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Short Report

DFNB16 is a frequent cause of congenital hearing impairment: implementation of *STRC* mutation analysis in routine diagnostics

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Increasing attention has been directed toward assessing mutational fallout of stereocilin (*STRC*), the gene underlying DFNB16. A major challenge is due to a closely linked pseudogene with 99.6% coding sequence identity. In 94 *GJB2/GJB6*-mutation negative individuals with non-syndromic sensorineural hearing loss (NSHL), we identified two homozygous and six heterozygous deletions, encompassing the *STRC* region by microarray and/or quantitative polymerase chain reaction (qPCR) analysis. To detect smaller mutations, we developed a Sanger sequencing method for pseudogene exclusion. Three heterozygous deletion carriers exhibited hemizygous mutations predicted as negatively impacting the protein. In 30 NSHL individuals without deletion, we detected one with compound heterozygous and two with heterozygous pathogenic mutations. Of 36 total patients undergoing *STRC* sequencing, two showed the c.3893A>G variant in conjunction with a heterozygous deletion or mutation and three exhibited the variant in a heterozygous state. Although this variant affects a highly conserved amino acid and is predicted as deleterious, comparable minor allele frequencies (MAFs) (around 10%) in NSHL individuals and controls and homozygous variant carriers without NSHL argue against its pathogenicity. Collectively, six (6%) of 94 NSHL individuals were diagnosed with homozygous or compound heterozygous mutations causing DFNB16 and five (5%) as heterozygous mutation carriers. Besides *GJB2*/*GJB6* (DFNB1), *STRC* is a major contributor to congenital hearing impairment.

Conflict of interest

The authors have no conflicts of interest to disclose.

Key words: chromosome 15q15.3 – congenital hearing impairment – deafness-infertility syndrome (DIS) – DFNB16 – non-syndromic hearing loss (NSHL) – *STRC*

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Hearing impairment is an extremely heterogeneous disorder affecting approximately 1 of 1000 newborns (1). At present, 42 genes and 69 loci (http://hereditaryhearingloss.org) are implicated in non-syndromic autosomal recessive deafness (locus notation DFNB). In the European population, 20–40% of non-syndromic hearing loss (NSHL) is due to mutations in *GJB2* (MIM: 121011) and *GJB6* (MIM: 604418), together comprising the DFNB1 locus (2). With few exceptions, autosomal-recessive NSHL has similar manifestations, wherein hearing loss is severe to profound with prelingual onset (3).

An initial candidate gene approach assigned *STRC* (MIM: 606440) to chromosome 15q15.3 encompassing the DFNB16 locus (4). Stereocilia form crosslinks necessary for longitudinal rigidity and outer hair cell structure, and upon mechanical deflection, stereociliary transduction sensitive channels open for cellular

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depolarization (5, 6). Reverse transcriptase polymerase chain reaction (RT PCR) from several mouse tissues showed strong, nearly exclusive expression in the inner ear (4) and upon knockout, these key structures were absent (7).

STRC deletion frequencies of >1% have been calculated in mixed deafness populations (8, 9) and the incidence of *STRC* hearing loss is an estimated 1 in 16,000 (10). Accumulating evidence suggests that DFNB16 constitutes a significant proportion of the otherwise genetically heterogeneous etiology comprising NSHL. One challenge impeding diagnostic implementation of *STRC* screening is the presence of a non-processed pseudogene with 98.9% genomic and 99.6% coding sequence identity (9) residing less than 100 kb downstream from *STRC* in a region encompassing a segmental duplication with four genes, *HISPPD2A* (MIM: 610979), *CATSPER2* (MIM: 607249), *STRC*, and *CKMT1A* (MIM: 613415). Apart from *CKMT1A*, these pseudogenes have mutations rendering them inactive (10). In the case of *pSTRC*, inactivity is due to a nonsense mutation in exon 20 (4). Homozygous deletions of *STRC* and *CATSPER2* result in deafness infertility syndrome (DIS; MIM: 611102), characterized by deafness in both males and females, and exclusive male infertility, as *CATSPER2* is required for sperm motility. Not only is it challenging to generate accurate sequencing data without pseudogene inclusion, it is even more difficult interpreting such data without the usual reliable resources for mutation interpretation, as these databases are 'polluted' with pseudogene data as well.

Materials and methods

The study was approved by the Ethics Committee at the Medical Faculty of Würzburg University. Informed written consent was obtained from all participants/parents.

Subjects

Our study cohort consisted of primarily pediatric individuals. Patients 1–94, with NSHL were recruited through the Comprehensive Hearing Center at Würzburg University Hospital. All patients had mild to profound sensorineural hearing loss (SNHL). Although study participants were counselled primarily for NSHL, additional symptoms were found in a limited minority. Patient 95 with syndromic SNHL was recruited through Charité Universitätsmedizin Berlin. Genomic DNA (gDNA) was extracted from whole blood using standard salt extraction methods.

STRC copy number counting

Individuals 1–93 were screened for copy number variations (CNVs) using the Omni1-Quad v1.0 array (Illumina, San Diego, CA) and analyzed using GenomeStudio version 2011.1. CNV calling was performed with QuantiSNP 2.2 (11) and cnvPartition 3.2.0 (Illumina). Syndromic patient 95 was tested by array CGH using the Agilent 4x180K (Agilent Technologies, Santa Clara, CA) platform. Individual 94 was tested for *STRC* CNVs by quantitative real-time PCR (qPCR), using unique *STRC* exon 22 primers excluding the pseudogene (Table S1, Supporting Information; exon 22 primers without M13 tags) and the SensiMix SYBR Green Kit (Bioline, Luckenwalde, Germany).

Primer design and Sanger sequencing of *STRC*

To exclude pseudogene sequences, two long-range (LR) PCR products were generated for subsequent nested PCR. Primers (Table S1) were designed using Primer3 (version 0.4.0) software (12) or obtained from the literature (9). The RefSeq *STRC* sequence annotation corresponds to NM_153700.2 and Ensembl ENSG00000166763 (hg 18). *STRC* and *pSTRC* sequences were aligned in UCSC Genome Browser (http://genome.ucsc.edu). *STRC*-specific sequences were verified using BLAT. Although confined to few divergent bases up- and downstream from *STRC*, we targeted these regions for LR-PCR primer design, placing the divergent nucleotides at the terminal 3′ end if possible (Fig. S1).

LR-PCR was performed with the Qiagen LongRange PCR Kit (Qiagen, Hilden, Germany) using cycling profiles in Table S2. Amplification products were diluted 1:1000 to reduce pseudogene carryover from gDNA and then used for nested PCR. A sequencing control in intron 18 overlapping with both LR products was included for pseudogene exclusion confirmation. Nested PCRs (Table S2) and sequencing continued after LR-PCR products were verified negative for a three-nucleotide frameshift, indicative of *pSTRC* sequence.

Bidirectional sequencing, performed with an ABI 3130xl 16-capillary sequencer (Applied Biosystems, Carlsbad, CA), was analyzed using Gensearch (Phenosystems, Lillois Witterzee, Belgium) and CodonCode Aligner (CodonCode, Dedham, MA). SIFT (13) and PolyPhen-2 (14) predicted amino acid substitution and disease causing potential.

Results

Individuals 1–93 were run on Illumina Omni1-Quad microarrays. We identified 2 cases with homozygous deletions, 5 with heterozygous deletions, and 10 with copy-neutral loss of heterozygosity (LOH) (Fig. 1; Table S3). Using the Agilent 4x180K array, we detected an additional homozygous deletion in syndromic patient 95. None of these individuals displayed disease-relevant CNVs elsewhere. The homozygous deletions were verified via PCR in exon 22 and the heterozygous deletions via qPCR. By qPCR, we also detected heterozygous deletions in both parents of the homozygous patients 1 and 95. Individual 94 did not have a microarray performed to simulate a diagnostic setting for NSHL patients where copy number counting is performed by qPCR. This individual showed a heterozygous

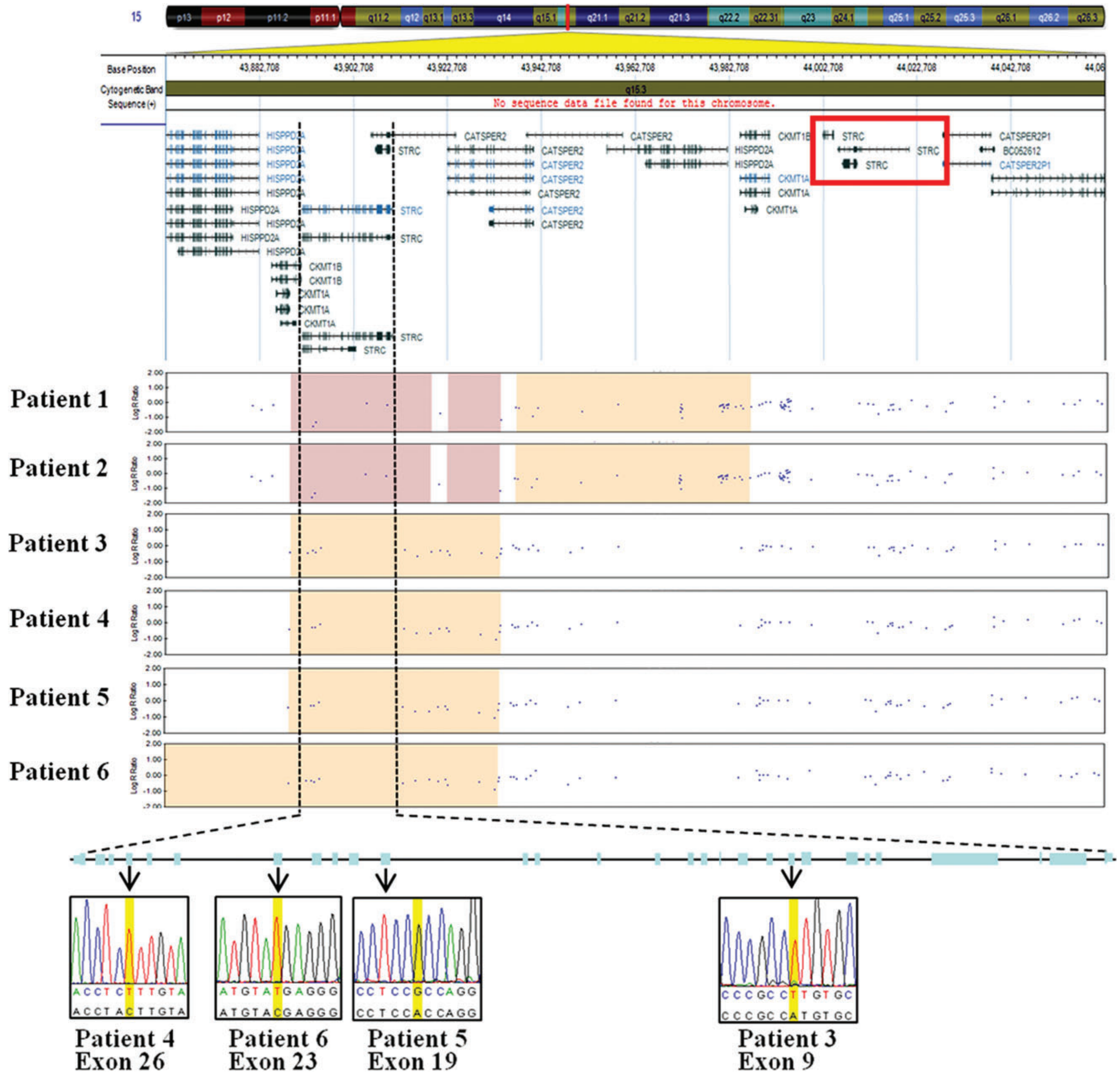

Fig. 1. Overview of patients with biallelic mutations in *STRC*. The upper part of the figure shows a map of the analyzed region. *pSTRC* transcripts are boxed in red. Illumina Omni1-Quad array data in the middle depict deletions in relation to the *STRC* and *pSTRC* genes. Regions with altered signal intensity are marked in pink representing homozygous deletions and orange indicating heterozygous deletions. The lower part of the figure shows Sanger sequencing chromatograms of the four heterozygous deletion patients with hemizygous sequence changes in relation to exonic position within the gene.

deletion, yielding a combined six heterozygous deletions (Table S3).

Thirty-six NSHL individuals, including six heterozygous deletion, 10 LOH and 20 cases without deletion or LOH, were selected for subsequent Sanger sequencing. Of the six heterozygous deletion carriers, three (nos. 3, 4, and 6) presented hemizygous pathogenic *STRC* mutations following pseudogene exclusion, with an additional patient (no. 5) exhibiting a heterozygous deletion in conjunction with a candidate mutation c.3893A>G (Fig. 1; Table 1). Table S4 summarizes PolyPhen-2 and SIFT prediction outcomes. None of the 10 individuals with copy-neutral LOH exhibited a homozygous pathogenic mutation; one (no. 16) had a heterozygous mutation. Of the 20 individuals without microdeletion or LOH, 1 (no. 24) displayed compound heterozygous pathogenic mutations, 1 (no. 25) a pathogenic mutation in conjunction with the homozygous c.3893A>G variant, and 3 (nos. 22, 23, and 26) heterozygous c.3893A>G variants (Table 1). The remaining cases were mutation negative. The minor allele frequency (MAF) of the c.3893A>G variant in our NSHL cohort is 9%. In 100 normally hearing adults, we identified 18 heterozygous and 2 homozygous

Table 1. SNHL individuals with *STRC* deletions and/or sequence changes in respective exons

No.	Allele 1	Allele 2	Exon	Interpretation
DFNB16 patients				
1, 2, 95	*STRC* gene deletion	*STRC* gene deletion		Homozygous deletions
3	*STRC* gene deletion	c.2726A>T, p.H909L	9	Heterozygous deletion and hemizygous pathogenic mutation
4	*STRC* gene deletion	c.4918C>T, p.L1640F	26	Heterozygous deletion and hemizygous pathogenic mutation
6	*STRC* gene deletion	c.4402C>T, p.R1468X	23	Heterozygous deletion and hemizygous pathogenic mutation
24	c.2303_2313+1del12, p.G768Vfs*77	c.5125A>G, p.T1709A	6, 28	Compound heterozygous pathogenic mutations
Heterozygous deletion, mutation, and variant carriers				
5	*STRC* gene deletion	c.3893A>G, p.H1298R	19	Heterozygous deletion and hemizygous variant
7, 94	*STRC* gene deletion	Normal		Heterozygous deletion
25	c.2640G>T, p.E880D; c.3893A>G, p.H1298R	c.3893A>G, p.H1298R	8, 19	Heterozygous pathogenic mutation and homozygous variant
16	c.5180A>G, p.E1727G	Normal	28	Heterozygous pathogenic mutation
22, 23, 26	c.3893A>G, p.H1298R	Normal	19	Heterozygous variant

variant carriers, corresponding to an MAF of 11%. Orthologous alignments illustrate strong evolutionary conservation in mutated positions, including the recurrent variant c.3893A>G (Fig. S2).

All patients with biallelic mutations underwent clinical evaluation and, with few exceptions, had audiogram(s) available (Fig. 2). Audiological, clinical and family history descriptions are detailed in Table 2. Besides the 7 DFNB16 patients here, 32 additional patients with biallelic *STRC* mutations (including 13 cases from four families) have been published so far (8–10, 15, 16) (Table S5). Many of these patients have sloping high-frequency audiometric profiles and together show an age of onset spectrum ranging from birth to childhood.

Discussion

We analyzed a cohort of 94 NSHL and one syndromic patient and determined three homozygous and six heterozygous *STRC* deletions. Deletions of 30 kb (two cases), 45 kb (four cases) and 82 kb (two cases) are recurrent (Fig. 1; Table S3), suggesting non-homologous recombination events (17) between highly similar short DNA elements in chromosome 15q15.3. The homozygous *STRC* deletions described here extend into *CATSPER2* and are responsible for male DIS, contributing to the limited cases in the literature (Table S5). Two of the three homozygous deletion patients are pre-pubertal boys unevaluated for fertility. One of them (no. 95) displayed congenital abnormalities and comorbidities (Table 2), which are probably independent of DFNB16. Three of the six heterozygous deletion patients exhibited hemizygous pathogenic mutations in the second allele, consistent with DFNB16. Among 10 patients with LOH >1 Mb, we identified a single heterozygous mutation, indicating that at least small stretches of LOH are not useful predictors of homozygous *STRC* mutations. Among 20 patients without heterozygous deletion or LOH, one exhibited biallelic mutations. Although microdeletions are the most frequent mutation type, Sanger sequencing for the detection of point mutations or smaller intragenic deletions/duplications is mandatory in all SNHL patients displaying appropriate DFNB16 audiogram configurations.

There are different methods for *STRC* CNV detection. Multiplex ligation-dependent probe amplification (MLPA) (10) and qPCR successfully distinguish copy numbers, but are limited to small non-homologous regions harboring divergent nucleotides. The Illumina SNP array employed here covers *STRC* with seven single-nucleotide polymorphism (SNP) probes (three of them lacking 100% identity with *pSTRC*), which is conducive to CNV detection using standard diagnostic reporting algorithms. Array CGH similarly shows adequate resolution to detect a 45 kb deletion.

One previous study (9) employed a Sanger sequence approach to detect small sequence changes, but was unable to differentiate the *STRC* gene from the pseudogene, which is a drawback we have overcome. There are limited divergent nucleotides between *STRC* and *pSTRC* toward the 3′ portion of the gene. The absence of these in our LR exon 12–29 sequences confirmed specificity. In addition, we implemented an LR-PCR control in intron 18, whereby a three-nucleotide frameshift is present if *pSTRC* is amplified. This control verifies pseudogene exclusion for each LR-PCR, since this region overlaps with both LR products. This is important because *pSTRC* amplifies with unoptimized annealing temperatures and unintended gDNA carryover.

Interpretation is especially challenging for *STRC* analysis since we cannot rely on customary mutation and allele frequency data. Thousand Genomes Project and dbSNP index variants from Next Generation

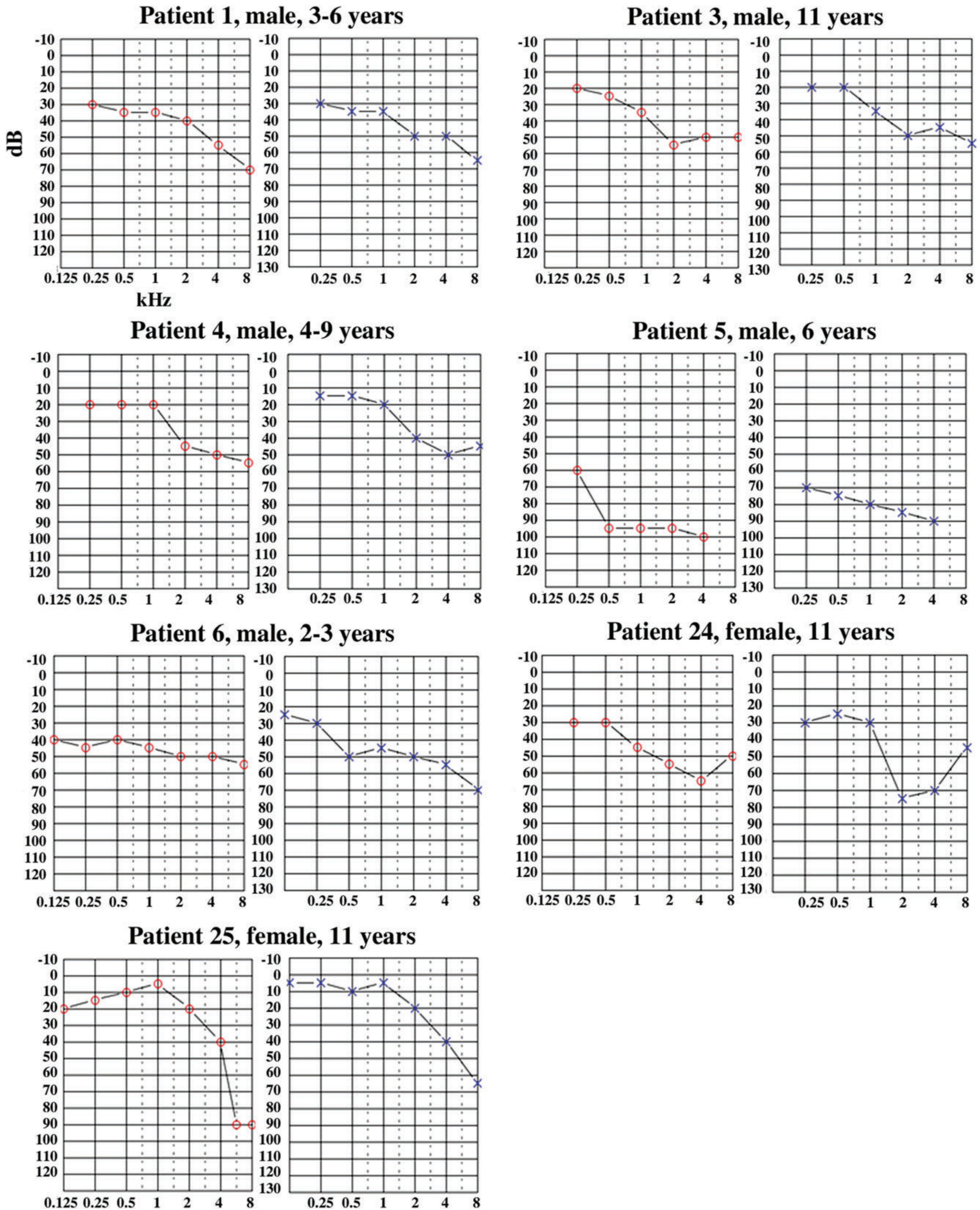

Fig. 2. Bilateral pure tone audiograms from individuals with biallelic *STRC* mutations. Above the audiograms are the patient number, sex, and age. Circles in the audiograms represent the right, and crosses, the left ear, respectively. If multiple audiograms were present, an age range is listed and the average of all thresholds is depicted. Created with AudiogramMaker software (http://www.jacobhaskins.com/).

Table 2. Audiological, clinical and family histories of biallelic *STRC* mutation patients

No.	Onset	Audiological description[a]	Clinical description	Family history of HL
Patients with homozygous *STRC* deletions				
1	2 years	HL in all frequencies with mild HL in low- to mid-frequencies until 4 kHz, moderate to severe HL to 8 kHz	No additional symptoms	None; parents are confirmed heterozygous deletion carriers
2	Childhood	High tone HL, no further description available	Delayed speech and language development	Sister has HL since birth; two additional sisters are without HL; maternal grandmother and paternal grandfather have reported age-related HL
95	Birth	Auditory brainstem response measurements from the newborn hearing test indicated maximal thresholds of 65 dB	Born in the 35th week of gestation presenting facial dysmorphisms (long eye lashes, flat nasal bridge, and epicanthus), hypoplastic widely spaced nipples, atrial septal defect, and delayed speech development; also diagnosed with hydroxylysinuria, hydroxylysinemia, and severe recurrent infections	No family history of HL; parents are first-degree cousins and confirmed heterozygous deletion carriers; one sibling died at 3 months because of an infection of unknown etiology
Patients with *STRC* deletion and pathogenic DNA sequence mutation				
3	5 years	Audiogram from age 10 indicated moderate HL, mildly sloping at higher frequencies	Oral motor skills and vocabulary developed normally per age; bifid uvula and sigmatism interdentalis diagnosed at 5 years of age; underwent intensive ambulant therapy for 1 year for dyslalia	Family history of HL
4	3 years	High frequency HL with normal hearing thresholds until 1 kHz and mild to moderate HL from 2 to 8 kHz	None available	None
6	Birth	Mild to middle grade HL; free field and PTA between 2 and 4 years of age demonstrated mild HL in all frequencies and sloping audiometric profiles, more pronounced between 2 and 8 kHz	Hypothyroidism with HL prompted Pendred syndrome screening with *SLC26A4* mutation negative result	None
Patient with compound heterozygous DNA sequence mutations				
24	6 years	PTA with sloping high frequency configuration in the right ear and cookie bite pattern in the left ear	No additional symptoms	Father and paternal uncle have mild HL; parents are first-degree cousins with two additional normally hearing children

HL, hearing loss; PTA, pure tone audiometry.
[a]All patients are bilaterally affected.

Sequencing (NGS) platforms that generate amplicon libraries indiscriminate of pseudogene counterparts. A well-rounded approach for *STRC* mutation assessment calls for consideration of evolutionary conservation of variants (18), as well as utilizing audiograms as helpful diagnostic tools, since high-frequency sloping appears a uniting feature of DFNB16. The c.3893A>G variant, which is predicted to be deleterious, was found with comparable MAFs (around 10%) in SNHL individuals and controls. Although we cannot entirely exclude the formal possibility that c.3893A>G in conjunction with an *STRC* deletion or pathogenic mutation contributes to SNHL, it should be considered as non-pathogenic as long as functional analyses are missing.

Conclusions

Our data confirm that *STRC* biallelic mutations significantly contribute to NSHL, particularly in children with mild to moderate hearing impairment with greater affection in higher frequencies. The frequency of DFNB16 in children with NSHL may be even higher than 6%

(6 of 94), considering we did not sequence all patients without *STRC* deletion. Gathering evidence implies that in addition to *GJB2*/*GJB6*, mutation analysis of *STRC* should be implemented as part of routine differential diagnostics for NSHL. Unfortunately, targeted NGS of deafness genes or exome sequencing does not reliably detect *STRC* mutations. As the prevalence of heterozygous deletion carriers at this locus is high, incidental CNVs could be detected in diagnostic and prenatal cases requiring microarray analysis. Initiation of mutational screening in *STRC* should be indicated in these cases for the detection of possible mutations in trans. The presentation of our sequencing assay allows the full disclosure of *STRC* mutations that will translate to improved NSHL diagnostics.

Supporting Information

The following Supporting information is available for this article:

Fig. S1. Long-range primer selection based on divergent nucleotides existing between *pSTRC* (top sequence) and *STRC* (bottom sequence). Dots represent deleted nucleotides; vertical dashes identical nucleotide bases. Primer sequences are boxed in red; divergent nucleotides due to deletions or divergent sequences are boxed in black. The upper part presents the long-range primers amplifying exons 1–19; the lower part, the primers amplifying exons 12–29.
Fig. S2. Conservation of STRC residues in mutated positions. Human wild-type residues are aligned against those of 36 species. Blosum62 coloring was used to notate conservation levels. The analyzed residue is highlighted in dark blue. If residue and consensus sequences match, they are colored in medium blue. If they do not match but they have a positive Blosum62 score indicating weaker conservation, then they are colored in light blue. Gaps are marked with a dot. Annotation tracks were obtained from PolyPhen-2.
Table S1. Primers for *STRC* long-range PCR, nested PCR, and Sanger sequencing
Table S2. PCR cycling information
Table S3. Patients with *STRC* deletions or copy neutral LOH
Table S4. *STRC* sequence changes with in silico predictions
Table S5. Summary of patients with biallelic *STRC* mutations (DFNB16) listed in publications to date

Additional Supporting information may be found in the online version of this article.

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