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Maternally Inherited Aminoglycoside-Induced and Nonsyndromic Deafness Is Associated with the Novel C1494T Mutation in the Mitochondrial 12S rRNA Gene in a Large Chinese Family

Hui Zhao,[1,2,*] Ronghua Li,[1,*] Qiuju Wang,[2] Qingfeng Yan,[1] Jian-Hong Deng,[3] Dongyi Han,[2] Yidong Bai,[3] Wie-Yen Young,[2] and Min-Xin Guan[1,2]

[1]Division and Program in Human Genetics and Center for Hearing and Deafness Research, Cincinnati Children's Hospital Medical Center, Cincinnati; [2]Department of Otolaryngology, Head and Neck Surgery, Chinese People's Liberation Army General Hospital, Beijing; and [3]Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio

We report here the characterization of a large Chinese family with maternally transmitted aminoglycoside-induced and nonsyndromic deafness. In the absence of aminoglycosides, some matrilineal relatives in this family exhibited late-onset/progressive deafness, with a wide range of severity and age at onset. Notably, the average age at onset of deafness has changed from 55 years (generation II) to 10 years (generation IV). Clinical data reveal that the administration of aminoglycosides can induce or worsen deafness in matrilineal relatives. The age at the time of drug administration appears to be correlated with the severity of hearing loss experienced by affected individuals. Sequence analysis of mitochondrial DNA in this pedigree identified a homoplasmic C-to-T transition at position 1494 (C1494T) in the 12S rRNA gene. The C1494T mutation is expected to form a novel U1494-1555A base pair, which is in the same position as the C1494-1555G pair created by the deafness-associated A1555G mutation, at the highly conserved A site of 12S rRNA. Exposure to a high concentration of paromomycin or neomycin caused a variable but significant average increase in doubling time in lymphoblastoid cell lines derived from four symptomatic and two asymptomatic individuals in this family carrying the C1494T mutation when compared to four control cell lines. Furthermore, a significant decrease in the rate of total oxygen consumption was observed in the mutant cell lines. Thus, our data strongly support the idea that the A site of mitochondrial 12S rRNA is the primary target for aminoglycoside-induced deafness. These results also strongly suggest that the nuclear background plays a role in the aminoglycoside ototoxicity and in the development of the deafness phenotype associated with the C1494T mutation in the mitochondrial 12S rRNA gene.

Introduction

Hearing loss is a very common congenital disorder affecting 1 in 1,000 newborns (Nance and Sweeney 1975; Morton 1991). In the pediatric population, >50% of patients with deafness have a genetic predisposition, with autosomal dominant, autosomal recessive, X-linked, or mitochondrial patterns of inheritance (Petit et al. 2001; Morton 2002). Deafness can result from a mutation in a single gene or from a combination of mutations in different genes. Hearing loss can also be caused by environmental factors, including perinatal infection, acoustic or cerebral trauma affecting the cochlea, or ototoxic drugs, such as aminoglycoside antibiotics, or it can be a result of interactions between genetic and environmental factors (Petit et al. 2001; Morton 2002). Specifically, in familial cases of ototoxic deafness, the aminoglycoside hypersensitivity is often maternally transmitted, suggesting mitochondrial involvement (Fischel-Ghodsian 1999).

Mutations in mitochondrial DNA (mtDNA) have been found to be associated with both aminoglycoside-induced and nonsyndromic deafness (Fischel-Ghodsian 1999; Van Camp and Smith 2000). Among the identified nonsyndromic deafness-causing mtDNA mutations are A7445G (Reid et al. 1994; Fischel-Ghodsian et al. 1995), 7472insC (Tiranti et al. 1995; Verhoeven et al. 1999), T7510C (Hutchin et al. 2000), and T7511C (Sue et al. 1999) in the $tRNA^{Ser(UCN)}$ gene and the A1555G mutation in the 12S rRNA gene (Prezant et al. 1993; Estivill et al. 1998; Li et al. 2004). In particular, the A1555G mutation has been found to be responsible for nonsyndromic deafness in many families of different ethnic backgrounds (Hutchin et al. 1993; Prezant et al. 1993; Matthijs et al. 1996; Estivill et al. 1998; Li et al. 2004). In the absence of exposure to aminoglycosides, the A1555G mutation produces a clinical phenotype

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Address for correspondence and reprints: Dr. Min-Xin Guan, Division and Program in Human Genetics, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. E-mail: min-xin.guan@chmcc.org

* These authors contributed equally to this work.

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that varies considerably among family members, ranging from severe congenital deafness, to moderate progressive hearing loss of later onset (Estivill et al. 1998; Li et al. 2004), to completely normal hearing (Prezant et al. 1993; Estivill et al. 1998; Li et al. 2004). More severe biochemical defects were observed in the mutant lymphoblastoid cell lines derived from symptomatic individuals from an Arab-Israeli family than from cell lines derived from asymptomatic individuals in the same family (Guan et al. 1996). These genetic and biochemical data strongly indicate that the A1555G mutation is a primary factor underlying the development of deafness and that nuclear modifier genes play a role in modulating the phenotypic expression of the hearing loss associated with the A1555G mutation (Guan et al. 1996, 2001; Li and Guan 2002; Li et al. 2002).

The A1555G mutation has been also found in a number of families and sporadic patients with aminoglycoside-induced severe or profound hearing loss (Hutchin et al. 1993; Prezant et al. 1993; Estivill et al. 1998; Li et al. 2004). This mutation lies in the conserved decoding region of small ribosomal RNA, which is important for the action of aminoglycosides (Moazed and Noller 1987; Purohit and Stern 1994). In fact, the new C1494-1555G pair in 12S rRNA created by the A1555G mutation facilitates the binding of aminoglycosides to mitochondrial 12S rRNA (Hamasaki and Rando 1997). Functional studies demonstrated a decrease in the growth rates of lymphoblastoid cells derived from symptomatic and asymptomatic members of the Arab-Israeli pedigree (mentioned above) in the presence of a high concentration of neomycin or paromomycin (Guan et al. 1996, 2000). In addition, a C insertion or deletion at position 961 of the 12S rRNA gene has been shown to be associated only with aminoglycoside-induced deafness (Bacino et al. 1995; Casano et al. 1999). These data strongly indicate that the human mitochondrial 12S rRNA, particularly that carrying the A1555G mutation, is the main ototoxic target for aminoglycoside antibiotics.

The mutations at positions 1555 and 961 in the 12S rRNA gene only account for a small portion of patients with aminoglycoside-induced hearing impairment (Fischel-Ghodsian 1999). 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 the large clinical population of the otology clinic at the Chinese People's Liberation Army (PLA) General Hospital. As a consequence of this study, 34 pedigrees with a maternally inherited pattern of aminoglycoside-induced hearing loss have been identified, including 15 pedigrees carrying the A1555G mutation in mitochondrial 12S rRNA gene (Yuan et al. 1999; Li et al. 2001). In the present study, we report the clinical, molecular, and genetic characterization of a large Chinese family with maternally transmitted aminoglycoside-induced and nonsyndromic deafness (fig. 1*A*). Despite sharing some common features, including bilateral, symmetric, and sensorineural hearing loss, 39 matrilineal relatives in this five-generation family exhibited the variable severity and age at onset in hearing impairment. Molecular analysis has led to the identification of a novel C-to-T transition at position 1494 in the mitochondrial 12S rRNA gene. The C1494T mutation is expected to form a new 1494-1555 U-A base pair at the highly conserved A site of the 12S rRNA (Zimmermann et al. 1990; Neefs et al. 1991), which is in the same position as the 1494-1555 C-G pair caused by the A1555G mutation (Hutchin et al. 1993; Prezant et al. 1993; Li and Guan 2002). To examine if the C1494T mutation leads to aminoglycoside toxicity, lymphoblastoid cell lines derived from six individuals of the Chinese family (including four individuals exhibiting both the mutation and hearing loss and two carrying the mutation but lacking a clinical phenotype) and four genetically unrelated controls lacking the mutation have been analyzed for the sensitivity to drugs by exposure of cells to a high concentration of paromomycin or neomycin. Furthermore, the respiration capacity of those cell lines was measured by determining the oxygen (O_2) consumption rate in intact cells.

Material and Methods

Patients

We ascertained a Chinese family (fig. 1*A*) through the Department of Otolaryngology, Head and Neck Surgery, Chinese PLA General Hospital. Informed consent, blood samples, and clinical evaluations were obtained from all participating family members, under protocols approved by the Cincinnati Children's Hospital Medical Center institute review board and the Chinese PLA General Hospital ethics committee. Members of this pedigree were interviewed at length to identify either personal or family medical histories of hearing loss, the use of aminoglycosides, and other clinical abnormalities. The 364 control DNA samples used for screening for the presence of mtDNA mutations were obtained from a panel of unaffected individuals from Chinese ancestry.

Audiological and Neurotological Examinations

The audiological and neurotological examinations of the proband and other members of this family were conducted, including pure-tone audiometry (Madsen GSI61), immittance (Madsen GSI33), and auditory brain-stem response (ABR) (IHS6002). The degree of hearing loss was defined according to the pure-tone averages (PTA), which were based on the three frequencies (500, 1,000, and

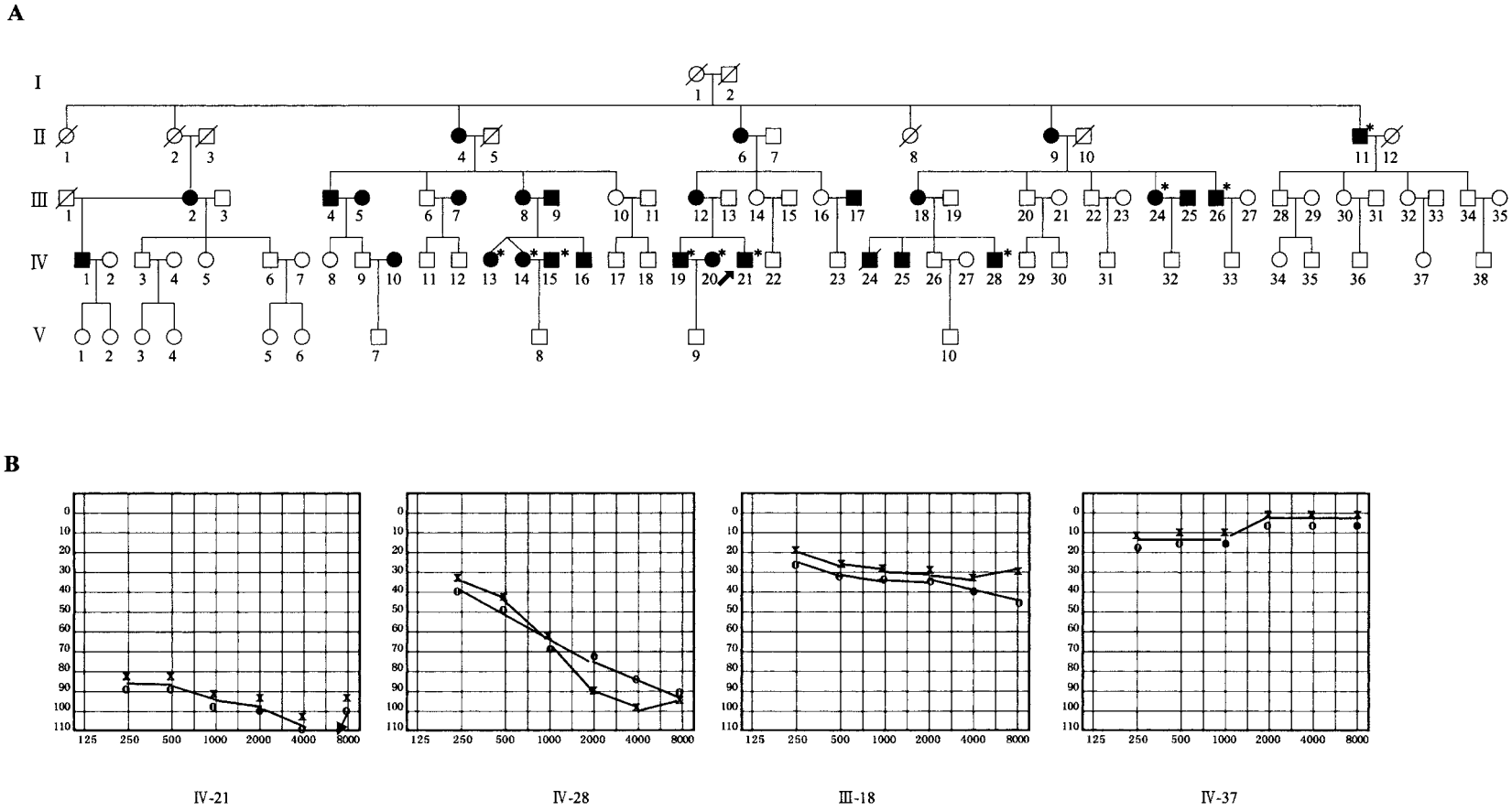

Figure 1 The Chinese pedigree with maternally inherited aminoglycoside-induced and nonsyndromic deafness. *A*, Pedigree of five generations of the Chinese family. Hearing-impaired individuals are indicated by filled symbols. Generations are indicated on the left by Roman numerals, and the numbers under the individuals represent identification numbers for each generation. Asterisks denote individuals who had a history of exposure to aminoglycosides. The proband is indicated by arrow. *B*, Audiograms of three deaf subjects and one married-in control of the Chinese pedigree. IV-21 and IV-28 were treated with streptomycin and gentamicin, respectively, before hearing impairment. III-18 had a hearing impairment at the age of 50 years and had no history of exposure to aminoglycosides. IV-37 had normal hearing.

2,000 Hz), as follows: normal <26 dB, mild 26–40 dB, moderate 41–70 dB, severe 71–90 dB, and profound >90 dB.

Mutational Analysis of the Mitochondrial Genome

Genomic DNA was isolated from whole blood of participants by use of the Puregene DNA Isolation Kits (Gentra Systems). First, affected individuals' DNA fragments spanning the entire mitochondrial 12S rRNA gene or $tRNA^{Ser(UCN)}$ gene were amplified by PCR using oligodeoxynucleotides corresponding to the mitochondrial genome at positions 618–635 and 1589–1606 (Rieder et al. 1998) and 7151–7170 and 8504–8623 (Fischel-Ghodsian et al. 1995). For the detection of the A1555G mutation, the amplified segments were digested with the restriction enzyme *Bsm*AI (Prezant et al. 1993; Guan et al. 1996; Li et al. 2004). For the examination of A7445G mutation, PCR fragments were digested with the restriction enzyme *Xba*I (Fischel-Ghodsian et al. 1995; Guan et al. 1998), whereas the presence of the T7511C mutation was examined by digesting PCR products with *Mbo*II (Sue et al. 1999).

The entire mitochondrial genome of the affected patient IV-21 was PCR amplified in 24 overlapping fragments by use of sets of the light-strand and the heavy-strand oligonucleotide primers, as described previously (Rieder et al. 1998). Each fragment was purified and subsequently analyzed by direct sequencing in an ABI 3700 automated DNA sequencer, using the BigDye Terminator Cycle sequencing reaction kit. The BLAST homology searches were performed using the programs available on the National Center for Biotechnology Information Web site (Altschul et al. 1997). DNA and protein-sequence alignments were carried out using the SeqWeb program GAP (GCG).

All family members and 364 controls were screened for the presence of the C1494T mutation, by direct sequencing of a PCR product spanning bases 1245–2007. Furthermore, the PCR products spanning bases 1245–2007 in an affected member IV-21 and a control were cloned into a PCR 2.1-TOPO vector (Invitrogen) and then were directly sequenced. The allele frequency of variants in 12S rRNA, 16S rRNA, ND1, ND4, and ND5 genes was determined by PCR amplification of fragments spanning the corresponding regions, using the genomic DNA derived from Chinese controls as templates and performing subsequent sequence analysis of PCR products, as described above. These sequence results were compared with the updated consensus Cambridge sequence (Anderson et al. 1981).

Quantification of the Mitochondrial 12S rRNA C1494T Mutation

Quantification of the C1494T mutation in the mitochondrial 12S rRNA gene was carried out by the allele-specific termination of primer extension, as described elsewhere (Bai and Attardi 1998). For this purpose, a 1,075-bp mtDNA segment at positions 697 and 1,771 was amplified by PCR reaction. The amplified fragment was purified with the QIAEX II gel extraction kit (Qiagen). The PCR product was then used for allele-specific primer-extension termination, using Sequenase (USB) and the corresponding ^{32}P-5′-end-labeled primer (Bai and Attardi 1998). Nucleotide concentrations were 33 μM dCTP and 500 μM ddTTP for the quantification of the C insertion, and 33 μM dCTP, 33 μM dATP, and 500 μM ddTTP (USB) for the quantification of the C-to-T mutation. The mixtures were heated to 95°C for 2 min, annealed at 50°C for 10 min, and finally chilled on ice. After the addition of Sequenase, they were incubated for 5 min at 45°C. Electrophoretic analysis of the products and quantification of the intensity of the bands were carried out as described elsewhere (Bai and Attardi 1998). The primer used for primer extension was 5′-GAAGCGCGTACACACCGCCCGT-3′.

Cell Cultures

Lymphoblastoid cell lines were immortalized by transformation with the Epstein-Barr virus, as described elsewhere (Miller and Lipman 1973). Cell lines derived from six members of the Chinese family (two individuals [III-14, III-16] with normal hearing, four affected individuals [III-12, III-18, IV-21, IV-28]), and four genetically unrelated control individuals (A3, A6, A7, A8) were grown in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS).

To test the various cell lines for sensitivity to paromomycin and neomycin, cells were grown for 4 d in RPMI 1640 medium (Invitrogen), supplemented with 10% FBS, in the presence or absence of 2 mg of the antibiotics per ml. The population doubling time (DT) of the cell lines in RPMI 1640 medium, supplemented with 10% FBS, was determined from the growth curves or by using the formula $DT = (t - t_0)\log 2/(\log N - \log N_0)$, where DT is the doubling time, t and t_0 are the times at which the cells were counted, and N and N_0 are the cell numbers at times t and t_0, respectively (Guan et al. 1996).

Oxygen Consumption Measurements

Rates of O_2 consumption in intact cells were determined with a YSI 5300 oxygraph (Yellow Springs Instruments) on samples of 1×10^7 cells in 1.5 ml of special Dulbecco modified Eagle medium (DMEM) lack-

Table 1

Summary of Clinical Data for Some Maternal Members of the Chinese Pedigree

Patient	Sex	Age (years)		Use of Aminoglycosides	PTA[a] (dB)		Level of Hearing Impairment
		At Testing	At Onset		Right Ear	Left Ear	
II-4	F	81	>65	No	42	37	Mild
II-6	F	78	>65	No	35	32	Mild
II-9	F	74	>65	No	42	35	Mild
II-11	M	68	24	Yes	90	82	Severe
III-2	F	60	50–60	No	40	42	Mild
III-4	M	58	50–60	No	80	32	Mild
III-8	F	50	40–50	No	37	43	Mild
III-10	F	45	...	No	25	23	Normal
III-12	F	52	40–50	No	32	57	Mild
III-14	F	50	...	No	22	27	Normal
III-16	F	46	...	No	20	20	Normal
III-18	F	57	40–50	No	28	28	Mild
III-24	F	46	1–10	Yes	103	100	Profound
III-26	M	42	30	Yes	68	65	Moderate
IV-1	M	44	20–30	No	78	78	Severe
IV-13	F	26	1	Yes	107	102	Profound
IV-14	F	26	1	Yes	98	97	Profound
IV-16	M	21	10–20	No	27	27	Mild
IV-17	M	21	...	No	22	18	Normal
IV-18	M	16	...	No	23	20	Normal
IV-19	M	29	1.5	Yes	108	98	Profound
IV-21	M	26	1.5	Yes	91	91	Profound
IV-22	M	20	...	No	22	18	Normal
IV-23	M	25	...	No	13	13	Normal
IV-28	M	30	21	Yes	62	67	Moderate
IV-32	M	21	...	No	23	22	Normal
V-8	M	1	...	No	NA	NA	Normal

[a] PTA = pure-tone averages.

ing glucose, supplemented with 10% dialyzed fetal bovine serum (FBS) (King and Attardi 1989).

Computer Analysis

Statistical analysis was performed by the unpaired, two-tailed Student *t* test contained in the Microsoft Excel program for Macintosh (version 5).

Results

Clinical Presentation

The proband (IV-21) received streptomycin (0.75 g/d for 3 d) for pneumonia at the age of 18 mo. He began suffering bilateral hearing impairment 3 d after therapy. He came to the otology clinic at the Chinese PLA General Hospital at the age of 26 years. As illustrated in figure 1*B*, the audiological evaluation, including the pure-tone audiometry, immittance, and ABR, showed that he had profound hearing loss. Karyotype analysis and a CT scan of temporal bones were normal. In addition, he had no other significant medical history.

The family originated from Liaoning Province in northeastern China, and the majority of family members live in the same area. As shown in figure 1, this familial history is consistent with maternal inheritance. None of the offspring of deaf fathers has a hearing impairment, whereas 20 of 39 matrilineal relatives, who are the offspring of subject I-1, exhibit bilateral and sensorineural hearing impairment as the sole clinical symptom. All affected individuals showed the loss of high frequencies. In 18 of the 20 affected subjects, hearing impairment was symmetric. Of two individuals with asymmetric hearing impairment, the more severe dysfunction in the left ear of III-12 was due to otitis media at the age of 34 years and the severe defect in the right ear of III-4 was due to sudden deafness at the age of 50 years. In the absence of aminoglycosides, matrilineal relatives of this family exhibited late-onset/progressive, but not congenital, hearing impairment. As shown in table 1, audiometric studies showed a variable severity of hearing impairment in the maternal kindred, ranging from severe hearing impairment, to moderate hearing impairment, to mild hearing impairment, to completely normal hearing. In addition, there was a wide range in the age at onset of hearing impairment in this family, varying from 10 years to 65 years. Notably, the average age at onset of hearing impairment in this family, excluding matri-

lineal relatives who had a history of exposure to aminoglycosides, has changed from 55 years (generation II) to 10 years (generation IV).

As shown in table 1 and figure 1, eight matrilineal relatives of this family, who had a history of exposure to gentamicin and/or streptomycin had subsequent moderate-to-profound hearing loss. Hearing impairment usually occurred 3 d after injection. The age at the time of administration varied among those individuals. Five individuals (III-24, IV-13, IV-14, IV-19, and IV-21) who received a regular dose of aminoglycosides (3–5 mg/kg/dose every 8 h for gentamicin or 15–25 mg/kg/dose every 12 h for streptomycin) at <10 years of age developed profound hearing loss. By contrast, three other individuals (II-11, III-26, and IV-28) suffered moderate-to-severe hearing loss after administration of a regular dose of aminoglycosides at >20 years of age. In particular, the subject IV-28 had mild hearing impairment at the age of 20 years. After administering a regular dose of gentamicin (80 mg/dose every 12 h) for appendicitis at the age of 21 years, he suffered moderate hearing loss (fig. 1*B*). Clearly, the age at the time of drug administration appears to be correlated with the severity of the hearing loss experienced by affected individuals.

There is no evidence that any member of this family had any other known cause to account for hearing impairment. Comprehensive family medical histories of these individuals showed no other clinical abnormalities, including diabetes, muscular diseases, visual problems, and neurological disorders. Furthermore, these matrilineal relatives, who were examined by karyotype analysis and a CT scan of temporal bones, revealed no abnormal findings.

Mitochondrial DNA Analysis

The maternal transmission of aminoglycoside-induced and nonsyndromic hearing loss in this family suggested mitochondrial involvement and led us to analyze the mitochondrial genome of matrilineal relatives. First, we examined the known mtDNA mutations associated with deafness by PCR amplification and subsequent restriction-enzyme digestion analysis of PCR fragments derived from four matrilineal relatives (the deaf proband [IV-21], his deaf mother [III-12], an unaffected female [III-14], and an affected male [IV-28]) and two unrelated Chinese controls. We failed to detect either the presence of A1555G mutation in the 12S rRNA gene or the A7445G, T7510C, and T7511C mutations in the t-RNA$^{Ser(UCN)}$ gene in those patients.

To elucidate the molecular basis for the mutations found in this family with maternally transmitted deafness, 24 overlapping DNA fragments spanning the entire mitochondrial genome of proband (IV-21) 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 a number of nucleotide changes, as shown in Table 2. All of those nucleotide changes were verified in 12 additional matrilineal relatives of this family (6 symptomatic and 6 asymptomatic individuals) by sequence analysis and appeared to be in homoplasmic form.

Of these nucleotide changes, there are three variants in the 12S rRNA gene and three variants in the 16S rRNA gene, as mitochondrial rRNAs were proposed to be the target sites for aminoglycoside otoxicity. The A663G mutation in the 12S rRNA gene and the A1736G mutation in the 16S rRNA gene were previously identified in the control population (Kogelnik et al. 1998), whereas the 961insC in the 12S rRNA gene has been implicated to have a role in the phenotypic expression of A1555G mutation (Li et al. 2004). On the other hand, the C1494T mutation in the 12S rRNA gene (fig. 2*A*) and the G1709A and C2572G mutation in the 16S rRNA gene appear to be novel variants. These variants were then examined to determine the frequency in the Chinese control population by sequencing the PCR fragments spanning the 12S rRNA and 16S rRNA genes, derived from the Chinese controls. As shown in table 2, the C1494T mutation was absent in 364 controls, whereas other variants in the 12S rRNA and 16S rRNA gene occurred in the Chinese controls. To determine if the C1494T mutation is present in homoplasmic form in the matrilineal relatives and controls, a more sensitive experiment, involving allele-specific termination of primer extension, was carried out. As can be seen in figure 2*B*, there is no detectable wild-type DNA in the six mutant individuals, indicating that the C1494T mutation appears to be homoplasmic. Furthermore, the PCR products spanning bases 1245–2007 in an affected family member (IV-21) and a control were cloned into a PCR 2.1-TOPO vector (Invitrogen) and then were directly sequenced. Sequence data showed that all 16 clones derived from patient IV-21 contain the C1494T mutation, whereas 16 clones derived from a control lack the C1494T mutation. Further analysis showed that this mutation was present in all matrilineal relatives of this family in the homoplasmic form. These variants in the rRNA genes were further evaluated by phylogenetic analysis of these mtDNA variants and mtDNAs from other organisms. The five variants in the 12S rRNA and 16S rRNA genes were not highly conserved, whereas the C1494T mutation in the 12S rRNA is localized at a highly evolutionarily conserved site from bacteria to vertebrate, including human (Anderson et al. 1981), mouse (Bibb et al. 1981), bovine (Gadaleta et al. 1989), and *Xenopus laevis* (Roe et al. 1985). As shown in figure 3, the C at position 1494 (equivalent to C at position 1409 of *Escherichia coli* 16S rRNA) is localized at the highly

Table 2

mtDNA Mutations in the Chinese Pedigree

Gene and Position	Mutation	Conservation in H/B/M/X[a]	Frequency[b]	Previously Reported[c]
D-Loop:				
73	A→G		NA	Yes
235	A→G		NA	Yes
263	A→G		NA	Yes
310	T→CTC		NA	Yes
515	AC deletion		NA	Yes[d]
16223	C→T		NA	Yes
16290	C→T		NA	Yes
16319	G→A		NA	Yes
16362	T→C		NA	Yes
12S rRNA:				
663	A→G		6/364	Yes[d,e]
961	C insertion		4/364	Yes[d]
1494	C→T	C/C/C/C	0/364	No
16S rRNA:				
1709	G→A	G/G/T/T	1/108	No
1736	A→G	A/T/C/A	2/108	Yes[c,d]
2572	C→G	C/C/C/G	1/108	No
ND1:				
4247	T→C (Ile to Thr)	I/I/V/L	1/108	No
ND2:				
4769	A→G		NA	Yes
4824	A→G (Thr to Ala)	T/T/T/L	NA	Yes
CO1:				
7028	C→T		NA	Yes
A6:				
8794	C→T (His to Tyr)	H/H/H/Y	NA	Yes
8860	A→G (Thr to Ala)	T/A/A/T	NA	Yes
ND4:				
11639	A→C (Met to Leu)	M/M/M/M	1/108	No
11718	G→A (Gly to Glu)	G/G/G/G	NA	Yes
ND5:				
12705	C→T		NA	Yes
14075	A→C (Gln to Pro)	Q/Q/Q/Q	1/108	No
cytochrome *b*:				
15326	A→G (Thr to Ala)	T/M/I/I	NA	Yes

[a] Conservation of amino acid for polypeptides or nucleotide for rRNAs, in human (H), bovine (B), mouse (M), and *Xenopus laevis* (X).
[b] Numbers of controls with mutation/total controls.
[c] See the online mitochondrial genome database MITOMAP.
[d] Li et al. 2004.
[e] Prezant et al. 1993.

conserved A site of human mitochondrial 12S rRNA (Zimmermann et al. 1990; Neefs et al. 1991), which is the main target site for aminoglycoside otoxicity (Moazed and Noller 1987; Purohit and Stern 1994). The C-to-U transition at position 1494 in this Chinese family would be base-paired with A at position 1555 of A site of the 12S rRNA. This new U-A pair, located at the same position as the C-G base pair generated by the deafness-associated A1555G mutation, is expected to create a new binding site for aminoglycosides, as described in a study of *E. coli* (De Stasio and Dahlberg 1990), thus causing the sensitivity to these drugs.

Of the other nucleotide changes in this mitochondrial genome, nine variants in the D-loop region and eight variants in the protein encoding genes were previously found in the Chinese control population (Kogelnik et al. 1998; Li et al. 2004). Furthermore, the T4247C (I314T) mutation in the ND1 gene, the A11639C (M294L) mutation in the ND4 gene, and the A14075C (G580P) mutation in the ND5 gene are probably novel missense polymorphisms in the Chinese population. As shown in table 2, sequencing analysis of the PCR fragments spanning the ND1, ND4, and ND5 genes revealed that these variants indeed occur in the Chinese control population. Furthermore, the I314T mutation in the ND1 gene was not highly conserved, whereas the M294L mutation in

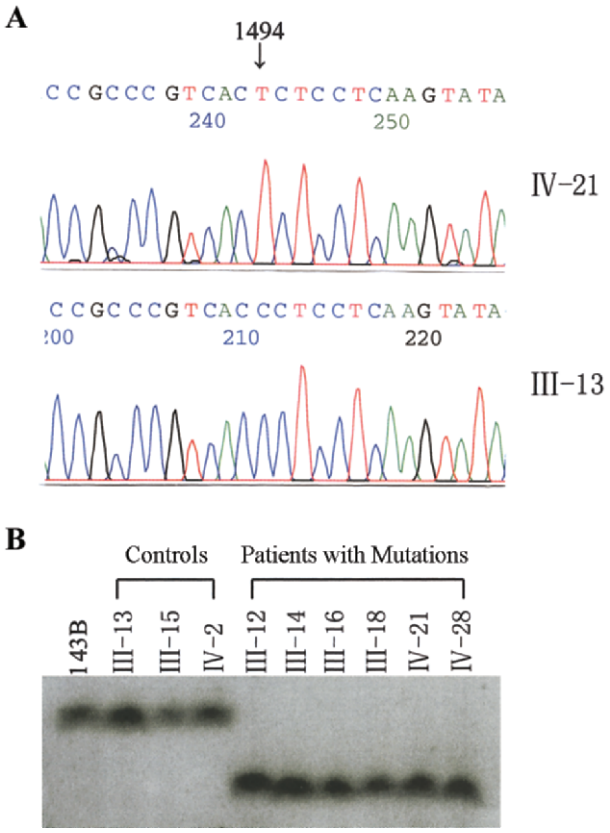

Figure 2 Identification and qualification of C1494T mutation in the mitochondrial 12S rRNA gene. *A*, Partial-sequence chromatograms of the 12S rRNA gene from an affected individual, IV-21, and a married-in control, III-13. An arrow indicates the location of the base changes at position 1494. *B*, Quantification of C1494T mutation in the 12S rRNA gene of individuals with mutations and controls derived from the Chinese family. Allele-specific termination of primer extension was carried out as detailed in the "Material and Methods" section. The products were separated on a 20% polyacrylamide/7M urea sequence gel.

the ND4 gene and the M294L mutation in the ND5 gene are localized at sites that are highly conserved in human (Anderson et al. 1981), mouse (Bibb et al. 1981), bovine (Gadaleta et al. 1989), and *Xenopus* (Roe et al. 1985).

Effect of Aminoglycosides on the Growth of Cell Lines Carrying the C1494T Mutation

To examine the role of the C1494T mutation in the effect of aminoglycosides on cell growth, lymphoblastoid cell lines derived from six individuals carrying the C1494T mutation and from four genetically unrelated Chinese controls were grown in RPMI 1640 medium in the presence of drugs, or in their absence, for 4 d. As shown in figure 4, in the presence and absence of 2 mg paromomycin or 2 mg neomycin per ml, the growth rates of the mutant cell lines carrying the C1494T mutation exhibited a significant average decrease, relative to the growth rates of control cell lines. In particular, in the mutant cell lines carrying the C1494T mutation, the ratios of doubling times (DT) in the presence and absence of paromomycin were increased by an average of 24% ± 7% (standard error of the mean [SEM]), relative to the average DT ratio in the control cell lines ($P = .01$). However, the sensitivity to paromomycin among those cell lines varied greatly: patient III-18 exhibited a DT ratio comparable with the mean control value, and patient IV-28 showed a 55% increase in DT ratio, relative to the average DT ratio in controls. Similarly, the DT ratios of mutant cell lines carrying the C1494T mutation in the presence and absence of the neomycin were increased by an average of 18% ± 5% (SEM), relative to the average DT ratio in the control cell lines ($P = .02$). Among these mutant cell lines, III-18 had a DT ratio comparable with the mean control value, whereas patient IV-28 displayed a 43% increase in DT ratio, relative to the average DT ratio in controls. From these data, we conclude that the C1494T mutation plays a role in the sensitivity to aminoglycoside of the growth rate of the cell lines derived from matrilineal relatives in this Chinese pedigree.

Oxygen Consumption Rate Measurements

The endogenous respiration rates of lymphoblastoid cell lines derived from two asymptomatic individuals and from four symptomatic individuals of the Chinese family carrying the C1494T mutation and from four genetically unrelated Chinese controls were measured by determining the O_2 consumption rate in intact cells, as described by King and Attardi (1989). As can be seen in figure 5, the rate of total O_2 consumption in six mutant lymphoblastoid cell lines exhibited a variable decrease, ranging from ~11% to ~27%, relative to the mean value measured in the control cell lines, with an average reduction of ~19% ($P = .0109$). In particular, a ~24% average reduction in the rate of total O_2 consumption was observed in two lymphoblastoid cell lines derived from the asymptomatic individuals that exhibited a variable decrease, relative to the mean value measured in the control cell lines, whereas the four lymphoblastoid cell lines from the symptomatic individuals also displayed a variable reduction in the rate of O_2 consumption, ranging from 11% to 21%, when compared with the mean control value, with a ~17% average reduction.

Discussion

In the present study, we have performed the clinical, genetic, and molecular characterization of a large Chi-

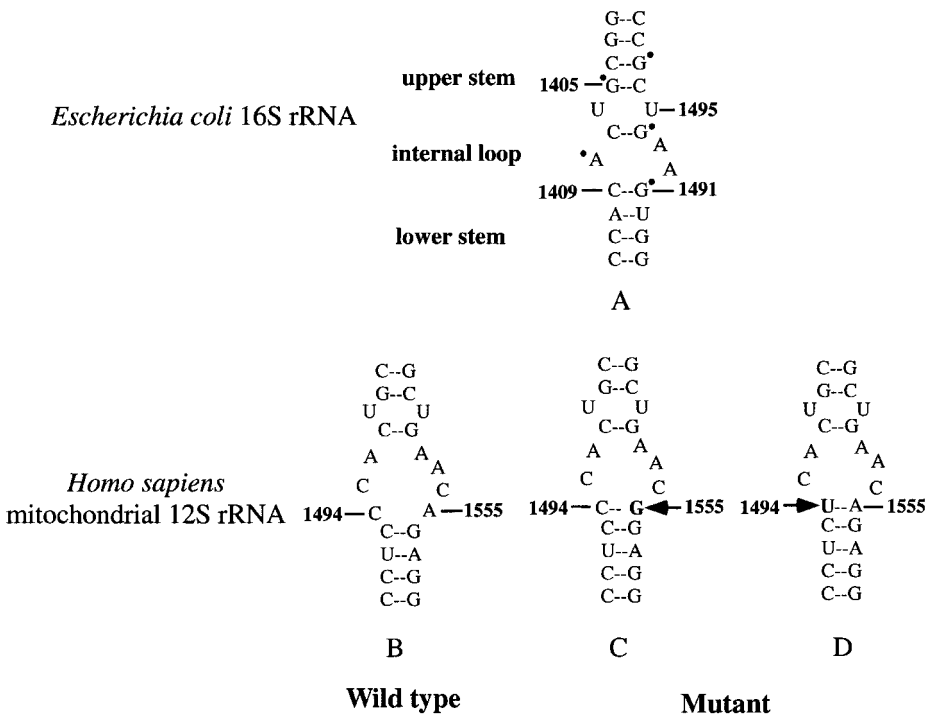

Figure 3 The site of the C1494T mutation in the decoding region of small ribosomal RNAs. The A site of the *E. coli* 16S rRNA oligonucleotide showing the dimethyl sulfate (DMS) footprints, observed in the presence of the aminoglycosides neomycin and paromomycin (Moazed and Noller 1987; Purohit and Stern 1994), is marked with a dot (*A*). The corresponding region of human mitochondrial 12S rRNA is shown as the wild-type version (*B*) (Li and Guan 2002) and in the version containing the A1555G mutation (*C*) and C1494T mutation (*D*). The sites for the A1555G and C1494T mutations are indicated by arrows.

nese family with aminoglycoside-induced and nonsyndromic hearing loss. The clinical phenotypes, including aminoglycoside-induced and nonsyndromic hearing impairment, were only present in the maternal lineage of this five-generation pedigree, suggesting that the mtDNA mutation is the molecular basis for this disorder. Here, we have identified a homoplasmic C-to-T transition at position 1494 in the mitochondrial 12S rRNA gene. The following evidence suggests that the C1494T mutation is a primarily pathogenic mtDNA mutation that causes a genetic predisposition to aminoglycoside ototoxicity and nonsyndromic deafness. This mutation is present only in matrilineal relatives of this family in the homoplasmic form, and not in the 364 Chinese controls. The C1494T mutation is localized at the A site of the mitochondrial 12S rRNA (Moazed and Noller 1987; Zimmerman et al. 1990; Neefs et al. 1991; Purohit and Stern 1994), which has been implicated to be associated with aminoglycoside ototoxicity (Prezant et al. 1993; Hamasaki and Rando 1997). The phylogenetic analysis of this mutation and mtDNAs from other organisms revealed that the nucleotide C at the position 1494 in the decoding region of 12S rRNA was extremely evolutionarily conserved from bacteria to human mitochondria (Zimmerman et al. 1990; Neefs et al. 1991). Finally, lymphoblastoid cell lines derived from members of the Chinese family carrying the C1494T mutation, compared with the wild-type cell lines, exhibited a significant reduction in the growth rate in the presence of a high concentration of paromomycin or neomycin, as well as in the rate of total O_2 consumption.

The region of 16S rRNA in *E. coli*, corresponding to that of the human 12S rRNA C1494T mutation, forms an essential part of the decoding site of the ribosome (De Stasio and Dahlberg 1990; Zimmermann et al. 1990) and is crucial for subunit association either by RNA-protein or RNA-RNA interactions (Zwieb et al. 1986). Furthermore, the sensitivity to paromomycin, neomycin, and related aminoglycosides in bacteria involves their direct binding to a C-G base pair at positions 1409–1491 of the penultimate helix in the small ribosomal subunit rRNA (fig. 3) (Moazed and Noller 1987; Purohit and Stern 1994). In particular, in wild-type *E. coli* sensitive to these aminoglycosides, the nucleotide at position 1491 (G) in 16S rRNA is base-paired with a C at position 1409; mutation or methylation of the 1491 nucleotide disrupts the G-C pairing, producing resistance to aminoglycosides (Li et al. 1982; Spangler and Blackburn 1985; De Stasio and Dahlberg 1990). On the other hand, the reverse mutations C1409U-

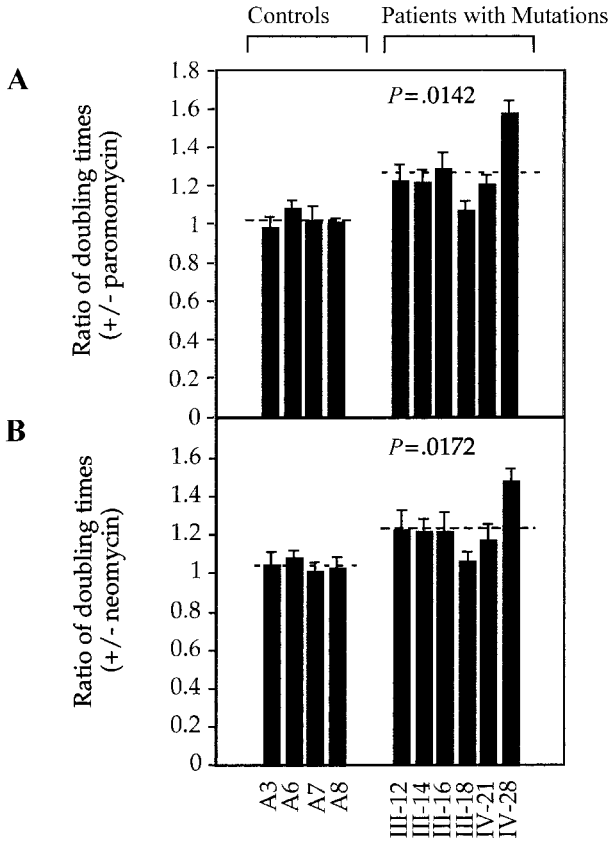

Figure 4 Growth properties of lymphoblastoid cell lines. The population doubling time (DT) during 4 d of growth was determined in RPMI 1640 medium in the presence and absence of paromomycin (*a*) and neomycin (*b*). The ratios of DTs in the presence and absence of 2 mg paromomycin or neomycin per ml are shown. The average of four to five determinations for each cell line is shown, with error bars representing 2 SEM. The horizontal dashed lines represent the average value for each group; *P* indicates the significance, according to the Student *t* test, of the differences between the mutant mean and the control mean.

G1491A, which create the new U-A pairing in the *E. coli* 16S rRNA, also confer sensitivity to a variety of aminoglycoside antibiotics, including paromomycin and neomycin (De Stasio and Dahlberg 1990). In human mitochondria, the nucleotide at position 1555 (corresponding to position 1491 in *E. coli* 16S rRNA) in wild type is A, which would be expected to pair with the C at position 1494 (Prezant et al. 1993; Li and Guan 2002) but instead mutates to a G, as in the Arab-Israeli and other families. It makes the secondary structure of the 12S rRNA similar to the corresponding region of the *E. coli* 16S rRNA (Prezant et al. 1993), thus facilitating the binding and sensitivity to aminoglycosides (Guan et al. 1996, 2000; Hamasaki and Rando 1997) and causing defects in mitochondrial protein synthesis and respiration (Guan et al. 1996, 2001). In the case of this Chinese family, the C-to-T transition at position 1494 in the human 12S rRNA (equivalent to position 1409 in the *E. coli* 16S rRNA) would be expected to pair with the A at position 1555. The new U-A base pair created by the C1494T mutation, corresponding to the U-A pairing at positions 1409–1491 in the *E. coli* 16S rRNA, was anticipated to lead to the binding and sensitivity to aminoglycosides. This alteration in the tertiary structure of the 12S rRNA may result in the impairment of mitochondrial protein synthesis. As a consequence, the C1494T mutation in the mitochondrial 12S rRNA produces a respiratory deficiency and could subsequently result in a decline in ATP production in the cochlear cells (hair cells and/or stria vascularis), which are essential for hearing function (Prezant et al. 1993; Guan et al. 1996).

Table 3 shows the aminoglycoside sensitivity and respiration defect detected in a previous analysis of the A1555G mutation and in the present work for the C1494T mutation. The rate of O_2 consumption of the lymphoblastoid cell lines derived from 10 symptomatic and 9 asymptomatic members of the Arab-Israeli family carrying the A1555G mutation revealed an average reduction of 30% and 25%, relative to the mean values measured in wild-type cell lines (Guan et al. 1996). In the present work, ~17% and ~24% average reductions in the rate of oxygen consumption were observed in the lymphoblastoid cell lines derived from four symptomatic and two asymptomatic individuals of the Chinese family carrying the C1494T mutation, respectively. Notably, there was the small difference in endogenous respiration rate between two groups of cell

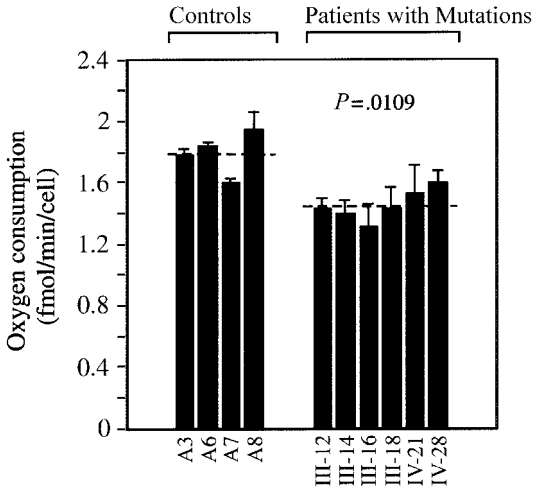

Figure 5 Respiration assays. Average rates of endogenous O_2 consumption per cell measured in different lymphoblastoid cell lines are shown, with error bars representing 2 SEM. Five to seven determinations were made for each cell line. The graph details and symbols are explained in the legend to figure 4.

Table 3

Oxygen Consumption Rate and DT Ratio in the Presence and Absence of Aminoglycosides of Lymphoblastoid Cell Lines Carrying the A1555G and C1494T Mutations

Rate or Ratio	Average Value ± SD in A1555G Cell Lines, Relative to controls[a,b] (%)		*P* for A1555G Cell Lines	Average Value ± SD in C1494T Cell Lines, Relative to controls (%)		*P* for C1494T Cell Lines
	Asymptomatic Individuals	Symptomatic Individuals		Asymptomatic Individuals	Symptomatic Individuals	
O_2 consumption rate	75 ± 9	70 ± 8	.4558	76 ± 4	83 ± 6	.0954
DT ratio (+/− paromomycin)	138 ± 3	137 ± 4	.8542	121 ± 5	123 ± 8	.8486
DT ratio (+/− neomycin)	132 ± 5	142 ± 9	.3284	123 ± 6	125 ± 11	.8473

Note.—*P* = the significance, according to the Student *t* test, of the differences between the symptomatic and asymptomatic means. The presence/absence of aminoglycosides is denoted by +/−.

[a] Guan et al. 1996, 2000.

[b] Guan, unpublished data.

lines. These strongly indicate that the C1494T mutation produces a milder biochemical defect than that of the A1555G mutation. A comparison between the DT ratios of presence and absence of paromomycin or neomycin in the lymphoblastoid cell lines derived from matrilineal relatives of the Chinese family carrying the C1494T mutation and those of the Arab-Israeli family carrying the A1555G mutation revealed that the cell lines carrying the C1494T mutation have less sensitivity to drugs than those carrying the A1555G mutation. These results are in agreement with the fact that the *E. coli* strains carrying the C1409U-G1491A mutation in the 16S rRNA exhibited less sensitivity to aminoglycosides than those carrying the wild-type 1409C-1491G pair at the A site of 16S rRNA (De Stasio and Dahlberg 1990). These data strongly suggest that sensitivity to aminoglycosides in the cell lines appears to be dependent on the presence of the mitochondrial 12S rRNA C1494T mutation. In contrast to the pedigrees carrying the A1555G mutation, the age at the time of drug administration has been shown to be correlated with the severity of the hearing loss experienced by affected individuals in this Chinese family. Furthermore, the considerable variability in sensitivity to aminoglycosides was observed among the six cell lines derived from this Chinese pedigree. These data are strong evidence that aminoglycosides can induce or worsen the deafness phenotype associated with the C1494T mutation. These observations also provide the first direct evidence that other factors, specifically in a nuclear background, play a role in the sensitivity to the drugs in those individuals carrying the C1494T mutation.

The fact that only a portion of patients with the C1494T mutation developed hearing impairment (31% penetrance when the effect of aminoglycosides was excluded, but 51% penetrance when aminoglycoside-induced deafness was included) in this Chinese family clearly indicates that the C1494T mutation alone is not sufficient to produce the clinical phenotype. As in the families carrying the A1555G mutation (Prezant et al. 1993; Li et al. 2004), the matrilineal relatives in this Chinese pedigree displayed phenotypic variability, including in the severity and age at onset in the absence of aminoglycosides. In particular, the members of the Chinese family with the C1494T mutation always exhibited progressive and/or later-onset hearing impairment, whereas the hearing impairment of members in the Arab-Israeli family carrying the A1555G mutation was mainly congenital (Prezant et al. 1993). This discrepancy likely reflects the difference between the genetic backgrounds of the Arab-Israeli and Chinese family. The other striking clinical feature in the Chinese family is that the average age at onset of hearing impairment in this family, excluding matrilineal relatives who had a history of exposure to aminoglycosides, has changed from 55 years (generation II) to 10 years (generation IV). These observations strongly suggest the involvement of nuclear modifier genes in the development of the deafness phenotype associated with the C1494T mutation, similar to that in the Arab-Israeli family carrying the A1555G mutation (Prezant et al. 1993; Guan et al. 1996, 2000). Furthermore, it is possible that other environmental factors, besides aminoglycosides, may contribute to the penetrance of the C1494T mutation, especially in late-onset cases, in this family. The products of a putative nuclear gene, which may functionally interact with mutated 12S rRNA, could enhance the effect of the C1494T mutation to produce the hearing impairment or to suppress it (Guan et al. 1996).

Furthermore, mitochondrial haplotypes have been implicated in influencing the penetrance of the primary mtDNA mutations. For instance, mtDNA mutations at positions 4216 and 13708 can increase the penetrance of the primary Leber hereditary optic neuropathy (LHON) mutations, including the 11778 mutation in the ND4 gene, the 14484 mutation in the ND6 gene (Wallace et al. 1988; Howell et al. 1991; Torroni et al. 1997), and the deafness-linked A7445G mutation in the precursor of $tRNA^{Ser(UCN)}$ gene (Guan et al. 1998). In this Chinese

family, several mtDNA mutations may also play a role in the deafness phenotypic manifestation of the C1494T mutation. In particular, the C insertion at position 961 was found to coexist with the C1494T mutation in this family. Despite a lack of biochemical evidence, genetic data have indicated that the mutations in 961 are associated only with aminoglycoside ototoxicity (Bacino et al. 1995; Casano et al. 1999). It has been suggested that the C insertion at position 961 may enhance the biochemical defect associated with the A1555G mutation, thus increasing the age at onset, as well as the severity of hearing impairment, in the large Chinese family (Li et al. 2004). Here, the cosegregation of the 961 C insertion with the C1494T mutation in the 12S rRNA gene in this family also raises the possibility that the 961 mutation may play a role in the phenotypic manifestation of the C1494T mutation. Other mutations, showing high evolutionary conservation, including the M294L mutation in the ND4 gene and the M294L mutation in the ND5 gene, may also contribute to the penetrance of the C1494T mutation in this large Chinese family.

In conclusion, the results reported here convincingly demonstrate that the C1494T mutation in the 12S rRNA gene is a novel primary mtDNA mutation associated with both aminoglycoside-induced and nonsyndromic deafness. This mutation is clearly not sufficient to produce the clinical phenotype, since several individuals who are carrying the mutation have normal hearing. Other factors, including nuclear modifier genes, mitochondrial haplotypes, and aminoglycosides, contribute to the penetrance and expressivity of deafness associated with the C1494T mutation in this Chinese large family. Specifically, our data strongly support the idea that the A site of mitochondrial 12S rRNA is the primary target for aminoglycoside ototoxicity. In addition, our results also provide the first direct evidence that the nuclear background plays a role in the sensitivity to the aminoglycosides in those individuals carrying the C1494T mutation in the mitochondrial 12S rRNA gene.

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Electronic-Database Information

The URLs for data presented herein are as follows:

Accelrys (GCG) SeqWeb GAP program, http://www.accelrys.com/products/seqweb/
MITOMAP: A Human Mitochondrial Genome Database, http://www.mitomap.org/
National Center for Biotechnology Information Home Page, http://www.ncbi.nlm.nih.gov/

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