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GJB2 Mutations and Genotype-Phenotype Correlation in 335 Patients from Germany with Nonsyndromic Sensorineural Hearing Loss: Evidence for Additional Recessive Mutations Not Detected by Current Methods

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Key Words
Nonsyndromic sensorineural hearing loss · *GJB2* · *GJB6* · Progression

Abstract
We report on 335 patients (319 families) with mild-to-profound nonsyndromic sensorineural hearing loss. We identified 178 mutated *GJB2* alleles representing 29 different sequence changes (including 3 novel mutations: Q7P, N14D, H100Q), and 2 alleles with the deletion del(GJB6-D13S1830) of the *GJB6* gene. Eleven *GJB2* mutations (119 mutated alleles) were truncating (T), and 18 mutations (59 alleles) were nontruncating (NT). Biallelic *GJB2* mutations were found in 71 patients (21.2%; 67 families; 25 different genotypes). Audiograms of 62 patients (56 families) with biallelic *GJB2* mutations typically indicated a profound hearing loss with T/T mutations, moderate hearing loss with T/NT mutations, and mild hearing impairment with NT/NT mutations ($p < 0.01$, Student's t test). From 37 patients (34 families) with biallelic *GJB2* mutations, audiograms at different ages were available and indicated progressive hearing loss (>15 dB) in 10 patients (27.0%, 10 families). Interestingly, we identified an unexpectedly large subset of patients (n = 29; 8.7%) presenting with only one *GJB2* mutation (n = 14 T/wild-type; n = 15 NT/wild-type). This strongly suggests the presence of additional recessive mutations that are not detected by current *GJB2* mutation and *GJB6* deletion analyses.

Introduction

Sensorineural hearing loss (SNHL) is extremely common, occurring in 1 in 500–1000 infants [Kenneson et al., 2002; Tranebjaerg, 2008]. Approximately 70% of this hearing loss is nonsyndromic, and over 130 loci for nonsyndromic deafness are known in humans [Kenneson et al., 2002; Ballana et al., 2008; Tranebjaerg, 2008]. Despite this extreme genetic heterogeneity, mutations in one particular gene *(GJB2)* predominate, and may cause 30–40% of genetic hearing impairment worldwide [Estivill et al., 1998; Tranebjaerg, 2008]. *GJB2* encodes the connexin 26 (Cx26) protein, a major regulator of potassium homeostasis in the cochlea of the inner ear. *GJB2* sequence analysis is widely performed in patients with congenital hearing loss [Estivill et al., 1998; Azaiez et al., 2004; Snoeckx et al., 2005]. In populations of European origin, one par-

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ticular *GJB2* mutation (c.35delG) accounts for up to 80% of *GJB2* alleles [Estivill et al., 1998; Janecke et al., 2002; Cryns et al., 2004], being most frequent in Southern European countries close to the Mediterranean [Gasparini et al., 2000; Janecke et al., 2002]. Apart from recessive and dominant mutations in the *GJB2* gene, the simultaneous presence of heterozygous mutations in 2 different genes *(GJB2* and *GJB6)* has been described in a small subset of patients [del Castillo et al., 2002; Snoeckx et al., 2005]. The clinical relevance of digenic inheritance of a *GJB2* mutation and a 309-kb deletion [del(GJB6-D13S1830)] or the less frequent 232-kb deletion [del(GJB6-D13S854)] of the *GJB6* gene has been well documented [del Castillo et al., 2005; Snoeckx et al., 2005]. Here, we report on a 6-year survey of molecular analyses of the *GJB2* and *GJB6* genes in a cohort of 335 patients studied at Mainz, Germany.

Material and Methods

This study was approved by the ethics committee of the Medical Council Rhineland-Palatinate, Germany. From April 2002 to March 2008, a total of 335 patients (319 families) with nonsyndromic sensorineural hearing loss, aged 1 month to 46 years, were analyzed at our molecular genetic laboratory. All patients underwent ear microscopy and audiometric testing; most children (>90%) were tested in our department for pediatric audiology. Pure-tone audiometry and speech audiometry were carried out in a sound-proof booth using a MAICO audiometer ST 36 (headphones Holmberg type 9501) with age- and language-adapted tests. Additionally, brain stem response using a Nicolet Pathfinder was measured. Frequency-specific information was assessed using the notched noise option, and the normalized hearing level was used for comparison. Pure tone averages (PTAs) in the conversational frequencies (0.5, 1, 2, and 4 kHz) were calculated for each ear, and the severity of hearing loss was defined using the data from pure tone audiometry in older children and adults, or the results of notched noise brainstem audiometry in children under 2 years of age and in cases of developmental delay and lacking cooperation. Hearing loss was classified by the hearing impairment in the better ear: mild (PTA 21–40 dB), moderate (41–70 dB), severe (71–90 dB), or profound (>90 dB). According to Orzan et al. [1999], progressive hearing loss was diagnosed when audiograms of the initial better ear indicated a PTA loss of >15 dB in at least 2 frequencies. All audiograms resulting from audiometric testing with most likely normal middle ear conditions were included in the analysis.

All patients underwent standard karyotype analysis (GTG banding at the 400-band level) and were screened for *GJB2* mutations and the deletion del(GJB6-D13S1830) of the *GJB6* gene as described later.

GJB2 *Sequence Analysis*

DNA was isolated from blood by standard salting-out procedures. The *GJB2* gene coding sequence was PCR-amplified using primers 5′-TGCTTACCCAGACTCAGAGAA-3′ (forward) and 5′-CGACTGAGCCTTGACAGCTGA-3′ (reverse). PCR conditions included initial denaturation at 94°C for 90 s, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were purified using the ExoSAP method and sequenced on a Beckman CEQ 8000 Genetic Analysis System. The presence of the *GJB2* variants c.457G→A (p.V153I) and c.493C→T (p.R165W) on the same allele was confirmed by cloning the PCR product with the Topo TA cloning kit (Invitrogen, Karlsruhe, Germany) and sequencing the inserts of individual plasmid clones.

PCR Analysis of the Deletion del(GJB6-D13S1830) of the GJB6 *Gene*

To determine the incidence of deaf individuals heterozygous or homozygous for the deletion del*(GJB6*-D13S1830) in the German population, all patients without biallelic *GJB2* mutations were tested for the presence of the deletion using a duplex PCR. The binding sites of the 2 forward primers Cx30del_for2 (5′-CATTGTTGTGAACTAACCTCC-3′) and Cx30_for1 (5′-GCCATGCATGTGGCCTACTA-3′) lay 131 bp telomeric from the distal and 170 bp telomeric from the proximal deletion breakpoint, respectively. The reverse primer Cx30_rev1 (5′-ACTATCTGAAATCAGCTCATT-3′) binds 310 bp centromeric from the proximal deletion breakpoint. PCR conditions were: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 40 s, 58°C for 45 s and 72°C for 45 s, and final extension at 72°C for 5 min. The wild-type allele produces a 480-bp fragment from primers CX30_for1 and CX30_rev1, and the del(GJB6-D13S1830) allele gives a 441-bp product from primers CX30del_for2 and CX30_rev1.

Results

This series of 335 patients included 284 (84.8%) individuals from Germany and Europe, 35 (10.4%) from the Middle East, 8 (2.3%) from Africa, 3 (0.9%) from East or South-East Asia, 3 (0.9%) from the USA, and 2 (0.6%) from the Indian subcontinent; 299 patients (89%) were children aged 0–12 years. We identified 178 mutated alleles with 29 different alterations, including 3 novel and 26 known *GJB2* mutations (fig. 1; table 1). Eleven mutations were truncating and 18 mutations were nontruncating. The most frequent mutations c.35delG, p.L90P, and p.M34T accounted for 102 (57.3%), 13 (7.3%), and 12 (6.7%) of the 178 mutated alleles, respectively (table 1). Table 2 summarizes the different *GJB2* or *GJB6* mutations and genotypes identified in this study. Table 3 relates the mutations with the degree of hearing loss.

Seventy-one (21.2%) of the 335 patients (319 families) with nonsyndromic SNHL exhibited biallelic *GJB2* or digenic *GJB2*/*GJB6* mutations, and of these 37 displayed homozygous *GJB2* mutations, 32 were *GJB2* compound heterozygotes, and 2 patients carried a heterozygous *GJB2*

Table 1. Summary of *GJB2* and *GJB6* mutations

	Alleles (this study), n	Alleles [Snoeckx et al., 2005], n
Truncating (11 different mutations)		
p.W24X	3 (1.7)	47 (1.53)
c.31_38del	2 (1.1)	3 (0.10)
c.35delG	102 (57.3)	2218 (72.44)
p.E47X	2 (1.1)	43 (1.40)
c.167delT	3 (1.7)	91 (2.97)
c.269delT	1 (0.6)	1 (0.03)
c.299_300delAT	1 (0.6)	4 (0.13)
c.310_323del	1 (0.6)	52 (1.70)
c.312_325del	1 (0.6)	–
c.333_334delAA	1 (0.6)	5 (0.16)
del(GJB6)-D13S1830	2 (1.1)	51 (1.67)
Nontruncating (18 different variants)		
p.Q7P	1 (0.6)	–
p.N14D	2 (1.1)	–
p.V27I (polymorphism)	4 (2.2)	10 (0.33)
p.R32H	1 (0.6)	4 (0.13)
p.M34T	12 (6.7)	123 (4.01)
p.V37I	4 (2.2)	75 (2.45)
p.W77R	1 (0.6)	15 (0.49)
p.I82M	3 (1.7)	4 (0.13)
p.L90P	13 (7.3)	57 (1.86)
p.V95M	1 (0.6)	16 (0.52)
p.H100Q	1 (0.6)	–
p.E120del	4 (2.2)	23 (0.75)
p.R127H	1 (0.6)	2 (0.07)
p.S139N	1 (0.6)	3 (0.10)
p.E147K	1 (0.6)	9 (0.29)
p.V153I (polymorphism)	3 (1.7)	5 (0.16)
p.V153I+p.R165W (in *cis*)	2 (1.2)	–
p.R184P	4 (2.2)	21 (0.74)

Figures in parentheses are percentages. In our cohort of 335 patients (319 families) with nonsyndromic SNHL, we identified 29 different sequence variants (178 alleles) including 11 truncating mutations (119 alleles) and 18 nontruncating variants (59 alleles; 16 mutations and 2 polymorphisms).

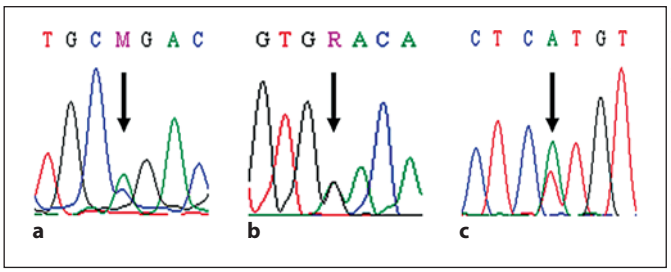

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Fig. 1. Sequence chromatograms of 3 novel *GJB2* mutations, all found in a compound heterozygous state: c.20A→C (p.Q7P) (**a**), c.40A→G (p.N14D) (**b**), and c.300T→A (p.H100Q) (**c**). Arrows indicate base substitutions.

mutation, c.35delG and p.E47X, respectively, in combination with a heterozygous 309-kb deletion del(*GJB6*-D13S1830) of the *GJB6* gene (table 2). Except these 2 patients, no further patients heterozygous for del(*GJB6*-D13S1830) were identified. In addition, patients homozygous for del(*GJB6*-D13S1830) were not present in the analyzed cohort. Forty-three (60.6%) of the 71 patients with biallelic mutations demonstrated 2 truncating *GJB2* mutations (T/T; table 2). Of these, 33 (46.5%) were homozygous c.35delG/c.35delG. Nineteen patients (26.8%) demonstrated 1 truncating and 1 nontruncating mutation each (T/NT), including 18 cases (25.4%) with c.35delG in combination with a nontruncating mutation. Nine patients (12.7%) had 2 non-truncating mutations (NT/NT; table 2).

Homozygosity for a non-c.35delG mutation was found in 4 patients/families: p.W24X/p.W24X in a nonconsanguineous Sinti family, p.V37I/p.V37I in a nonconsanguineous Vietnamese family, p.E120del/p.E120del in a consanguineous Turkish family, and p.R184P/p.R184P in an adult German patient (possibly endogamy) (table 2). One woman from Sri Lanka and her newborn daughter carried a *GJB2* allele with 2 mutations, p.V153I and p.R165W, in *cis* (table 2). Heterozygous *GJB2* mutations were detected in 29 (8.7%) of the 335 patients and at least 14 of these individuals had moderate to profound hearing loss (table 3). This heterozygosity rate, 8.7%, is far higher than that expected in normal European populations [Green et al., 1999; Gasparini et al., 2000; Janecke et al., 2002].

Audiograms were available for 62 patients (from 56 families) with biallelic *GJB2* mutations (fig. 2); most T/T mutations (20 out of 37; 54.1%) were associated with profound hearing impairment, most T/NT mutations (10 of 18; 55.6%) with moderate HI, and most NT/NT mutations (4 of 7; 57.1%) with mild HI (table 3). In the patients with T/T mutations, T/NT mutations, and NT/NT mutations, medians of PTAs were located at 100 dB (profound HI), 54.4 dB (moderate HI), and 36.3 dB (mild HI), respectively. Statistic analysis indicated a more pronounced hearing loss with T/T mutations as compared to T/NT and NT/NT mutations ($p < 0.00001$, Kruskal-Wallis test).

Further, we found a more profound hearing impairment in subjects with a monoallelic *GJB2* mutation of the truncated type (T/wild-type) than in individuals with biallelic nontruncating *GJB2* mutations (NT/NT) (fig. 2).

Table 2. Summary of *GJB2* and *GJB6* genotypes

Genotype and mutation	Patients with biallelic mutations (n = 71)
Truncating/truncating (43 patients, 40 families)	
p.W24X/p.W24X	1 (1.4)
c.35delG/c.35delG	33 (46.5)
c.35delG/p.E47X	1 (1.4)
c.35delG/c.167delT	2 (2.8)
c.35delG/c.269delT	1 (1.4)
c.35delG/c.299_300delAT	1 (1.4)
c.35delG/c.310_323del	1 (1.4)
c.35delG/c.333_334delAA	1 (1.4)
c.35delG/del(GJB6)-D13S1830	1 (1.4)
p.E47X/del(GJB6)-D13S1830	1 (1.4)
Truncating/nontruncating (19 patients, 16 families)	
c.35delG/p.N14D	2 (2.8)
c.35delG/p.R32H	1 (1.4)
c.35delG/p.M34T	3 (4.2)
c.35delG/p.I82M	3 (4.2)
c.35delG/p.L90P	6 (8.5)
c.35delG/p.E120del	1 (1.4)
c.35delG/p.R184P	2 (2.8)
c.312_325del/p.V95M	1 (1.4)
Nontruncating/nontruncating (9 patients, 8 families)	
p.Q7P/p.M34T	1 (1.4)
p.M34T/p.V37I	1 (1.4)
p.M34T/p.L90P	2 (2.8)
p.M34T/p.E120del	1 (1.4)
p.V37I/p.V37I	1 (1.4)
p.L90P/p.E147K	1 (1.4)
p.E120del/p.E120del	1 (1.4)
p.R184P/p.R184P	1 (1.4)

Genotype and mutation	Patients with heterozygous variants (n = 36)
Truncating/wild-type (14 patients, 14 families)	
c.31_38del	2
c.35delG	10
p.W24X	1
c.167delT	1
Nontruncating/wild-type (22 patients, 21 families)	
p.V27I (polymorphism)	4
p.M34T	4
p.V37I	1
p.W77R	1
p.L90P	4
p.H100Q	1
p.S139N	1
p.R127H	1
p.V153I (polymorphism)	3
p.V153I+p.R165W (in *cis*)	2

Figures in parentheses are percentages. In our cohort of 335 patients (319 families) with nonsyndromic SNHL, we identified 71 patients (21.2%, 64 families) with biallelic mutations.

Table 3. Correlation of genotypes and audiometric data in 100 patients (92 families) with biallelic and monoallelic *GJB2* and *GJB6* mutations

Genotype and mutation	Total patients	Mild HI	Moderate HI	Severe HI	Profound HI	No data
Truncating/truncating (43 patients, 40 families)						
p.W24X/p.W24X	1				1	
c.35delG/c.35delG	33		12	3	13	5
c.35delG/p.E47X	1			1		
c.35delG/c.167delT	2				2	
c.35delG/c.269delT	1				1	
c.35delG/c.299_300delAT	1	1				
c.35delG/c.310_323del	1				1	
c.35delG/c.333_334delAA	1				1	
c.35delG/del(GJB6-D13S1830)	1				1	
p.E47X/del(GJB6-D13S1830)	1					1
Truncating/nontruncating (19 patients, 16 families)						
c.35delG/p.N14D	2	2				
c.35delG/p.R32H	1		1			
c.35delG/p.M34T	3	2	1			
c.35delG/p.I82M	3		1		1	1
c.35delG/p.L90P	6		6			
c.35delG/p.E120del	1			1		
c.35delG/p.R184P	2		1		1	
c.312_325del/p.V95M	1				1	
Nontruncating/nontruncating (9 patients, 8 families)						
p.Q7P/p.M34T	1		1			
p.M34T/p.V37I	1	1				
p.M34T/p.L90P	2	2				
p.M34T/p.E120del	1		1			
p.V37I/p.V37I	1		1			
p.L90P/p.E47K	1	1				
p.E120del/p.E120del	1					1
p.R184P/p.R184P	1					1
Truncating/wild-type (14 patients, 14 families)						
c.31_38del/wt	2		1		1	
c.35delG/wt	10	5	1		3	1
p.W47X/wt	1		1			
c.167delT/wt	1				1	
Nontruncating/wild-type (15 patients, 14 families)						
p.M34T/wt	4	2	2			
p.V37I/wt	1		1			
p.W77R/wt	1		1			
p.L90P/wt	4		2		1	1
p.H100Q/wt	1		1			
p.R127H/wt	1					1
p.S139N/wt	1		1			
p.V153I+p.R165W/wt	2			1		1

Figures in parentheses are percentages. Where several audiograms were available, the most recent was used. HI = Hearing impairment.

These heterozygous individuals were, by trend, even more severely affected than patients with biallelic T/NT mutations. Correspondingly, subjects with monoallelic nontruncating mutations (NT/wild-type) demonstrated a more marked hearing loss than patients with biallelic nontruncating mutations (NT/NT) (fig. 2).

Twenty-two patients with biallelic mutations demonstrated profound hearing impairment at first testing, excluding a further progression, and of these, 17 received bilateral cochlear implants, 3 received 1 cochlear implant, and 2 received no cochlear implants. Twenty (91%) of the patients with initially profound hearing loss had T/T mutations, including 14 with homozygous c.35delG, 1 patient with homozygous p.W24X, and 5 patients with compound heterozygous truncating mutations: c.35delG/c.167delT (2 patients), c.35delG/c.269delT, c.35delG/c.310_323del, c.35delG/del(GJB6-D13S1830). Two patients (9%) showed T/NT mutations (c.35delG/p.I82M and c.35delG/p.R184P).

Of 37 biallelic *GJB2* mutation cases with initially nonprofound hearing loss, we could compare audiometric data from 2 or more years. In 30 patients, we obtained at least 2 audiograms for both ears each at different ages of the patients, and 9 of these patients demonstrated progressive hearing loss, as defined by a loss of >15 dB in at least 2 of 4 frequencies tested (fig. 3). In 4 children, only free field audiometry was available several times, including 1 child with progression and 3 children without. For 3 patients, repeated brainstem audiometry measurements were available, and none of these showed progressive hearing loss. Altogether, 26 patients presented a stable hearing loss on their better hearing ear or only temporary threshold shifts because of middle ear affections, and 10 patients demonstrated a progression of >15 dB. The audiometric data of the patients with progressive hearing loss indicate a hearing loss progression of 18.1 dB (mean) in 39.6 months, or 5.5 dB per year (mean of 0.5, 1, 2, and 4 kHz).

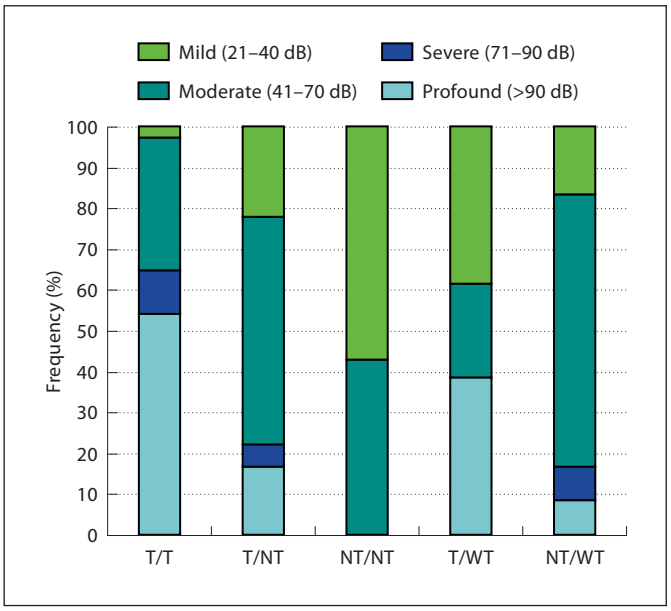

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Fig. 2. Relative frequencies of the degree of hearing loss in the 5 classes of genotypes. The proportions of patients in each subgroup are shown. T/T genotypes were associated with a significantly more pronounced hearing loss ($p < 0.00001$).

Discussion

In our population of 335 patients (319 families) with mild-to-profound nonsyndromic SNHL, we identified 178 mutant alleles with 29 different sequence changes including 27 mutations and 2 polymorphisms (table 1). Approximately 90 recessive and 9 dominant *GJB2* mutations have been reported so far; in addition, some 50 sequence changes have been assessed as possible mutations or polymorphisms [Ballana et al., 2008].

All *GJB2* mutations in this study are recessive ones, with the possible exception of the p.V153I+p.R165W double mutation in *cis*, which occurred in combination with a wild-type allele in a profoundly deaf woman from Sri Lanka and her newborn daughter. First, the p.V153I+p.R165W double mutation was tentatively classified as dominant [Rickard et al., 2001], but later the same allele was also found in normal-hearing parents of affected individuals [Santos et al., 2005]. Unfortunately, the newborn was not available for hearing tests and our data do not clarify whether p.V153I+p.R165W is another recessive or a dominant mutation.

Different publications classified p.M34T as a nonpathogenic variant polymorphism [Feldmann et al., 2004; Ballana et al., 2008]. In our series, p.M34T represented the second most frequent *GJB2* alteration (12 alleles; vs. c.35delG, 102 alleles), and we identified 8 patients with mild to moderate hearing loss and biallelic mutations including p.M34T (table 3). This strongly supports the view that p.M34T is a weak recessive mutation [Snoeckx et al., 2005; Putcha et al., 2007]. Two other sequence variants, p.V27I and p.V153I, were only observed in combination with wild-type alleles, and may represent non-pathogenic polymorphisms [Roux et al., 2004].

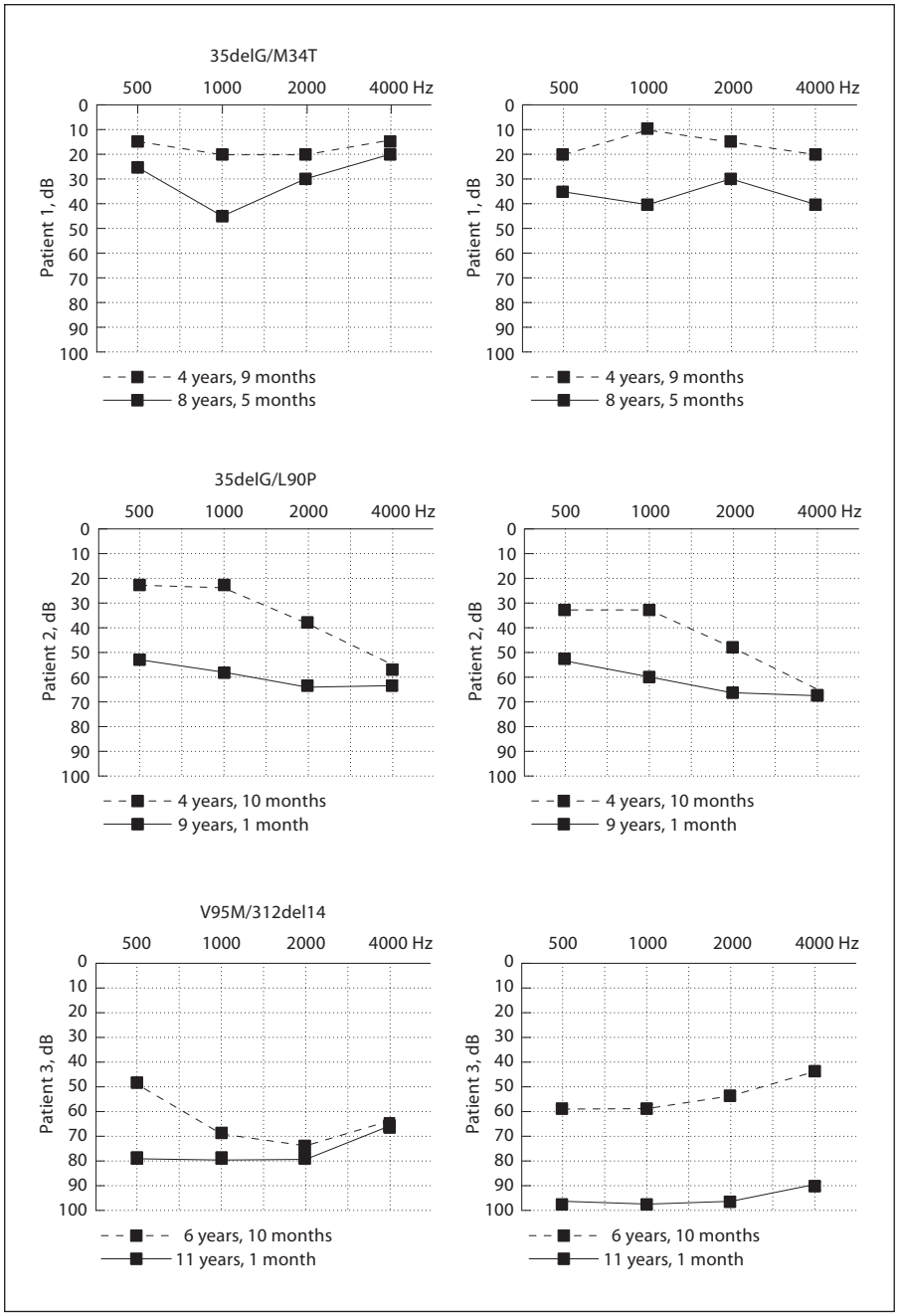

Fig. 3. Bilateral audiometric data from 3 of 10 patients with *GJB2* mutations and definitive progression of hearing loss.

The 29 sequence changes in our patient population comprised 11 truncating mutations (119 alleles, including 2 with the 309-kb deletion of the *GJB6* gene), 18 nontruncating mutations (52 alleles, including 2 with p.V153I+p.R165W), and 2 polymorphisms (7 alleles). The *GJB2* mutations p.Q7P (c.20A→C, present on 1 allele) and p.H100Q (c.300T→A, 1 allele) are first described here. In an earlier paper, we already demonstrated pathogenicity of the novel mutation p.N14D (c.40A→G; 2 alleles in siblings) [Haack et al., 2006]. The p.Q7P and p.N14D mutations occurred in combination with a p.M34T and a c.35delG mutation, respectively, and were associated with mild hearing impairment (table 3); evidently, these are recessive mutations. p.H100Q was identified in combination with a wild-type allele in a 4-year-old girl with moderate hearing loss. We could not formally prove the patho-

genicity of this sequence change, but other alterations at this residue such as p.H100L, p.H100P, and p.H100Y represent known recessive mutations [Green et al., 1999; Snoeckx et al., 2005; Putcha et al., 2007; Ballana et al., 2008].

The frequency of 21.2% (71/335) of biallelic *GJB2* mutations in our patient population is well within expected limits. Of the 26 observed allele combinations (genotypes), 10 were all T/T, 8 were NT/NT, and 8 T/NT (table 2). Audiograms indicated a more pronounced hearing loss of biallelic T/T mutations, compared to T/NT and NT/NT mutations. This is consistent with a previous report on a large collection of patients from 16 different countries (Europe, Israel, the USA, and Australia) [Snoeckx et al., 2005] and another multicenter study from North America [Putcha et al., 2007].

Interestingly, there have been reports that *GJB2* deafness may be non-penetrant at birth in a subset of cases and progressive [Ravecca et al., 2005; Norris et al., 2006; Welch et al., 2007]. Based on observations in 7 individuals with homozygous c.35delG mutations and a meta-analysis of 7 hearing loss studies, Gopalarao et al. [2008] estimated the frequency of *GJB2* cases with a progression of hearing loss to be approximately 20%. Our findings support these data. Ten of 37 patients with biallelic *GJB2* mutations and at least 2 audiograms at different ages demonstrated progression of >15 dB (fig. 3), and therefore the subset of patients with initially nonprofound hearing loss and progression amounted to 27% in this study. The median hearing loss in the patients showing progression was 5.5 dB per year.

Interestingly, the monoallelic presence of a mutated *GJB2* allele, without a second detectable mutation, was identified in 29 out of 335 patients (8.7%) [c.35delG mutation only; 10 out of 335 individuals (3%)], clearly exceeding the carrier frequencies in central and northern European populations with similar ethnicities. Gasparini et al. [2000] detected the 35delG mutation in 23 out of 1826 (1.25%) unrelated random subjects in central and northern Europe ($\chi^2 = 5.60$, df = 1, $p < 0.02$ as compared to this study), and Janecke et al. [2002] reported a carrier frequency for all *GJB2* mutations in western Austria of approximately 1/77 (1.3%), and the 35delG mutation in 11 out of 1212 (0.9%) random controls ($\chi^2 = 8.45$, $p < 0.005$ compared to this study).

Further, the clinical relevance of apparently monoallelic *GJB2* mutations (T/wild-type and NT/wild-type) represents a novel observation. In this study, T/wild-type mutations were associated with a severer hearing impairment than NT/NT and, by trend, T/NT mutations. Similarly, patients with NT/wild-type mutations were more severely affected than patients with NT/NT mutations (fig. 2). Our data suggest that only a subset of the subjects (by estimate ~40% in this study) with nonsyndromic SNHL and monoallelic *GJB2* mutations (identified by present techniques) are heterozygous carriers of *GJB2* mutations by chance coincidence. In the remaining patients, we assume the presence of additional recessive mutations that remain undetected by current *GJB2* mutation and *GJB6* deletion analyses. These patients provide an excellent starting material for future genetic analyses on *GJB2* promoter methylation or monoallelic silencing, splice mutations, and/or other connexin genes. We identified only 2 individuals (0.6%) showing *GJB2*/*GJB6* digenic inheritance. This is consistent with previous reports that *GJB6* deletions are rare except in populations originating from Spain [del Castillo et al., 2002; Snoeckx et al., 2005; Putcha et al., 2007]. Possibly, mutation analyses of other genes causing nonsyndromic deafness such as *GJB1*, *GJB3*, and *GJA1* could also be worthwhile in patients showing only one *GJB2* mutation.

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