Two truncating USH3A mutations, including one novel, in a German family with Usher syndrome

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Purpose: To identify the genetic defect in a German family with Usher syndrome (USH) and linkage to the USH3A locus.

Methods: DNA samples of five family members (both parents and the three patients) were genotyped with polymorphic microsatellite markers specific for eight USH genes. Three affected family members underwent detailed ocular and audiologic characterization.

Results: Symptoms in the patients were compatible with Usher syndrome and show intrafamilial variation, for both hearing loss (ranging from severe to profound with non-linear progression) and vision. Genotyping of microsatellite markers for the different USH loci was in line with a defect in the USH3A gene on chromosome 3q25. Sequence analysis of the USH3A gene revealed two truncating mutations: c.149_152delCAGGinsTGTCCAA T, which has been described previously, and a novel mutation, c.502_503insA, segregating with the phenotype.

Conclusions: To date, only 11 USH3A mutations have been described. This is the first description of a German family with USH due to USH3A mutations, including one novel. Our findings indicate that also in the Central European population, USH3A mutations should be considered in cases of USH.

Usher syndrome (USH) is an autosomal recessive condition characterized by sensorineural hearing loss, vestibular dysfunction, and visual impairment due to retinitis pigmentosa (RP). It is the leading cause of deafblindness, with a general prevalence of 2.6-2.100,000 [1,2]. There are three clinical subtypes, with type 1 (USH1) representing the most severe subtype with profound congenital deafness, vestibular dysfunction, and prepubertal onset of RP. USH type 2 (USH2) is characterized by moderate to severe hearing impairment, and onset of RP in the first or second decade of life. Vestibular function is not impaired in this subtype. USH type 3 (USH3) presents with progressive, post-lingual hearing loss, RP of variable onset and severity, and with or without vestibular impairment. To date, nine causative USH genes have been identified [3-16].

Following the initial description of the USH3A gene [12], additional transcripts were identified and the genomic structure of USH3A was updated: the longest isoform consists of three coding exons (exons 0, 2, and 3 in Adato et al. [4]) and encodes clarin-1, a 232 residue four-transmembrane domain protein of the clarin protein family, comprising only three members [4,17]. Homology to stargazin, a protein from the cerebellar synapse, suggests a role for clarin-1 at hair cell and photoreceptor cell synapses. A C-terminal TNV-peptide signature constitutes a putative PDZ-binding motif that could eventually tie clarin-1 to the network of USH1 and USH2 proteins [4].

Compared to other USH genes, USH3A mutations are a rare cause of Usher syndrome outside Finland, where the USH3 subtype accounts for roughly 40% of USH cases due to a founder effect [18]. Moreover, there is a high prevalence of the p.N48K mutation among Ashkenazi Jews [19]. To date, only 11 mutations have been published [4,12,17,20,21]. USH3A mutations cause a wide spectrum of phenotypes, and some cases are clinically compatible with USH1 [22].

In this study, we genetically evaluated a German family with USH and established linkage data compatible with the USH3A locus.

METHODS

Patients: This study was approved by the ethics committee of the University Hospital of Cologne, and written informed consent was obtained from all five participants from the family investigated herein. DNA from 106 healthy Caucasian control individuals (all had negative family history for Usher syndrome) was extracted from EDTA blood (10 ml). Audiometric examination of the three patients consisted of otoscopic exploration, pure-tone and speech audiometry. Testing of the vestibular system was done by electronystagmography (ENG). Ocular examinations included measurement of visual acuity, fundus ophthalmoscopy, visual field examination, color vision testing, course of dark adaptation, Ganzfeld-electroretinogram (ERG) and multifocal ERG (mfERG; II:2, and II:3) according to ISCEV-standards. DNA samples were available from the three patients and both parents.

Genotyping/linkage analysis: DNA was isolated as follows: 5-10 ml of EDTA-blood were added with lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA in aqua dest., pH 7.4) to a total volume of 40 ml and incubated on ice.
for 15 min. After centrifugation (15 min, 1,500 rpm, 4 °C), pellets were resuspended in 5 ml nucleus lysis buffer (10 mm Tris, 400 mM NaCl, 2 mM EDTA in H2O, pH 8.2). After addition of 330 µl 10% SDS and 250 µl proteinase K (20 mg/ml in H2O), the solution was incubated as 37 °C over night. After addition of 1,66 ml saturated NaCl (about 6 M), supernatants were centrifuged twice (10 min, 2,683 xg, room temperature). DNA was precipitated by adding 7 ml isopropanol to the supernatant, washed in 70% ethanol and solved in 400 µl TE.

PCR fragments of polymorphic microsatellite markers were amplified using the tailed primer method [23]: A universal oligonucleotide tail (5’-CAT CGC TGA TTC GCA CAT-3’) was added to the 5’-part of the sequence-specific primer (either the forward or reverse primer) for automatic fragment analysis. The amplification was performed using three oligonucleotides: (1) tailed locus-specific primer (tailed F or R), (2) un-tailed locus-specific primer (F or R) and (3) labeled tail corresponding to the 5’-tail sequence of the tailed locus-specific primer. PCR fragments were then analysed on an ABI-377 DNA sequencer (Applied Biosystems, Foster City, CA).

Genotyping for eight USH gene loci was done using locus-specific microsatellite markers: MYO7A_1VS15_CA for USH1B, D11S902 and D11S4130 for USH1C, D10S1759 and D10S1694 for USH1D, D10S1643 and D10S546 for USH1F, D17S1535 and D17S1839 for USH1G, D1S474, USH2A_1VS24_CA, USH2A_1VS44_CA, and D1S490 for USH2A, D5S618 and D5S1463 for USH2C, and D3S3022, D3S1299, D3S1315, D3S1279, and D3S4531 for USH3A. Data provided by the Genome database were used as reference for allele sizes. Aside from those known markers, we identified two intragenic polymorphic repeat markers for the USH2A locus, USH2A_IVS24_CA and USH2A_IVS44_CA, and amplified them with primers USH2A_IVS24_CA-F (5’-GGA GAA GGA TAT AGT TGA ATC CTC-3’), USH2A_IVS44_CA-R (5’-CTT AGT ATC AGG GTA TTC TCT CC-3’), USH2A_IVS44_CA-F (5’-GTT TTC CAG TTT CAT CAA GGT TTTC-3’), and USH2A_IVS44_CA-R (5’-GCA GCA CAG TTC ACA ATT GTG-3’). We previously described the intragenic marker MYO7A_1VS15_CA [24]. A novel USH2 subtype (USH2D), which is defined by mutations in the DFNB31 gene, was described by us just recently [10] and was therefore not tested in the family presented herein.

Mutation analysis for the USH3A gene: The three coding exons [4,17] and adjacent intronic sequences of the USH3A gene were amplified with primers as given in Table 1 and se-

<table>
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<th>Exon</th>
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<tr>
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<td></td>
<td>R: GCCTCCCTTCTGCTCC</td>
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Coding sequence length and genomic amplicon size are given for each exon. Exon numbering is based on Adato et al. [4]. GenBank accession number: AF495720.
quenced. Numbering of exons is as in Adato et al. [4]. PCR was carried out following standard protocols DNA from 106 healthy Caucasian control individuals (all had negative family history for Usher syndrome) was extracted from EDTA blood (10 ml) and screened for the presence of the novel mutation described herein by direct sequencing. PCR products were amplified using 100 ng of genomic DNA in a 25 µl reaction mixture containing 10 pmol of forward and reverse primers, 0.2 mM dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, and 0.5 units of Taq polymerase (Invitrogen Corp.,

Figure 2. Fundus photographs from study patients. A: II:2 at 27 years of age, B: II:3 at 22 years of age, and C: II:4 at 22 years of age. Note slight waxy pallor of the optic disc (left column in A, B, and C), and thinning/loss of pigment epithelium with dense bone-spicule pigmentation in the middle periphery (right column in A, B, and C).
Carlsbad, CA). After initial denaturation at 95 °C for 4 min, 30 cycles were performed, which consisted of 95 °C for 1 min, 55-62 °C (depending on the fragment; details available on request) for 1 min, and 72 °C for 1 min, with a final extension step of 72 °C for 10 min for all exons. DNA mutation numbering of identified mutations was based on cDNA sequence of the longest isoform of *USH3A* [4], with +1 corresponding to the A of the ATG translation initiation codon (codon 1) in the respective reference sequence (GenBank accession number AF495717). For the description of sequence variations, we followed the recommendations of the Human Genome Variation Society (HGVS).

**RESULTS**

**Clinical assessment:** Three patients from the family investigated herein were diagnosed as having USH based on their clinical history and audiometric and ophthalmologic tests.

Hearing loss was first noted at five years of age in all three patients. Progression was noted the next year. Afterwards, the hearing loss was stable (1-4 kHz). Remarkably, hearing loss was profound in the monozygotic twins (II:3 and II:4), while it was severe in their older brother, II:2 (Figure 1). Neither clinical history (no delay in reaching motor milestones) nor ENG indicated vestibular dysfunction.

The retinal phenotype was progressive in all three patients. Fundus examinations showed slight waxy pallor of the optic disc, severe retinal degeneration as indicated by thinning and loss of pigment epithelium in combination with dense bone-spicule pigmentation as well as attenuated retinal vessels in the middle periphery (Figure 2). Night blindness was noticed.
at age of 17 (II:2; as represented by monophasic dark adaptation function) and 12 years of age (II:3 and II:4), respectively. Visual acuity was 0.8/0.7, 1.0/0.8, and 1.0/1.0 in II:2, II:3, and II:4, respectively. Visual fields (Goldmann targets III/4e) were significantly reduced to 5° concentric field and temporal island fields in II:3, and 5° and temporal island fields in II:4. The older brother (II:2) showed many paracentral and midperipheral absolute and relative scotomata but had maintained a surprisingly large nasal end temporal outer border up to 60° and 90°, respectively, for Goldmann target III 4e in both eyes. Automated static perimetry within 30° angle (Figure 3A-C) showed that visual field loss was more severe in II:4. The older brother (II:2) showed many paracentral and midperipheral absolute and relative scotomata but had maintained a surprisingly large nasal end temporal outer border up to 60° and 90°, respectively, for Goldmann target III 4e in both eyes. Automated static perimetry within 30° angle (Figure 3A-C) showed that visual field loss was more severe in

Figure 4. Multifocal electroretinograms of the right and left eye. A: II:3 at 22 years of age and B: II:2 at 26 years of age. The following abbreviations were used: left eye (LE) and right eye (RE). In contrast to II:3, there is a significant affection of macular function in II:2, again demonstrating intrafamilial variability and showing that retinal degeneration can involve the center, and even at an early stage.
Figure 5. Genetic data from the study family.  

A: Pedigree of the German USH3 family investigated in this study. DNA was available from all persons displayed except II:1. Alleles for five microsatellite markers specific for the USH3A locus are given. The position of the USH3A gene relative to the markers is indicated. The three patients share the same haplotypes.

B and C: Electropherograms for the heterozygous USH3A mutations c.149_152delCAGGinsTGTCCAA T and c.502_503insA, respectively. Deleted or inserted nucleotides are boxed.

D: Drawing of the USH3A protein and position of the mutations identified in this study. In contrast to II:3, there is a significant affection of macular function in II:2, again demonstrating intrafamilial variability and showing that retinal degeneration can involve the center, and even at an early stage. The star designates the putative PDZ-binding motif.
the twin brothers than in II:2, who is four years older and whose kinetic fields are shown in Figure 3D. Ganzfeld-ERG was assessed in all three patients. In each case, only cone flicker responses of less than 15% of the normal mean were recordable under photopic conditions while all other reponses were below noise level, a typical finding for patients with retinitis pigmentosa. In the multifocal ERG (mERG) recorded in II:3, an almost normal response was found in the central field (Figure 4A) in both eyes with remnant responses in the area up to 10° eccentricity, indicating that foveolar cones are not noticeably affected, except of a slightly prolonged latency. In contrast, in the older brother (II:2), such maintained central responses could not be recorded anymore (Figure 4B). Color vision, as assessed by the Panel D-15 test in II:3, revealed repeatedly a clear protan axis with multiple errors in the saturated as well as in the desaturated version of the test. In contrast, in the older brother (II:2), a mild tritan defect was seen without indication of protan errors.

**Linkage analysis for known USH genes and USH3A mutation screening:** Genotyping of microsatellite markers excluded all investigated loci except USH1F and USH3A (for USH3A locus, see Figure 5A). The USH2D locus was identified by us after completion of this study [10] and was therefore not tested. Genotypes of II:3 and II:4 for all loci were in line with the assumption of monozygotic twins (data not shown). Because of the small size of the USH3A gene compared to the USH1F gene, PCDH15, we started with sequence analysis of USH3A, revealing compound heterozygosity for two truncating mutations, c.149_152delCAGGinsTGTCCAAAT in exon 0, and c.502_503insA in exon 3, in all three patients (Figure 5B,C). While c.149_152delCAGGinsTGTCCAAAT has been described previously, c.502_503insA is novel. This novel mutation was absent in 106 healthy control individuals. The deletion/insertion mutation c.149_152delCAGGinsTGTCCAAAT was predicted to result in a truncated protein of 60 amino acids with the inclusion of 11 unrelated residues or in an unstable transcript. The insertion of an adenine after nucleotide position 503 of the coding sequence should lead to a shortened protein of 171 amino acids with the inclusion of four unrelated residues (p.Ile168AsnfsX5; Figure 5B-D), or an unstable transcript. Genotyping of the parents revealed that the mother carried c.149_152delCAGGinsTGTCCAAAT while the father was a carrier of c.502_503insA.

**DISCUSSION**

USH3A mutations have so far been described in patients from Finland [12], Spain [20], United States citizens with Scotch-Irish, Dutch, Jewish, and Swedish ancestry [17], Jews from Israel, Hungary, Russia, Spain, and Yemen [4,19], and in a French-Canadian patient [21]. In this report, we describe a novel USH3A mutation and the first case of USH3 in Central Europe. Both changes identified in the family investigated herein were predicted to result in a truncated protein or in an unstable transcript. In case of stable transcripts, the novel mutation, c.502_503insA, would lead to a truncation after the third transmembrane domain, whereas c.149_152delCAGGinsTGTCCAAAT would result in a protein truncated immediately behind the putative N-glycosylation site and the first transmembrane domain (Figure 5D).

The deletion/insertion, previously described in patients of Scotch-Irish and English ancestry, respectively [20], was transmitted by the mother in our case study. She descends from German parents from North Rhine-Westfalia. As there were no sufficient haplotype data available for the two previously published cases with this mutation, it is not possible to decide whether c.149_152 is a mutational hot spot or if this is a founder allele. The haplotypes in our family, however, provided data for comparison should other patients with this mutation be identified in future studies (Figure 5A).

The clinical presentation of the three patients showed significant intrafamilial variability in hearing loss and visual impairment. While the peripheral visual field was surprisingly well maintained in II:2 (Figure 3A), macular function in this patient was more severely affected than in his brother II:3 (see multifocal ERG recordings, Figure 4B). Digenic inheritance has been suggested for a previously reported family in which two brothers, who were homozygous for an USH3A mutation, displayed different USH phenotypes. In one brother, a severe phenotype (USH1) resulted from additional heterozygosity for a truncating MYO7A mutation [4,25]. Marker analysis for the USH1B locus showed that in our family, the twins inherited different MYO7A alleles than their brother who had a milder phenotype. Although this could reflect a similar constellation as reported by Adato et al., we think this is rather unlikely as the twins in our family did not have an USH1 phenotype.

As in previous descriptions of Finnish USH3 patients, patients in this study showed poor visual field function [26]. The lack of vestibular dysfunction, once considered a key criterion to distinguish between clinical diagnoses “USH2” and “USH3,” was in line with earlier reports (e.g. [27] describing vestibular dysfunction in only half of the tested patients) and exemplifies the difficulties in defining USH3 clinically. Plantinga et al. have pointed out that progressive sensorineural hearing impairment is highly variable, ranging from normal to moderate hearing impairment at young ages as seen in case of USH2A mutations to profound and even “USH1B-like” hearing impairment at more advanced ages. They point out a non-linear progression of hearing loss, with highest progression during the first two decades of life, gradually slowing down with further aging, which may be unique among USH patients [28]. The initially progressive hearing impairment in our family with subsequent stability is in line with those findings.

Although possibly rare, our results show that USH3A mutations have to be taken into consideration in patients with USH from Central Europe.

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