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Mutation analysis of mitochondrial *12S rRNA* gene in Polish patients with non-syndromic and aminoglycoside-induced hearing loss

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ABSTRACT

Mutations in mitochondrial DNA have been reported as associated with non-syndromic and aminoglycoside-induced hearing loss. In the present study, we have performed mutational screening of entire *12S rRNA* gene in 250 unrelated patients with non-syndromic and aminoglycoside-induced hearing loss. Twenty-one different homoplasmic sequence variants were identified, including eight common polymorphisms, one deafness-associated mutation m.1555 A>G and three putatively pathogenic variants: m.669 T>C, m.827 A>G, m.961 delT+C(n)ins. The incidence of m.1555 A>G was estimated for 3.6% (9/250); however, where aminoglycoside exposure was taken as a risk factor, the frequency was 5.5% (7/128). Substitution m.669 T>C was identified only in patients with hearing impairment and episode of aminoglycoside exposure, which may suggest that such additional risk factors must appear to induce clinical phenotype. Moreover, two *12S rRNA* sequence variants: m.988 G>A and m.1453 A>G, localized at conserved sites and affected RNA secondary structure, may be new candidates for non-syndromic and aminoglycoside-induced hearing loss associated mutations.

1. Introduction

Hearing loss (HL) is the most frequent sensory disorder affecting 1–3 in 1000 newborns [1]. According to the report of the Polish Universal Neonatal Hearing Screening Program, 1/560 newborns suffered hearing impairment, including profound and severe sensorineural hearing loss [2]. It was estimated that over 50% of HL have a genetic cause with autosomal dominant, autosomal recessive, X-linked or mitochondrial pattern of inheritance [3,4].

Several mutations in mitochondrial DNA (mtDNA) have been found to be associated with syndromic, non-syndromic (NSHL) and aminoglycoside-induced hearing loss [5, MITOMAP: http://www.mitomap.org]. Sequence analysis of the mitochondrial genome in families and sporadic cases from various ethnic origins provides strong evidence that *12S rRNA* gene is the hot spot for NSHL and genetically-determined susceptibility to aminoglycoside ototoxicity. Several sequence variants were reported as associated with HL, including m.872 A>G, m.961 delT+insC, m.961 T>C, m.1095 T>C, m.1494 C>T and m.1555 A>G [6–10]. However, mutations that have clear contribution to non-syndromic and aminoglycoside-induced hearing impairment are the m.1494 C>T and m.1555 A>G substitutions [7,11,12]. Other sequence variants are still considered as provisional and their pathogenic nature remains controversial, mainly due to a variable frequency in different populations, mtDNA phylogeny and occurrence in normal hearing individuals.

Up to date, little is known about the incidence of *12S rRNA* mutations in Polish patients. Our previously published data were focused only on the m.1555 A>G substitution, however the incidence of other deafness-associated mutations were not determined [13]. Thus, it is anticipated that additional, known or novel mutations in *12S rRNA* associated with NSHL and susceptibility to aminoglycoside ototoxicity can be found in our population. In this study, we performed mutational screening of the entire mitochondrial *12S rRNA* gene to estimate the involvement and the frequency of known and putative mutations in Polish patients with non-syndromic and aminoglycoside-induced hearing loss.

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2. Materials and methods

2.1. Patients

We recruited 250 unrelated Polish patients with NSHL, negative for deafness-associated mutations in *GJB2* gene [14,15] as well as *GJB6* del(*GJB6*-D13S1830). Clinical information, such as the age of onset of HL, severity, the history of aminoglycoside (AG) or other kind of ototoxic drugs exposure and family history were obtained from the patients through questionnaire. In studied group, 128 subjects had a history of aminoglycosides exposure and 29 had a family history with maternally inherited hearing impairment.

Hearing loss was age-appropriate quantified by pure-tone audiometry and/or auditory brainstem response. The severity of hearing impairment was defined by pure-tone threshold average (PTA) in frequencies: 500, 1000, 2000 and 4000 Hz. Hearing loss of $\leqslant$20 dB was considered as normal hearing, 21–40 dB – mild, 41–70 dB – moderate, 71–90 dB – severe and >90 dB profound.

In addition, 250 Polish individuals with normal hearing (including 134 with AG exposure and no ototoxic effect) were recruited as a control group.

From each individual enrolled to the study written informed consent was obtained. The study was approved by the Institutional Review Board at Poznan University of Medical Sciences.

2.2. DNA isolation and mutational screening of 12S rRNA

Total DNA was isolated from the peripheral lymphocytes by phenol–chloroform extraction and ethanol precipitation or exfoliated buccal mucosa using QIAamp DNA Mini Kit (Qiagen, Germany).

Mutational screening of entire *12S rRNA* gene was carried out using PCR–SSCP, followed by direct sequencing as previously reported [16]. The PCR products showing shifts in SSCP were purified (PCR Purification Kit, Qiagen, Germany) and analyzed by direct sequencing in the ABI 3730 automated DNA sequencer using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). The sequence data were compared with the revised Cambridge Sequence (rCRS), GeneBank Accession No. NC_012920 (http://www.ncbi.nlm.nih.gov/Genbank/).

The nature of all identified *12S rRNA* nucleotide changes (polymorphism, putative pathogenic variant, mutation) was checked by searching human mitochondrial genome databases: MITOMAP, mtDB (http://www.genpat.uu.se/mtDB/) and Giib-JST mtSNP (http://mtsnp.tmig.or.jp/mtsnp/index_e.shtml).

2.3. Sequence conservation and 12S rRNA secondary structure analysis and prediction

Sequence alignment of the *12S rRNA* gene in 50 different mammals (Supplementary Table 1) were performed to analyze conservation of the positions of identified sequence variants in human *12S rRNA* (NC_012920). The variants in *12S rRNA* gene were considered as conserved when showed more than 50% conservation rate. Alignment was generated by Clustal V align method.

The RNAfold software from Vienna RNA package (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) was used to generate and predict RNA secondary structure based on minimum energy requirements and pair probability. The folding way of mutated sequences was compared to the wild-type prediction.

2.4. Sequence of whole mtDNA genome and haplogroup defining

The entire mitochondrial genome of selected subjects carrying putative pathogenic variants were PCR amplified using mitoSEQr™-Resequencing System (Applied Biosystems, Foster City, USA), according to manufacture's instruction. PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, USA) and sequencing as described above. Haplogroups were defining using haplogroup finder and haplogroup trees from the Logan's mtDNA website (http://www.ianlogan.co.uk/).

3. Results

The mitochondrial *12S rRNA* gene sequence was analyzed in 250 Polish patients with non-syndromic and aminoglycoside-induced hearing loss, as well as in 250 individuals with normal hearing. In both studied groups, 25 different homoplasmic sequence variants were identified, including 10 common *12S rRNA* polymorphisms at positions m.709, m.750, m.769, m.813, m.930, m.961, m.1189, m.1243, m.1438 and m.1598. The characteristics of detected nucleotide changes are summarized in Table 1.

In the patients group, 21 different sequence variants were identified. Among them, one known deafness-associated mutation m.1555 A>G and three nucleotide changes reported as putatively pathogenic: m.669 T>C, m.827 A>G and m.961 delT+C(n)ins were established. Clinical and molecular characteristic of affected subjects carrying known and provisional mutations in *12S rRNA* gene is shown in Table 2. Substitution m.1555 A>G was identified in nine independent subjects, of these, seven had a history of aminoglycoside exposure. A variable level of hearing impairment was observed among m.1555 A>G carriers, from moderate to profound hearing loss (Table 2). In three cases, a family history of hearing loss was reported; however, an incomplete penetrance of m.1555 A>G was observed among the mutations carriers (Fig. 1A). Substitution m.669 T>C was observed in three independent cases with aminoglycoside exposure history. The G52 subject had a family history of aminoglycoside-induced hearing loss. Both her sons, harbored the same sequence variant, suffer bilateral moderate hearing impairment after gentamicin injection due to bronchitis (Fig. 1B). Patient M21 received one dose of 12 mg of gentamicin intratympanic into right ear due to unilateral Meniere's disease. After drug administration hearing deficiency was observed in an affected ear. There was no hearing problems observed in proband's family.

Besides known and putative HL causative mutations, we identified five *12S rRNA* sequence variants not found in the control group and with a low frequency in mitochondrial databases, including: m.723 T>C, m.960 C>insC, m.988 G>A, m.1453 A>G and m.1503 G>A (Table 1).

In order to speculate about provisional pathogenic nature of identified nucleotide changes, sequence conservation and 12S rRNA secondary structure analysis and folding prediction were performed. We found two nucleotides presenting conservation rate over 50% among 50 different mammals: m.988G and m.1453A (Table 1). Moreover, both nucleotide changes alter 12S rRNA secondary structure leading to more complicated organization of 12S rRNA compared to the wild-type (Supplementary Fig. 1). Clinical and molecular characteristics of affected subjects carrying new candidates for pathogenic sequence variants in *12S rRNA* gene is shown in Table 3. We have found four carriers of m.988 G>A substitution, including three without AG exposure history presented different degree of hearing impairment from mild to profound with an early age of onset. In one case (W61B) maternally inherited HL was observed (Fig. 1C). Patient M8 was treated with 6 intratympanic injections of 12 mg of gentamicin each to affected left ear due to unilateral Meniere's disease and a significant hearing impairment followed the treatment. Patient harboring m.1453 A>G sequence variant suffers bilateral severe hearing loss after amikacin treatment in an early childhood.

Table 1
Sequence variants detected in *12S rRNA* gene.

Position	Nucleotide change	Frequency[a]						Sequence conservation in 50 mammals[c] (%)	Previously reported	
		Patients	%	Controls	%	Polish population[b]	%		MITOMAP[d]	mtDB frequency[e]
m.669[f]	T>C	3/250	1.2	0/250	0.0	1/500	0.2	54	Yes	1/2703
m.709	G>A	37/250	14.8	37/250	14.8	64/500	12.8	34	Yes	444/2260
m.721	T>C	1/250	0.4	2/250	0.8	0/500	0.0	2.0	Yes	5/2699
m.722	C>T	0/250	0.0	1/250	0.4	0/500	0.0	20	Yes	1/2703
m.723	A>C	1/250	0.4	0/250	0.0	0/500	0.0	12	Yes	5/2694
m.750	G>A	4/250	1.6	1/250	0.4	6/500	1.2	2.0	Yes	22/2682
m.769	G>A	0/250	0.0	1/250	0.4	1/500	0.2	8.0	Yes	149/2555
m.813	A>G	1/250	0.4	0/250	0.0	0/500	0.0	30	Yes	44/2660
m.827[f]	A>G	1/250	0.4	0/250	0.0	1/500	0.2	94	Yes	54/2650
m.930	G>A	11/250	4.4	14/250	5.6	16/500	3.2	2.0	Yes	61/2643
m.951	G>A	4/250	1.6	1/250	0.4	2/500	0.4	88	Yes	8/2696
m.960	C>insC	2/250	0.8	0/250	0.0	3/500	0.6	8.0	Yes	n/d
m.961	T>G	8/250	3.2	5/250	2.0	4/500	0.8	34	Yes	37/2662
m.961[f]	delT+insC$_{(n)}$	1/250	0.4	0/250	0.0	0/500	0.0	34	Yes	n/d
m.988	G>A	4/250	1.6	0/250	0.0	1/500	0.2	72	Yes	4/2700
m.1040	T>C	0/250	0.0	1/250	0.4	0/500	0.0	4.0	No	2/2702
m.1047	A>G	1/250	0.4	1/250	0.4	1/500	0.2	44	Yes	n/d
m.1189	T>C	3/250	1.2	0/250	0.0	0/500	0.0	48	Yes	104/2600
m.1243	T>C	7/250	2.8	3/250	1.2	10/500	2.0	52	Yes	57/2647
m.1406	T>C	2/250	0.8	1/250	0.4	1/500	0.2	44	No	10/2694
m.1438	G>A	10/250	4.0	8/250	3.2	23/500	4.6	4.0	Yes	84/2620
m.1453	A>G	1/250	0.4	0/250	0.0	0/500	0.0	82	Yes	n/d
m.1503	G>A	1/250	0.4	0/250	0.0	0/500	0.0	46	Yes	5/2699
m.1555[f]	A>G	9/250	3.6	0/250	0.0	2/500	0.4	100	Yes	12/2692
m.1598	G>A	0/250	0.0	1/250	0.4	2/500	0.4	10	Yes	62/2637

[a] Number of individuals with the sequence variant/total individuals tested.
[b] Own results [16].
[c] The list of species and reference sequences used for alignment is shown in Supplementary Table 1.
[d] MITOMAP: a human mitochondrial genome database, http://www.mitomap.org.
[e] mtDB: human mitochondrial genome database, http://www.genpat.uu.se/mtDB/; n/d, no data.
[f] Known and putative mutations associated with non-syndromic and aminoglycoside-induced hearing loss (source MITOMAP).

Table 2
Clinical and molecular characteristics of affected subjects carrying known and putative pathogenic mutations in *12S rRNA* gene.

Patient	Sex	Age of onset (years)	PTA (dB)		AG exposure	Family history of HL	*12S rRNA* sequence variant	Haplogroup	2D structure change[a]	*GJB2* mutational status
			R	L						
G52	F	16	105	100	Yes (G)	Yes (2)[b]	m.669 T>C	N1a	Yes	WT/WT
G130	M	5	70	86	Yes (G)	No		N1a		WT/35delG
M21[c]	F	54	75	20	Yes (G)	No		N1a		WT/WT
G215	F	0.4	46	51	Yes (G)	No	m.827 A>G	H8	Yes	WT/WT
W150	M	20	40	80	No	No	m.961 delT+insC(n)	U4	n/a	WT/WT
G8	F	17	66	70	Yes (S)	Yes (1)	m.1555 A>G	Not determined	Yes	WT/WT
G29	F	4	70	58	Yes (G)	No				WT/WT
G38	F	9	56	59	No	No				WT/WT
G43	F	10	70	74	Yes (G)	No				WT/WT
G101	M	3	85	84	No	No				WT/WT
G104	M	3	73	80	Yes (G)	Yes (2)				WT/WT
G136	M	7	49	44	Yes (A)	No				WT/WT
G144	F	6	n/a	n/a	Yes (G)	Yes (1)				WT/WT
G244	F	15	108	112	Yes (G)	No				WT/WT

Note: PTA, pure-tone threshold average; R, right ear; L, left ear; AG, aminoglycoside antibiotic; HL, hearing loss; A, amikacin; G, gentamicin; S, streptomycin; WT, wild-typed; n/a, no available.
[a] See Supplementary Fig. 1.
[b] In bracket number of affected maternal relatives.
[c] Patient treated with intratympanic gentamicin injections due to unilateral Meniere's disease. Hearing loss observed only in an affected ear.

We have performed screening of the entire mitochondrial genome in 10 selected patients harboring putative and proposed pathological sequence variants of *12S rRNA* gene. The characteristic of detected mtDNA variants is shown in Supplementary Table 2. In each case, the coexistence of deafness-associated mutations in another mtDNA genes was excluded and haplogroup was defined. Nine of identified mtDNA sequence variants were not previously reported in human mitochondrial databases (Supplementary Table 2) and we have submitted all of them to MITOMAP.

4. Discussion

In the present study, we have analyzed the entire sequence of *12S rRNA* gene in 250 patients with NSHL and aminoglycoside-in-

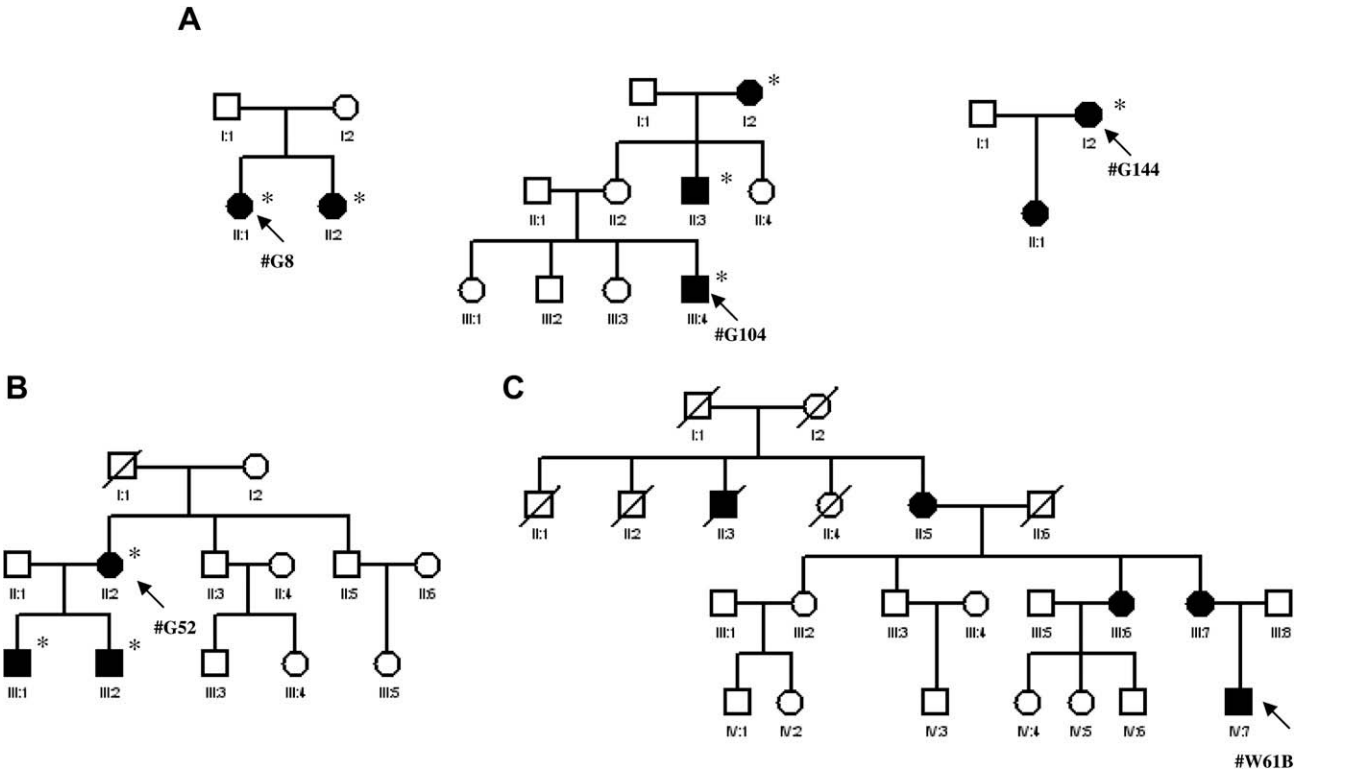

Fig. 1. Pedigrees of five families with maternally inherited non-syndromic and aminoglycoside-induced hearing loss carrying known and proposed pathogenic mutations in *12S rRNA* gene. (A) Families carrying m.1555 A>G mutation, (B) m.669 T>C and (C) m.988 G>A *12S rRNA* sequence variants. Hearing impairment individuals are indicated by filled symbols, arrows denote the proband in each family, while asterisks individuals with a history of aminoglycoside exposure.

Table 3
Clinical and molecular characteristic of affected subjects carrying new candidates for pathogenic sequence variants in *12S rRNA* gene.

Patient	Sex	Age of onset (years)	PTA (dB)		AG exposure	Family history of HL	*12S rRNA* sequence variant	Haplogroup	2D structure change[a]	*GJB2* mutational status
			R	L						
M8[b]	F	41	62	25	Yes (G)	No	m.988 G>A	U2e	Yes	WT/WT
G110	M	0	113	107	No	No		U2e		WT/WT
36A	M	0	>120	>120	No	No		U2e		WT/WT
W61B	M	3	33	33	No	Yes (4)[c]		U2e		WT/WT
G209	M	Early childhood	108	113	Yes (A)	No	m.1453 A>G	V	Yes	WT/WT

Note: PTA, pure-tone threshold average; R, right ear; L, left ear; AG, aminoglycoside antibiotic; HL, hearing loss; A, amikacin; G, gentamicin; WT, wild-typed.
[a] See Supplementary Fig. 1.
[b] Patient treated with intratympanic gentamicin injections due to unilateral Meniere's disease. Hearing loss observed only in an affected ear.
[c] In bracket number of affected maternal relatives.

duced hearing loss. We have identified nine unrelated patients positive for m.1555 A>G and the incidence of this mutation was estimated for 3.6%, what is in the range previously reported for Europeans [13,17]. However, when m.1555 A>G frequency was analyzed in patients positive and negative for AG treatment history, the observed incidence of m.1555 A>G substitution was 5.5% (7/128) and 1.6% (2/122) in studied cohorts with aminoglycoside-induced and non-syndromic hearing loss, respectively. The pedigree analysis (Fig. 1A) showed that m.1555 A>G penetrance is strongly associated with AG exposure. In 86% (6/7) of affected subjects hearing deficiency occurred after AG administration. This observation strongly supports the relationship between m.1555 A>G substitution and hearing loss after AG exposure. Mutation m.1555 A>G itself seems to be not sufficient for clinical phenotype manifestation and additional factors, such as nuclear background, mitochondrial haplotype and environment may modulate the phenotype [18–20].

In five HL patients we have found nucleotide changes, including: m.669 T>C, m.827 A>G and m.961 T>insC(n), previously reported as related to NSHL an aminoglycoside-induced hearing loss. m.669 T>C substitution has been proposed as new putatively pathogenic mitochondrial variant. It has been identified in one four-generation family with maternally inherited NSHL, however an incomplete penetrance, variable severity, as well as various age of onset were observed [21]. Reported frequency of m.669 T>C in mtDB database is low (0.04%), as well as in general Polish population (0.2%) [16], excluding this substitution as a common *12S rRNA* sequence variant. We found T at position m.669 to be relatively well conserved in mammals (Table 1). m.669 T>C substitution caused structural changes of 12S rRNA compared to the wild-type (Supplementary Fig. 1). However, a pathogenic nature of m.669 T>C still remains controversial. Except Lévêque et al. report [21], there is no other proof for m.669 T>C pathogenicity. In two studies m.669 T>C was identified in hearing controls [22,23] and

is associated with haplogroup N1a, which is also our subjects' haplogroup. However, it is worth to point out, that we have identified m.669 T>C only in subjects with hearing impairment after aminoglycoside exposure. Moreover, in one case maternally transmitted susceptibility to aminoglycoside ototoxicity was observed. This substitution was not identified in controls, where over 50% of studied cohort was treated with aminoglycoside without ototoxic effect. Incomplete penetrance of m.669 T>C may suggest that this nucleotide change itself is not sufficient to exhibit the clinical phenotype and aminoglycoside exposure appears to be the modifier and/or crucial factor conditioning pathogenic manifestation of m.669 T>C variant.

m.827 A>G substitution has been described as associated with NSHL, as well as aminoglycoside-induced hearing impairment [22,24,25]. Nevertheless, its pathogenic significant is still not clear. Li et al. reported this substitution in patients with NSHL and aminoglycoside-induced hearing loss, as well as in normal hearing controls [8]. It was also suggested that m.827 A>G may define haplogroups B4b and B4d [26,27]. Further, its frequency reported in mtDB is rather high (54/2650) suggesting, that m.827 A>G may be a common polymorphic variant. In our study, the patient carrying the m.827 A>G substitution suffers moderate HL after aminoglycoside treatment in an early childhood, and her haplogroup was defined as H8. Analysis and prediction of 12S rRNA secondary structure for gene sequence harbored m.827 A>G showed alterations of folding compared with the wild-type (Supplementary Fig. 1). Moreover, m.827 A>G was not found in the control group, and we have previously reported this substitution as a rare *12S rRNA* sequence variant in general Polish population [16]. It suggests that m.827 A>G may be a risk factor for HL after aminoglycoside exposure.

Nucleotide changes found at position m.961, including T>C, delT+C(n)ins, T>insC, have been reported as related to NSHL and aminoglycoside-induced hearing loss [8,28,29]. However, several authors have reported this variant both in NL patients and normal hearing individuals [22,23,30]. In our study, we have identified m.961 delT+insC(n) in one patient with bilateral asymmetric sensorineural hearing loss (Table 2). No family information or aminoglycoside exposure history was available for this patient. This sequence variant was not found in the control group, as well as in general Polish population, but we found T at position m.961 not well conserved among mammals (Table 1). The results obtained in the current study, do not provide any new evidences to support m.961 delT+insC(n) as a HL causing variant, and doubt about its pathogenic nature still remains.

We propose substitutions m.988 G>A and m.1453 A>G in *12S rRNA* as a new candidates for genetic risk factors of NSHL and aminoglycoside-induced hearing impairment. Both variants were identified only in HL patients, m.988 G>A and m.1453 A>G are well conserved among mammals and alter the secondary structure of 12S rRNA comparing with the wild-typed folding. We found m.988 A>G substitution as a rare sequence variant in general Polish population (1/500) [16], and its frequency in mtDB is also very low (4/2700). However, no evidence for m.988 G>A pathogenicity exists in the literature. Konings et al., reported m.988 A>G as a polymorphic variant of *12S rRNA* found in HL patient (1/466) and normal hearing individual (1/400) as well [22]. In Logan's mitochondrial database, m.988 A>G was reported in association with the U haplogroup, which is characteristic for Indo-European's. However, it is not one of the mtDNA variant defining the U haplogroup. In our study, all patients harboring this substitution belong to haplogroup U2e, what may suggest, that m.988 A>G is a single nucleotide polymorphism (SNP) rather then putative pathogenic variant.

Substitution m.1453 A>G has been reported in two unrelated individuals with NSHL (http://www.mitomap.org/bin/view/MITOMAP/Submissions/20081105001). In Logan's database, m.1453 A>G was reported in association with M2b haplogroup which is characteristic for East Asian's. Sequence analysis of entire mtDNA showed that the patient harbored m.1453 A>G belonging to V haplogroup, what may suggest, that this nucleotide change is not a part of set of mtDNA variants defining a single haplogroup.

In conclusion, we have found m.1555 A>G as the main genetically determined cause of HL, especially after aminoglycoside treatment. Our findings support m.669 T>C and m.827 A>G as putative pathogenic variants predisposing to hearing loss after aminoglycoside exposure. Though, two nucleotide changes in *12S rRNA*: m.988 G>A, m.1453 A>G, may be new candidates for HL causing variants. However, to find out and understand the nature of identified putative and proposed novel pathogenic *12S rRNA* variants and their association with NSHL and/or aminoglycoside-induced hearing impairment, further genetic and functional studies are needed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.03.149.

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