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Mutational analysis of the mitochondrial 12S rRNA gene in Chinese pediatric subjects with aminoglycoside-induced and non-syndromic hearing loss

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Abstract

Mutations in mitochondrial DNA (mtDNA) have been found to be associated with sensorineural hearing loss. We report here a systematic mutational screening of the mitochondrial 12S rRNA gene in 128 Chinese pediatric subjects with sporadic aminoglycoside-induced and non-syndromic hearing loss. We show that aminoglycoside ototoxicity accounts for 48% of cases of hearing loss in this Chinese pediatric population. Of the known deafness-associated mutations in this gene, the incidence of the A1555G mutation is ~13% and ~2.9% in this Chinese pediatric population with aminoglycoside-induced and non-syndromic hearing loss, respectively. Furthermore, mutations at position 961 in the 12S rRNA gene account for ~1.7% and 4.4% of cases of aminoglycoside-induced and non-syndromic hearing loss in this Chinese clinical population, respectively. The T1095C mutation has been identified in one maternally inherited family with aminoglycoside-induced and nonsyndromic hearing loss. However, the C1494T mutation was not detected in this clinical population. In addition, three variants, A827G, T1005C and A1116G, in the 12S rRNA gene, localized at highly conserved sites, may play a role in the pathogenesis of aminoglycoside ototoxicity. These data strongly suggest that the mitochondrial 12S rRNA is a hot-spot for deafness-associated mutations in the Chinese population.

Introduction

Aminoglycoside antibiotics, such as gentamicin, streptomycin and tobramycin, are clinically important drugs. In the developed countries, these drugs are mainly used in the treatment of hospitalized patients with aerobic Gram-negative bacterial infections, particularly in patients with chronic infections such as cystic fibrosis or tuberculosis (Sande and Mandell 1990; Lortholary et al. 1995). However, in developing countries aminoglycosides are more routinely used, even for relative minor infections. These drugs are known to exert their antibacterial

effects by directly binding to 16S ribosomal RNA (rRNA) in the 30S subunit of the bacterial ribosome, causing mistranslation or premature termination of protein synthesis (Davis and Davis 1968; Noller 1991; Chamber and Sande 1996). The use of these drugs frequently leads to toxicity, involving the renal, auditory and vestibular systems (Sande and Mandell 1990; Lortholary et al. 1995). The renal impairment is usually reversible, whereas the auditory and vestibular ototoxicity is frequently irreversible.

Mitochondrial ribosomes share more similarities to bacterial ribosomes than do cytosolic ribosomes (Hutchin et al. 1993; Fischel-Ghodsian 1999; Guan 2004). Therefore, it is proposed that the ototoxic site of aminoglycoside antibiotics is the mitochondrial ribosome. Specifically, in familial cases of ototoxic deafness, the aminoglycoside hypersensitivity is often maternally transmitted, suggesting the mitochondrial involvement (Fischel-Ghodsian 1999; Guan 2004). Hu et al. (1991) described 36 Chinese families with maternally transmitted predisposition to aminoglycoside ototoxicity, while Higashi (1989) reported that 26 of 28 families with streptomycin-induced deafness had maternally inherited transmission. Sequence analyses of the mitochondrial genome in patients with aminoglycoside ototoxicity have led to the identification of several ototoxic mtDNA mutations in the 12S rRNA gene. The homoplasmic A1555G mutation in the highly conserved decoding site of the 12S rRNA has been found to be associated with both aminoglycoside-induced and non-syndromic hearing loss in many families of different ethnic origins (Fischel-Ghodsian et al. 1993; Hutchin et al. 1993; Prezant et al. 1993; Matthijs et al. 1996; Pandya et al. 1997; Usami et al. 1997; Estivill et al. 1998; Li et al. 2004b; Young et al. 2005). Similarly, the homoplasmic C1494T mutation in the same gene has been associated with aminoglycoside-induced and non-syndromic hearing loss in a large Chinese family (Zhao et al. 2004a). In addition, mutations at position 961 of the 12S rRNA gene have been implicated to be associated with aminoglycoside induced and non-syndromic hearing loss (Bacino et al. 1995; Casano et al. 1999; Tang et al. 2002; Li et al. 2004a).

However, less is known about the incidence of those deafness-associated mtDNA mutations in the Chinese population. These known deafness-associated mutations in the 12S rRNA gene only account for a portion of patients with aminoglycoside-induced hearing impairment (Fischel-Ghodsian 1999; Guan 2004). Thus, it is anticipated that additional mutations causing drug susceptibility can be found in the same gene. Recently, a systematic and extended mutation screening of the mitochondrial 12S rRNA gene has been initiated in a clinical population of the otology clinic at the First Affiliated Hospital of Wenzhou Medical College, China. In the present study, we report the clinical, genetic and molecular characterization of 128 sporadic Chinese pediatric subjects with aminoglycoside-induced and non-syndromic hearing loss. In particular, a mutational analysis of the 12S rRNA gene has been performed in 128 pediatric hearing-impaired subjects from this clinic and 144 unaffected individuals from the Chinese ancestry.

Materials and methods

Subjects

One hundred and twenty-eight hearing-impaired Chinese subjects, who were from 7 to 17 years old at the Wenzhou City School for Deaf Children, 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, USA and the First Affiliated Hospital of Wenzhou Medical College, China. A comprehensive history and physical examination for these participating subjects was performed to identify any syndromic findings, the history of use of aminoglycosides (including drug, duration of therapy, the diseases for treatment, the age of onset), or genetic factors related to hearing loss. An age-appropriate audiological examination was performed and this included pure-tone audiometry (PTA) and/

or auditory brainstem response (ABR), immittance testing and transiently evoked otoacoustic emissions (TEOAEs). The PTA was calculated from the sum of the audiometric thresholds at 500, 1,000 and 2,000 Hz. The severity of hearing impairment was classified into five grades: mild (20–39 dB), moderate (40–54 dB), moderate severe (55–69 dB), severe (70–89 dB) and profound (90 dB or greater). The 144 control DNA samples used for screening for the presence of mtDNA mutations were obtained from a panel of unaffected individuals with Chinese ancestry.

Mutational screening of the mitochondrial genome

Genomic DNA was isolated from whole blood of participants using the Puregene DNA Isolation Kit (Gentra Systems). DNA fragments spanning the entire mitochondrial 12S rRNA gene were amplified by PCR using oligodeoxynucleotides corresponding to positions 618–635 and 1589–1606 (Rieder et al 1998; Li et al. 2004a). Each fragment was purified and subsequently analyzed by direct sequencing in an ABI 3700 automated DNA sequencer using the Big Dye Terminator Cycle sequencing reaction kit. The resultant sequence data were compared with the updated consensus Cambridge sequence (GenBank accession number: NC_001807) (Anderson et al. 1981). For the quantification of the A1555G mutation, the amplified segments were digested with a restriction enzyme *BsmAI* (Prezant et al. 1993; Guan et al. 1996, 2001; Li et al. 2004a). Equal amounts of various digested samples were then analyzed by electrophoresis through a 1.5% agarose gel. The proportions of digested and undigested PCR product were determined by laser densitometry after ethidium bromide staining to determine if the A1555G mutation is in the homoplasmy in these subjects.

Results and discussion

The study population consisted of 128 pediatric Chinese subjects with sensorineural hearing loss. Of these, 60 cases (32 males and 28 females) had a history of exposure to aminoglycosides, including gentamicin, streptomycin and kanamycin. 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, 15–25 mg/kg/dose every 12 h for streptomycin, 15 mg/kg/day every 8 h for kanamycin) at younger than 7 years old. Hearing impairment usually occurred within 1 month after the administration of drugs. Audiological evaluation revealed a variable severity of hearing loss in these subjects: ten subjects had severe hearing loss and 50 individuals exhibited profound hearing loss. Their hearing loss shared some common features: being bilateral and sensorineural. All affected individuals showed loss of high frequencies. Comprehensive family medical histories of these individuals showed no other clinical abnormalities, such as diabetes, muscular diseases, visual dysfunction or neurological disorders.

To elucidate the molecular basis of aminoglycoside ototoxicity, we have performed a mutational screening of the mitochondrial 12S rRNA gene in those 60 subjects demonstrating aminoglycoside ototoxicity. DNA fragments spanning this gene were PCR amplified and each fragment was purified and subsequently analyzed by DNA sequencing. The comparison of the resultant sequence with the Cambridge consensus sequence (Anderson et al. 1981) identified 15 nucleotide changes in the 12S rRNA gene of 60 subjects with aminoglycoside ototoxicity as shown in Table 1. All of those nucleotide changes were verified by sequence analysis of both strands and appeared to be homoplasmy. The deafness-associated 12S rRNA C1494T mutation was absent in this hearing-impaired population, indicating that this mutation is a rare ototoxic mtDNA mutation. However, the sequence analysis has identified that eight subjects with profound hearing loss carried the A1555G mutation. The restriction enzyme digestion and subsequent electrophoresis analysis indicated that the A1555G mutation was indeed present in homoplasmy in these subjects (Fig. 1). This translates to a frequency of ~13% for the A1555G mutation in this Chinese sporadic pediatric population with aminoglycoside-

induced deafness. This is comparable with previous reports that suggest that the frequency of this mutation ranges from ~5 to ~17% in other clinical populations with aminoglycoside ototoxicity (Hutchin et al. 1993; Fischel-Ghodsian et al. 1993; Bacino et al. 1995). Further clinical and genetic evaluations of some matrilineal relatives in eight subjects carrying the A1555G mutation were performed. The familial history of these subjects, as shown in Fig. 2, exhibited a pattern of maternally transmitted inheritance. As can be seen in Fig. 2, about half of affected matrilineal relatives had aminoglycoside-induced hearing loss, suggesting that the aminoglycoside is a major modifier factor in the phenotypic manifestation of the A1555G mutation in these families.

Interestingly, as shown in Fig. 3a, the T1095C mutation in the 12S rRNA gene was identified in subject #110 apparently in homoplasmy. This girl received gentamycin (80 mg/dose every 12 h) for pneumonia at the age of 20 months. She began suffering from bilateral hearing impairment 7 days after administration. Audiological evaluation at the age of 9 years showed that she had profound hearing loss (118 dB, left ear; 113 dB, right ear). As shown in Fig. 3b, the family history of this subject suggested a maternally transmitted pattern of inheritance. Further nucleotide sequence analysis revealed the presence of the T1095C mutation in other matrilineal relatives of this family but the absence of mutations in the mitochondrial tRNA^{Ser(UCN)} and *GJB2* (data not shown). The tRNA^{Ser(UCN)} and *GJB2* genes have been shown to be the hot-spots for deafness-associated mutations (Morton 2002). This T1095C mutation was also absent in 364 Chinese control (Zhao et al. 2004a). This mutation has been associated with hearing impairment in several genetically unrelated families including Italian (Tessa et al. 2001; Thyagarajan et al. 2001) and Chinese (Zhao et al. 2004b). This T to C transition disrupted an evolutionarily conserved base pair at the stem loop of helix 25 of 12S rRNA (Neefs et al. 1991). An alteration of tertiary or quaternary structure of this rRNA with the T1095C mutation may lead to impairing mitochondrial protein synthesis, thereby causing the mitochondrial dysfunction associated with hearing impairment. In fact, the occurrence of the T1095C mutation in these several genetically unrelated pedigrees affected by hearing impairment strongly indicates that this mutation is involved in the pathogenesis of hearing impairment. However, a functional characterization needs to be performed to elucidate the pathogenesis of the T1095C mutation.

Of other nucleotide changes, eight variants in the 12S rRNA gene were previously identified in the control population (Kogelnik et al. 1998). Furthermore, the 961 C-insertion and the A827G mutation have been implicated to be associated with aminoglycoside-ototoxicity (Bacino et al. 1995; Casano et al. 1999; Tang et al. 2002) or non-syndromic hearing loss (Li et al. 2004a), respectively. The T1005C and A1116G are probably novel variants in this Chinese population. These variants in the 12S rRNA gene were then examined for the allelic frequency by sequencing PCR fragments spanning the 12S rRNA gene derived from 144 Chinese controls. Indeed, two variants, T1005C and A1116G, were not present in 144 Chinese controls. All variants in the 12S rRNA gene were further evaluated by phylogenetic analysis of these mtDNA variants and mtDNAs from other organisms. Interestingly, the A827G, T1005C and A1116G variants in the 12S rRNA gene are localized at sites which are highly conserved in human (Anderson et al. 1981), mouse (Bibb et al. 1981), bovine (Gadaleta et al. 1989) and *Xenopus laevis* (Roe et al. 1985). However, other variants in the same gene are not evolutionarily conserved. An alteration of tertiary or quaternary structure of this rRNA by these variants may lead to mitochondrial dysfunction, thereby playing a role in the pathogenesis of aminoglycoside ototoxicity.

Sixty-eight subjects (38 males and 30 females) without a history of exposure to aminoglycosides developed hearing impairment prior to 7 years of age. Audiological evaluation showed that eight individuals exhibited moderate hearing impairment, 19 patients had severe hearing impairment and 41 subjects suffered from profound hearing loss. Their

hearing loss shared some common features: being bilateral and sensorineural. Comprehensive family medical histories of these cases exhibited no other clinical abnormalities. Mutational screening of the mitochondrial 12S rRNA gene in 68 subjects with non-syndromic hearing loss led to identification of 15 nucleotide changes, as shown in Table 2. All nucleotide changes were verified by sequence analysis of both strands and appeared to be homoplasmy. Of these, one subject carried the only A1555G mutation, while subject #3 had both A1555G and 961insC mutations in the 12S rRNA gene, similar to a large Chinese pedigree with aminoglycoside-induced and non-syndromic deafness (Li et al. 2004b). A restriction enzyme digestion and subsequent electrophoresis analysis indicated that the A1555G mutation was present in homoplasmy in these subjects (Fig. 1). The ~2.9% incidence of the A1555G mutation in this Chinese sporadic pediatric population with non-syndromic hearing loss seems to be higher than in other ethnic groups (Usami et al. 2000; Hutchin et al. 2001; Kupka et al. 2002; Tekin et al. 2003; Li et al. 2004a). Of other variants in this gene, the novel T961C mutation was identified in a patient with moderate hearing impairment, while the 961 C-insertion was found in two subjects with severe hearing impairment. There was a 4.4% incidence of mutations at position 961 in this Chinese hearing-impaired population. Furthermore, three subjects carry the A827G mutation and two subjects have the T1005C mutation. In addition, T1443C and T1119C appear to be novel variants in the Chinese population.

In summary, the results reported here demonstrated that aminoglycoside ototoxicity accounts for 48% of the cases of hearing loss in a Chinese pediatric population. The frequency of the A1555G mutation is ~13% and ~2.9% in this Chinese pediatric population with aminoglycoside-induced and non-syndromic hearing loss, respectively. The T1095C mutation has been found to be associated with aminoglycoside ototoxicity in the Chinese clinical population. Mutations at position 961 in the 12S rRNA gene account for ~1.7% and 4.4% cases of aminoglycoside-induced and non-syndromic hearing loss in this Chinese clinical population, respectively. Three variants in the 12S rRNA gene, A827G, T1005C and A1116G, localized at highly conserved sites, may play a role in the pathogenesis of aminoglycoside ototoxicity. In this clinical population, the C1494T mutation appears to be less frequent than the A1555G mutation in the 12S rRNA gene. These data strongly indicate that the mitochondrial 12S rRNA is a hot-spot for deafness-associated mutations in the Chinese population.

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Fig. 1. Qualification of the A1555G mutation in ten Chinese subjects with aminoglycoside-induced and non-syndromic hearing loss. PCR products around the A1555G region of mitochondrial genome were digested with *Bsm*AI and analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. F11B is a positive control from an Arab-Israeli family (Guan et al. 1996) and A2 is a Chinese control subject (Zhao et al. 2004a)

Fig. 2. Three Chinese pedigrees with maternally inherited aminoglycoside-induced and non-syndromic hearing impairment. Hearing impaired individuals are indicated by *filled symbols*. *Arrows* denote probands. *Asterisks* denote individuals who had a history of exposure to aminoglycosides

Fig. 3a,b.

Molecular and genetic analysis of an affected subject #110 carrying the T1095C mutation in the 12S rRNA gene. **a** Sequence chromatograms from an affected individual #110 and an unaffected control A2, showing the T to C transition at position 1095 (arrow). **b** A Chinese pedigree with aminoglycoside-induced and non-syndromic hearing impairment. Hearing impaired individuals are indicated by *filled symbols*. Arrow denotes subject #110

Variants in the mitochondrial 12S rRNA gene in 60 aminoglycoside-induced hearing-impaired Chinese pediatric subjects. Known and putative pathogenic mutations are indicated in *boldface*

Table 1

Position	Replacement	No. of subjects	Conservation (H/B/M/X) ^a	Frequency ^b	Previously reported ^c
709	G to A	5	G/A/A/-	29/144	Yes
750	A to G	60	A/A/A/-	144/144	Yes
752	C to T	1	C/C/A/-	5/144	Yes
827	A to G	2	A/A/A/A	2/144	Yes
961	C insertion	1	T/T/A/A	5/144	Yes
1005	T to C	1	T/T/T/T	0/144	No
1048	C to T	1	C/C/T/C	1/144	Yes
1095	T to C	1	T/T/T/T	0/144	Yes
1107	T to C	3	T/C/T/T	2/144	Yes
1116	A to G	1	A/A/A/A	0/144	No
1382	A to C	2	A/A/A/G	3/144	Yes
1438	A to G	60	A/A/A/G	144/144	Yes
1503	G to A	1	G/A/A/C	1/144	Yes
1555	A to G	8	A/A/A/A	0/354	Yes
1598	G to A	3	G/A/T/T	3/144	Yes

^aConservation of nucleotide for the 12S rRNA in human (H), bovine (B), mouse (M) and *Xenopus laevis* (X)

^bNumbers of controls with variant/total controls

^cSee <http://www.mitomap.org>

Table 2
Variants in the mitochondrial 12S rRNA gene in 68 non-syndromic hearing-impaired Chinese pediatric subjects. Known and putative pathogenic mutations are indicated in *boldface*

Position	Replacements	No. of subjects	Conservation (H/B/M/X) ^a	Frequency ^b	Previously reported ^c
709	G to A	15	G/A/A/-	29/144	Yes
750	A to G	68	A/A/A/-	144/144	Yes
752	C to T	1	C/C/A/-	5/144	Yes
827	A to G	3	A/A/A/A	2/144	Yes
961	C insertion	2	T/T/A/A	5/144	Yes
961	T to C	1	T/T/A/A	0/144	No
1005	T to C	2	T/T/T/T	0/144	No
1107	T to C	3	T/C/T/T	2/144	Yes
1119	T to C	4	T/T/T/C	1/144	No
1382	A to C	2	A/A/A/G	3/144	Yes
1420	T to C	1	T/A/A/C	2/144	Yes
1438	A to G	68	A/A/A/G	144/144	Yes
1443	T to C	1	T/T/T/A	0/144	No
1555	A to G	2	A/A/A/A	0/354	Yes
1598	G to A	2	G/A/T/T	3/144	Yes

^aConservation of nucleotide for the 12S rRNA in human (H), bovine (B), mouse (M) and *Xenopus laevis* (X)

^bNumbers of controls with variant/total controls

^cSee <http://www.mitomap.org>