMU* 10A>S variant also plays a role in phenotypic expression of the 1555A>G mutation and 12 putative 12S rRNA mutations, we carried out a mutational screening of exon 1 in *TRMU* gene in 65 Chinese subjects carrying the 1555A>G mutation and 21 subjects carrying one of 12 putative 12S rRNA mutations. We failed to detect any variant in *TRMU* exon 1 in these affected subjects.

4. DISCUSSION

4.1. Prevalence of known deafness-associated 12S rRNA 1555A>G and 1494C>T mutations

The large cohort of Chinese pediatric hearing-impaired subjects consisted of 461 subjects with aminoglycoside-induced hearing loss and 1181 subjects, who did not have a history of exposure to aminoglycosides. Of known deafness-associated 12S rRNA mutations, the 1555A>G mutation accounted for 3.96% cases of this entire Chinese clinical population. In particular, the incidences of the 1555A>G mutation were 1.43% and 10.41% in this Chinese pediatric population with nonsyndromic and aminoglycoside-induced hearing loss, respectively. In fact, the incidences of the 1555A>G mutation varied among different ethnic backgrounds. With regard to the subjects with aminoglycoside ototoxicity, the incidences of the 1555A>G mutation were 33% in a small Japanese cohort (Usami et al., 2000), 13% and 5% in two Chinese cohorts (Hutchin et al., 1993; Li et al., 2005) and ~17% in the two white cohorts from United States and Spain (Estivill et al., 1998; Fischel-Ghodisian et al., 1993). However, the incidence of 1555A>G mutation in nonsyndromic hearing loss was much lower than in those with aminoglycoside ototoxicity. In two white cohorts with nonsyndromic hearing loss, the frequency of the 1555A>G mutation varies from 0.6% to 2.5% (Casano et al., 1993; Li et al., 2004b; Jacobs et al., 2005), while the incidence of the 1555A>G mutation in several Asian cohorts ranged from 2.9% to 5.3% (Malik et al., 2003; Li et al., 2005). On the other hand, the incidences of the 1494C>T mutation appeared to be lower than those of the 1555A>G mutation. In this cohort, only 3 hearing-impaired subjects carried the 1494C>T mutation. Two of three subjects carrying the 1494C>T mutation had a history of exposure to aminoglycosides (Chen et al., 2007). This data was comparable with the previous report that three familial cases of 1340 sporadic Spanish hearing-impaired subjects carried the 1494C>T mutation (Rodriguez-Ballesteros et al., 2006). Therefore, these two known mutations account for at least 4% cases of this Chinese hearing-impaired population.

4.2. Mutation spectrum of 12S rRNA gene

A total of 68 (54 known and 14 novel) variants in 12S rRNA gene were identified in a cohort of 1642 hearing-impaired Chinese subjects. Similar to other mtDNA variations, these variants can be grouped into three categories: neutral, adaptive and deleterious (Ruiz-Pesini and Wallace, 2006). To identify putative deleterious mutation, these variants were further evaluated using following three criteria: 1). Absent in the 449 Chinese controls; 2). CI is >78%, proposed by Ruiz-Pesini and Wallace (2006); 3). Potential structural and functional alterations. Among these variants, 36 variant were absent in the 449 Han Chinese controls, while the frequency of other 32 variants ranged from 0.2% (eight variants such as 711T>C) to 99.6% (750A>G and 1438A>G variants) in this Chinese control population. In particular, some of these variants occurring at high frequencies of both control and patient populations were the mitochondrial haplogroup specific variants (Tanaka et al., 2004). These included the 663A>G variant of haplogroup A, the 827A>G and 1119T>C variants of haplogroup B4, the 709G>A and 1598G>A variants of haplogroup B5, the 1382A>C variant of haplogroup D4, the 681T>C, 752C>T, 1048C>T and 1107T>C variants of D5 haplogroup, the haplogroup F2 specific variant 1005T>C, the 1041A>G variant of haplogroup M9a, and the 1541T>C variant of

haplogroup R5b (Tanaka et al., 2004). Apparently, these haplogroup specific variants were adaptive or neutral but unlikely deleterious.

Phylogenetic analysis showed that CIs of 28 variants were more than 78%. Despite their higher CI, the 14 variants such as 663A>G, 681T>C, 752C>T, 735A>G, 827A>G, 1107T>C, 1382A>C and 1438A>G were present in the controls. On the other hand, the CIs for other 14 variants including 1555A>G and 1494C>T mutation were at least 78% but these variants were absent in 449 Chinese controls. Based on the predicted secondary structure of mitochondrial 12S rRNA (Neefs et al., 1991; De Rijk and De Wachter, 1997), 44 variants were located at the loops and 24 variants occurred in the stems of this rRNA. Among these variants, 17 variants including the 1095T>C mutation disrupted a WC base pairing(s) of 12S rRNA, while 7 variants including the 1555A>G and 1494C>T mutations created a novel WC base-pairing(s) of this rRNA (Neefs et al., 1991; De Rijk and De Wachter, 1997). In fact, the 1555A>G or 1494C>T mutation made the mitochondrial ribosome more bacteria-like (Prezant et al., 1993; Zhao et al., 2004a; Qian and Guan, 2009). Functional characterization demonstrated that the 1555A>G or 1494C>T mutation conferred sensitivity to aminoglycosides (Guan et al., 1996; 2001; Zhao et al., 2005a). Thus, individuals carrying either of mutations are predisposed to hearing loss. Indeed, the 745A>G, 792C>T, 839A>G, 856A>G, 1310C>T and 1452T>C variants, which resided at the stems of 12S rRNA, were fitted with three criteria for the pathogenic mutations as described above. Furthermore, the 801A>G, 1027A>G, 1192C>T, 1192C>A, 1331A>G and 1374A>G variants, whose location were at loops in the 12S rRNA and whose CI's were >78%, were absent in 449 Han Chinese controls. Thus, alterations of the tertiary or quaternary structure of 12S rRNA by these variants may lead to significant effects on function, thereby contributing to the deafness phenotype. However, the families carrying one of these 12 putative 12S rRNA mutation, similar to some pedigrees carrying the 1555A>G and 1494C>T mutations (Young et al., 2005; Chen et al., 2007; Dai et al., 2006; Tang et al., 2007), exhibited extremely lower penetrance of hearing loss. Indeed, 19 of 21 subjects carrying one of these mutations had a history of exposure to aminoglycosides. These suggested that these subjects carrying one of these mutations are susceptible to aminoglycosides, thereby causing hearing loss. Alternatively, aminoglycoside ototoxicity may result from other causes such as medical condition affecting aminoglycoside metabolism, or mutations in other genes. Furthermore, the hearing-impaired subject QZ149 carrying the 792C>T mutation did not receiving the treatment of aminoglycosides. Thus, it is possible that other genetic or environmental factors may contribute to the deafness phenotype of this individual.

Of other known putative deafness-mutations, the frequency of the 1095T>C mutation was 0.61% in this deafness population. The 1095T>C mutation, whose CI was 92.9%, occurred in one of 449 Chinese controls. In fact, this mutation has been found in several genetically-unrelated families with nonsyndromic and aminoglycoside-induced hearing loss, respectively (Tessa et al., 2002; Thyagarajan et al., 2002; Zhao et al., 2004b; Wang et al., 2005; Li et al., 2005). Furthermore, the incidences of putative mutations at position 961 including 961delT, 961insC and 961T>C were 1.8% in this pediatric population. Mutations at the position 961, whose CI was 42.9%, were present 4% of 449 Chinese controls. Indeed, putative mutations at this position have been implicated to be associated with hearing loss in different ethnic backgrounds (Bacino et al., 1995; Casano et al., 1999; Li et al., 2004a; 2004b). Furthermore, approximately 70% of subjects with aminoglycoside-induced hearing loss in this cohort did not carry the pathogenic 12S rRNA A1555G and C1494T mutations as well as putative deafness-associated 12S rRNA mutations. These data implicated the involvement of other nuclear genes, besides mitochondrial 12S rRNA mutations, in development of hearing loss in these subjects.

4.3. Clinical features of 65 Han Chinese subjects carrying the 1555A>G mutation

In the present study, 65 Han Chinese subjects carrying the 1555A>G mutation were composed of 48 subjects who had a history of exposure to aminoglycosides and 17 individuals who did not receive aminoglycoside treatments. Hearing impairment as a sole clinical phenotype was present in these subjects. Their hearing loss, similar to other affected individuals carrying the 1555A>G mutation (Usami et al., 1997; Estivill et al., 1998; de Castillo et al., 2003; Yuan et al., 2005; Dai et al., 2006; Young et al., 2006), shared some common features: being bilateral, sensorineural and the loss of hearing at the high frequencies. However, a wide range of severity, age-of-onset and audiometric configuration was observed among these subjects carrying the 1555A>G mutation, indicating the involvement of modifier factors. These subjects with the administration of aminoglycosides at the age of three months to 4 years old exhibited profound or severe hearing loss. These subjects with aminoglycoside ototoxicity exhibited variable patterns of audiometric configurations: 37 individuals with the sloping pattern, 8 subjects with the flat pattern, 2 individuals with the rising-shaped pattern and one subject with the U-shaped pattern. These data, similar to our previous studies (Usami et al., 1997; Yuan et al., 2005; Zhao et al., 2005b; Dai et al., 2006; Young et al., 2006), suggested that the sloping and flat shaped patterns were the common audiograms in the aminoglycoside-induced hearing loss subjects carrying the 1555A>G mutation. This discrepancy likely reflects the dosage and the age at the time of drug administration and different nuclear background in these subjects, similar to these carrying the A1555G mutation in other Chinese family (Yuan et al., 2005) and other subjects carrying the C1494T mutation (Zhao et al. 2004a; 2005a).

In the absence of aminoglycosides, 17 individuals exhibited a variety of severity, age-of-onset and audiometric configuration of hearing loss. In terms of severity of hearing loss, 8 individuals suffered from profound hearing loss, 5 subjects exhibited severe hearing impairment, 3 probands had moderate hearing impairment and 1 subjects exhibited mild hearing impairment. The age-of-onset of hearing loss in these subjects ranged from infant to 10 years old. There were variable patterns of audiograms in these hearing-impaired subjects: 10 individuals with the sloping pattern, 3 subjects with the flat pattern and 4 individuals with U-shaped pattern. The phenotypic variability of these subjects indicated the involvement of nuclear modifier genes and mitochondrial haplogroups in the phenotypic manifestation of the 1555A>G mutation, as in the case of other pedigrees carrying the 1555A>G mutation (Bykhovskaya et al., 1998; Guan et al., 2001; Young et al., 2006; Chen et al., 2008; Wang et al., 2008). Here, the absence of the nuclear modifier *TRMU* 10A>S variant suggested that *TRMU* may not play an important role in the phenotypic expression of the 1555A>G mutation in these Chinese subjects, similar to other Chinese families carrying the 1555A>G mutation (Chen et al., 2008; Wang et al., 2008). However, two subjects carried both 12S rRNA 1555A>G mutation and homozygous *GJB2* 235delC mutation, indicating that the *GJB2* 235delC mutation was responsible for deafness in these two subjects. The lack of *GJB2* mutation in other subjects implied that other nuclear modifier genes may contribute to the phenotypic variability in these Chinese subjects. On the other hand, the co-segregation of the 1555A>G mutation with haplogroups B5b specific variant 1598G>A here implied that the haplogroup B5b may also modulate the phenotypic manifestation of the 1555A>G mutation, as in the case of other Chinese families (Chen et al., 2008; Wang et al., 2008; Lu et al., 2010). Furthermore, it is possible that other environmental factors, besides aminoglycosides, such as the severity of illness, may also contribute to the phenotypic variability in these subjects.

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Figure 1. Geographic locations of hearing-impaired Han Chinese subjects
 Dot indicates the sites under this study.

Figure 2. Structure and sequence variants of human mitochondrial 12S rRNA

The secondary structure was predicted by using the RnaViz program (De Rijk and De Wachter, 1997). The variants were indicated by arrows.

Figure 3. Nine Han Chinese pedigrees with aminoglycoside-induced and nonsyndromic hearing impairment

Hearing impaired individuals are indicated by filled symbols. Arrowhead denotes probands. Asterisks denote individuals who had a history of exposure to aminoglycosides.

Table 1

Variants in the mitochondrial 12S rRNA gene in 1642 hearing-impaired pediatric subjects

Position	Replacement	Conservation index (%) ^a	WC base-pairs ^b	Previously reported ^c	Number of affected subjects	Percentage (%)	Number of controls (number/449)	Percentage (%)
663	A to G	78.6	↓A-U	Yes	16	1.0	5	1.1
681	T to C	85.7	↓U-A	Yes	30	1.8	8	1.8
709	G to A	64.3	↓G-C	Yes	330	20.1	102	22.7
710	T to C	28.6	↑C-G	Yes	2	0.1	0	0
711	T to C	35.7		Yes	3	0.2	1	0.2
714	A to T	42.9		No	1	0.1	0	0
722	C to T	71.4		Yes	1	0.1	1	0.2
723	A to G	28.6		Yes	4	0.2	2	0.4
735	A to G	78.6		Yes	10	0.6	5	1.1
737	C to T	71.4		No	1	0.1	0	0
745^d	A to G	85.7	↓A-U	Yes	1	0.1	0	0
750	A to G	100.0		Yes	1638	99.8	447	99.6
752	C to T	100.0		Yes	51	3.1	17	3.8
761	A to G	100.0		Yes	2	0.1	2	0.4
789	T to C	85.7		Yes	2	0.1	1	0.2
792	C to T	100.0	↓C-G	No	1	0.1	0	0
801	A to G	100.0		No	1	0.1	0	0
813	A to G	28.6		Yes	2	0.1	0	0
824	T to C	57.1		Yes	1	0.1	0	0
827	A to G	92.9		Yes	54	3.3	12	2.7
839	A to G	78.6	↓A-U	No	4	0.2	0	0
856	A to G	100.0	↓A-U	Yes	2	0.1	0	0
869	C to T	42.9		Yes	1	0.1	0	0
921	T to C	50.0	↑C-G	Yes	3	0.2	2	0.4
922	C to T	35.7	↑U-A	No	2	0.1	0	0
930	G to A	7.1		Yes	4	0.2	1	0.2
942	A to G	64.3		Yes	1	0.1	0	0
951	G to A	92.9	↓G-C	Yes	7	0.4	2	0.4

Position	Replacement	Conservation index (%) ^a	WC base-pairs ^b	Previously reported ^c	Number of affected subjects	Percentage (%)	Number of controls (number/449)	Percentage (%)
953	T to C	57.1		Yes	5	0.3	0	0
955	A to G	64.3		Yes	1	0.1	0	0
959	C to T	35.7		No	1	0.1	0	0
960	delC	42.9		No	1	0.1	1	0.2
961	insC	42.9		Yes	25	1.5	14	3.1
961	delT	42.9		Yes	1	0.1	0	0
961	T to C	42.9		Yes	3	0.2	4	0.9
980	T to C	64.3	↓U-A	Yes	2	0.1	0	0
1005	T to C	35.7		Yes	72	4.4	22	4.9
1007	G to A	50.0	↓G-C	Yes	3	0.2	0	0
1009	C to T	21.4		Yes	10	0.6	8	1.8
1027	A to G	92.9		No	5	0.3	0	0
1041	A to G	42.9		Yes	11	0.7	4	0.9
1048	C to T	57.1		Yes	46	2.8	11	2.4
1053	A to G	71.4		No	1	0.1	0	0
1095	T to C	92.9	↓U-A	Yes	10	0.6	1	0.2
1107	T to C	85.7		Yes	103	6.3	25	5.6
1116	A to G	57.1		Yes	1	0.1	0	0
1119	T to C	50.0		Yes	53	3.2	17	3.8
1189	T to C	57.1		Yes	2	0.1	0	0
1192	C to T	85.7		No	1	0.1	0	0
1192	C to A	85.7		Yes	1	0.1	0	0
1310	C to T	85.7	↓G-C	Yes	2	0.1	0	0
1331	A to G	100.0		No	1	0.1	0	0
1374	A to G	92.9		No	1	0.1	0	0
1382	A to C	92.9	↓A-U	Yes	43	2.6	9	2.0
1391	T to C	64.3		Yes	2	0.1	1	0.2
1393	G to A	28.6	↑A-U	Yes	1	0.1	0	0
1415	G to A	57.1		Yes	1	0.1	0	0
1438	A to G	100.0	↓A-U	Yes	1640	99.9	447	99.6
1442	G to A	42.9		Yes	1	0.1	0	0

Position	Replacement	Conservation index (%) ^a	WC base-pairs ^b	Previously reported ^c	Number of affected subjects	Percentage (%)	Number of controls (number/449)	Percentage (%)
1452	T to C	100.0	↓U-A	Yes	1	0.1	0	0
1462	G to A	50.0		Yes	1	0.1	1	0.2
1494	C to T	78.6	↑U-A	Yes	3	0.2	0	0
1503	G to A	50.0	↑A-U	Yes	7	0.4	0	0
1520	T to C	28.6	↓U-A	Yes	6	0.4	4	0.9
1535	T to C	42.9	↓U-A	No	1	0.1	0	0
1541	T to C	78.6		Yes	11	0.7	4	0.9
1555	A to G	85.7	↑A-U	Yes	65	4.0	0	0
1598	G to A	50.0		Yes	49	3.0	9	2.0

^aThe conservation index (CI) was then defined as the percentage of the human nucleotide variants with other 13 primates that have the wild-type nucleotide at that position

^bClassic Watson-Crick (WC) base pair: created (↑) or abolished (↓).

^cSee Ruiz-Pesini E, Wallace DC. (2006) and <http://www.mitomap.org>

^dKnown and putative pathogenic variants are indicated in boldface.

Table 2

Summary of clinical and molecular data for 21 Han Chinese subjects carrying the putative 12S rRNA mutations

12S rRNA mutation	GJB2 gene mutation	Subject	Gender	Audiometric configuration	Age-at-onset (years)	PTA (dB) ^a right ear	PTA (dB) left ear	Use of drugs	Level of hearing impairment	Family history of hearing loss
745A>G	polymorphism	LS068-III-1	F	Slope	<1	92	91	Yes	Profound	No
792C>T	polymorphism	QZ149III-4	M	Slope	3	87	89	No	Profound	No
801A>G	polymorphism	HZ151	M	Flat	2	101	108	Yes	Profound	No
839A>G	polymorphism	LS203-III-6	M	Flat	<1	93	95	Yes	Profound	No
839A>G	polymorphism	LC116	F	Slope	3	92	98	Yes	Profound	No
839A>G	polymorphism	WC28	M	Flat	2	105	110	Yes	Profound	No
839A>G	polymorphism	HA11	M	Flat	2	90	95	Yes	Profound	No
856A>G	polymorphism	HA023-III-1	M	Flat	<1	114	102	Yes	Profound	No
856A>G	polymorphism	HZ112	F	Flat	3	114	104	Yes	Profound	No
1027A>G	polymorphism	YJ067-III-7	M	Flat	3	99	99	Yes	Profound	No
1027A>G	polymorphism	QZ104-III-1	M	Slope	<1	78	79	Yes	Severe	No
1027A>G	polymorphism	QZ055-IV-6	F	Flat	2	110	105	Yes	Profound	No
1027A>G	235DeIc/235DeIc	YQ062-IV-3	F	Flat	2	101	98	No	Profound	Yes
1027A>G	polymorphism	LS109	M	Slope	<1	95	98	Yes	Profound	No
1192C>T	polymorphism	HA083-II-1	M	Flat	1	107	105	Yes	Profound	No
1192C>A	polymorphism	HZ29	F	Flat	2	98	94	Yes	Profound	No
1310C>T	polymorphism	TZ007	M	Flat	2	95	92	Yes	Profound	No
1310C>T	polymorphism	TZ095	M	Flat	1	91	115	Yes	Profound	No
1331A>G	polymorphism	HZ020	M	Flat	1	108	106	Yes	Profound	No
1374A>G	polymorphism	HA078-III-3	M	Flat	2	115	103	Yes	Profound	No
1452T>G	polymorphism	QZ093-III-5	F	Slope	<1	82	85	Yes	Severe	No

^aPTA: Pure-tone audiometry; dB: Decibel

Table 3
Summary of clinical data for 65 Chinese subjects carrying the A1555G mutation

Subject	Gender	Age-at-onset (years)	Use of aminoglycosides	PTA (dB) ^a right ear	PTA (dB) left ear	Audiometric configuration	Level of hearing impairment
HA062-V-1	M	2	Yes	103	99	Slope	Profound
HA087-IV-12	F	2	Yes	98	93	Slope	Profound
HA101-III-1	M	1	Yes	93	100	Slope	Profound
HA102-IV-2	M	<1	No	108	103	Flat	Profound
HA121-V-3	M	3	Yes	99	99	Slope	Profound
HA146-III-11	F	1	Yes	99	101	Slope	Profound
HZ023-IV-6	M	1	Yes	88	94	Slope	Profound
HZ142-III-12	F	<1	Yes	98	98	Slope	Profound
LC021-IV-1	F	<1	Yes	99	88	Slope	Profound
LC027-IV-1	F	3	No	101	92	Slope	Profound
LC063-IV-2	F	2	Yes	100	103	Slope	Profound
LS058-IV-9	M	2	Yes	97	103	Slope	Profound
QZ036-IV-17	M	<1	No	92	98	U	Profound
QZ069-IV-7	F	1	Yes	88	97	Flat	Profound
QZ085-IV-5	M	2	No	97	97	Slope	Profound
QZ097-IV-5	M	1	Yes	106	105	Slope	Profound
QZ107-IV-1	M	<1	Yes	93	94	Slope	Profound
RA036-IV-12	M	1	Yes	99	95	Slope	Profound
SX003-III-5	M	<1	Yes	106	108	Slope	Profound
SX012-III-1	M	<1	No	93	92	U	Profound
SX052-IV-8	F	1	Yes	103	106	Slope	Profound
SX070-IV-5	F	<1	No	98	102	Slope	Profound
SX072-IV-11	M	1	Yes	104	102	Slope	Profound
SX082-IV-3	F	4	Yes	100	102	Slope	Profound
WC012-IV-1	M	1	Yes	99	103	Slope	Profound
YJ042-IV-7	M	<1	No	92	93	Slope	Profound
YJ052-IV-8	M	<1	Yes	105	109	Slope	Profound
YQ016-III-2	F	1	Yes	96	98	U	Profound
YQ021-III-2	F	4	Yes	98	108	Flat	Profound

Subject	Gender	Age-at-onset (years)	Use of aminoglycosides	PTA (dB) ^a right ear	PTA (dB) left ear	Audiometric configuration	Level of hearing impairment
YQ023-III-2	M	4	Yes	93	91	Slope	Profound
YQ052-IV-5	F	<1	No	96	98	U	Profound
FE003-IV-1	M	2	Yes	99	101	Slope	Profound
FE008-IV-6	M	1	Yes	100	103	Flat	Profound
HA167-III-3	M	1	Yes	78	97	Flat	Severe
HZ171-IV-4	M	<1	Yes	75	85	Flat	Severe
LC07-IV-2	M	4	No	73	90	Slope	Severe
LS099-III-14	F	2	Yes	86	94	Slope	Severe
LS160-III-1	M	2	Yes	79	88	Slope	Severe
LS192-IV-1	M	<1	Yes	79	92	Slope	Severe
QZ081-IV-1	M	<1	No	74	74	U	Severe
RA035-III-6	F	4	Yes	84	83	Slope	Severe
RA060-VI-1	M	<1	No	78	74	Flat	Severe
RA098-IV-1	M	3	Yes	56	93	Flat	Severe
WC002-IV-1	M	<1	Yes	71	83	Flat	Severe
YJ049-IV-16	M	3	Yes	74	90	Slope	Severe
FE019-IV-1	F	<1	No	88	73	Slope	Severe
FE020-III-1	M	8	No	89	83	Slope	Severe
QZ09-IV-6	F	1	No	70	68	Slope	Moderate
FE036-III-1	M	10	No	56	61	Slope	Moderate
SX018-V-7	F	4	No	8	62	Flat	Mild
WZD1-IV-1	M	<1	Yes	68	77	Rising	Severe
WZD2-IV-1	M	<1	Yes	85	85	Rising	Severe
WZD3-III-1	F	2	Yes	80	92	Slope	Severe
WZD4-III-1	M	2	Yes	85	85	Slope	Severe
WZD5-III-1	M	1.5	Yes	92	98	Slope	Profound
WZD6-III-1	F	3	Yes	103	105	Slope	Profound
WZD7-IV-1	M	<1	Yes	102	103	Slope	Profound
WZD8-III-1	M	1	Yes	98	98	Slope	Profound
WZD9-IV-1	M	2	Yes	97	100	Slope	Profound
WZD10-III-2	F	2	Yes	67	82	Slope	Severe
WZD31-III-1	F	3	Yes	98	98	Slope	Profound

Subject	Gender	Age-at-onset (years)	Use of aminoglycosides	PTA (dB) ^a right ear	PTA(dB) left ear	Audiometric configuration	Level of hearing impairment
WZD32-IV-2	F	2	Yes	75	84	Flat	Severe
WZD33-V-1	M	2	Yes	102	98	Slope	Profound
WZD34-III-2	M	4	Yes	105	109	Slope	Profound
WZD11-IV-1	F	1	No	58	75	Slope	Moderate

^aPTA: Pure-tone audiometry; dB: Decibel