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ARTICLE

Partial *USH2A* deletions contribute to Usher syndrome in Denmark

This paper has been corrected since online publication and a corrigendum also appears in this issue

Shzeena Dad[1], Nanna D Rendtorff[2,3], Erik Kann[1], Anders Albrechtsen[4], Mana M Mehrjouy[2], Mads Bak[2], Niels Tommerup[2], Lisbeth Tranebjærg[2,3], Thomas Rosenberg[5], Hanne Jensen[5] and Lisbeth B Møller*[,1]

Usher syndrome is an autosomal recessive disorder characterized by congenital hearing impairment, progressive visual loss owing to retinitis pigmentosa and in some cases vestibular dysfunction. Usher syndrome is divided into three subtypes, USH1, USH2 and USH3. Twelve loci and eleven genes have so far been identified. Duplications and deletions in *PCDH15* and *USH2A* that lead to USH1 and USH2, respectively, have previously been identified in patients from United Kingdom, Spain and Italy. In this study, we investigate the proportion of exon deletions and duplications in *PCDH15* and *USH2A* in 20 USH1 and 30 USH2 patients from Denmark using multiplex ligation-dependent probe amplification (MLPA). Two heterozygous deletions were identified in *USH2A*, but no deletions or duplications were identified in *PCDH15*. Next-generation mate-pair sequencing was used to identify the exact breakpoints of the two deletions identified in *USH2A*. Our results suggest that USH2 is caused by *USH2A* exon deletions in a small fraction of the patients, whereas deletions or duplications in *PCDH15* might be rare in Danish Usher patients.

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INTRODUCTION

Usher syndrome (USH) is an autosomal recessive disorder characterized by congenital hearing impairment, progressive visual loss owing to retinitis pigmentosa (RP) and in some cases, vestibular dysfunction. USH accounts for 50% of the deaf–blind cases and the prevalence has been estimated to be 3.5–16.7 per 100.000.[1–4] USH is genetically and clinically a heterogeneous disorder. On the basis of the degree of hearing loss, onset of RP and effect on the vestibular function USH is clinically divided into three subtypes: USH1, USH2 and USH3. Patients with USH1 have severe to profound congenital hearing impairment, prepubertal onset of RP and vestibular dysfunction.[5] In USH2 patients, the hearing loss is moderate to severe, the onset of RP is pre- or postpubertal, and the vestibular function is normal. Patients affected with USH3 have a moderate to severe progressive loss of hearing. RP begins at puberty or later and vestibular function may be affected.[6,7]

To date, eleven genes for USH have been identified. Six genes have been identified for USH1 (OMIM 276900): *MYO7A* (OMIM 276903), *USH1C* (OMIM 605242), *PCDH15* (OMIM 605514), *USH1G* (OMIM 607696), *CDH23* (OMIM 605516) and *CIB2 (OMIM* 605564).[8] Three genes have been identified for USH2 (OMIM 276901): *USH2A* (OMIM 608400), *GPR98* (OMIM 602851) and *DFNB31* (OMIM 607084).[9] For USH3, two genes have been identified: *CLRN1* (OMIM 606397) and *HARS* (OMIM 614504).[10] Furthermore, the USH2 modifier gene, *PDZD7*, has been recognized.[11]

Sequence variants affecting the function of the *PCDH15* gene, located at chromosome 10q21-22, are identified in 11–19% of the USH1 patients.[12–14] Function-affecting variants in *PCDH15* may not only lead to USH1, but also to autosomal recessive nonsyndromic profound hearing impairment (*DFNB23*).[12]

Most patients with USH2, including patients from Denmark, have function-affecting variants in *USH2A*,[15–19] which is located on chromosome 1. Sequence variants in *USH2A* are also responsible for atypical Usher syndrome and recessive nonsyndromic RP.[20] Besides the frequent function-affecting variant, c.2299delG[18] in *USH2A*, a large number of private family-specific 'function-affecting', sequence variants have been identified in this gene (https://grenada.lumc.nl/LOVD2/Usher_montpellier/home.php?select_db=USH2A).

To date, only two published studies used multiplex ligation-dependent probe amplification (MLPA) to investigate for exon deletions and duplications in USH genes.[21,22] Investigation of *PCDH15* revealed two deletions of 55 kb (exon 3 including the flanking introns) and 82 kb (exon 4–6 including the flanking introns), respectively,[21] whereas investigation of *USH2A* revealed one duplication (from exon 4 to exon 13) and five deletions (exon 4, exons 22-23, exon 27, exon 40 or exon 70 respectively).[22]

Here we used MLPA to screen a cohort of 50 Danish USH patients (20 USH1 and 30 USH2) for exon deletions and duplications in *PCDH15* and *USH2A*.

[1]Clinical Genetics Clinic, The Kennedy Center, Rigshospitalet, University of Copenhagen, Glostrup, Denmark; [2]Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen, Denmark; [3]Department of Otorhinolaryngology, Head and Neck Surgery and Audiology, Bispebjerg Hospital/Rigshospitalet, Copenhagen, Denmark; [4]Department of Biology, Computational and RNA Biology, University of Copenhagen, Copenhagen, Denmark; [5]Department of Ophthalmology, The National Eye Clinic, Copenhagen University Hospital, The Kennedy Center, Glostrup, Denmark
*Correspondence: Dr LB Møller, Clinical Genetics Clinic, The Kennedy Center, Rigshospitalet, University of Copenhagen, Gl. Landevej 7, Glostrup 2600, Denmark. E-mail: Lisbeth.Birk.Moeller@regionh.dk
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MATERIALS AND METHODS

Patients

Fifty Danish unrelated probands diagnosed with USH were included in this study: 20 USH1 and 30 USH2 patients. The patients were diagnosed based on the diagnostic criteria for USH.[5]

The group of patients was unselected. All USH patients with DNA available at the National Eye Clinic for the Visual Impaired at the Kennedy Center were included. The majority of the patients had not previously been investigated for sequence variants in any USH genes: Thus only eight USH2 patients had two variants in *USH2A* affecting the function, three USH1 patients had one sequence variant in *MYO7A* affecting the function and four USH2 patients had one function-affecting variant in *USH2A* at the start of the study. The remaining 35 patients had no function-affecting sequence variants identified before this investigation. The eight patients with two sequence variants in *USH2A* were included, as patients with three different function-affecting sequence variants located in two different genes[19,23] as well as patients with three different sequence variants located in the same gene, including *USH2A*, have been published previously.[15,24]

The project was approved by the local ethics committee (H-3-2011-070) and carried out in accordance with the Declaration of Helsinki. Consent to genetic testing was obtained from the patients.

USH2A sequencing

Prior to MLPA, patient 1063 and 55555 were analyzed for sequence variants in *USH2A* by Sanger sequencing, as described previously.[17]

MLPA analysis

Genomic DNA was extracted from blood samples using standard methods. The MLPA *PCDH15* (P292-A2) kit and the *USH2A* kit (P361-A1, mix 1 and P362-A2, mix 2), respectively, were obtained from MRC-Holland (Amsterdam, Netherlands). The P292-A2 probe-mix contains two probes for exon 1 and 2, and one probe for each of the remaining 34 exons of *PCDH15* with the exception of exon 3, 13, 33 and 35. The two *USH2A* kits, P361-A1 and P362-A2, have overall probes for each of the 72 exons of *USH2A*. Details of probe sequences can be found at the company's website (https://www.mrc-holland.com). MLPA was performed according to the manufacturer's protocol using 50 ng of genomic DNA per reaction and 1 μl of each reaction product was separated on a POP7 polymer ABI 3130xl capillary sequencer (Applied Biosystems, Foster City, CA, USA). The results were analyzed by comparing the peak profiles using GeneMapper (Applied Biosystems).

Targeted next-generation sequencing of a panel of USH genes

Genomic DNA from patient 70353 was analyzed by targeted next-generation sequencing (NGS) of the USH genes *MYO7A*, *PCDH15*, *CDH23*, *USH1C*, *USH1G*, *USH2A*, *GPR98*, *DFNB31*, *CLRN1* and *PDZD7*. The targeted NGS analysis and initial bioinformatics data analysis were conducted by Otogenetics Corporation (Norcross, GA, USA) and delivered through DNAnexus.com together with a report of identified sequence variants. Identified variants were classified and annotated using Alamut v2.4 (Interactive Biosoftware, Rouen, France).

Affymetrix CytoScan HD analysis

The Affymetrix CytoScan HD array (AROS Applied Biotechnology AS, Aarhus, Denmark) was used to confirm the results obtained with MLPA. This array can detect 25–50 kb copy number changes with its 750 000 single-nucleotide polymorphisms (SNPs) and 1 900 000 nonpolymorphic (CNV) markers (Affymetrix, Santa Clara, USA). Data analysis was carried out with Affymetrix Chromosome Analysis Suite Software (ChAS). The data were normalized to the reference model (NA32.1) provided by Affymetrix (284 HapMap samples and 96 phenotypically healthy male and female individuals). The hidden Markov model available within the software package was used to determine the copy number states and to estimate the breakpoints. Thresholds of log2 ratio $\geqslant 0.58$ and $\leqslant -1$ were used to categorize altered regions as gains and losses, respectively. In order to exclude false-positive CNVs, only alterations that involved at least 10 consecutive probes for gains and 5 consecutive probes for losses were considered in the analysis. The confidence limit was set to 80%.

Next-generation2 mate-pair sequencing

Mate-pair libraries of DNA from patient 70353 and from patient 1063, respectively, were prepared using the Nextera Mate Pair Sample Preparation Kit (Illumina, San Diego, CA, USA) essentially according to the manufacturer's protocol. Libraries were quantified and ~40 million fragments from each library were paired-end sequenced (2×100 bp) on a HiSeq2000 (Illumina).

Molecular characterization of breakpoints identified by next-generation mate-pair sequencing

Read-pairs were aligned to the human genome (Hg19) using Burrows–Wheeler Aligner (http://bio-bwa.sourceforge.net/). Discordant reads were extracted and structural variations were identified using SVDetect.[25] The results allowed us to design primers for breakpoint spanning PCRs. In brief, genomic sequences flanking each of the breakpoints indicated by mate-pair analysis were extracted and masked for repetitive sequences using the UCSC Genomic Browser. The sequences were linked and primers were designed to amplify sequences spanning the breakpoints. PCRs were performed on genomic DNA from the respective probands and from normal controls. PCR conditions are available upon request. The primers used to identify the exon 4–10 deletion breakpoints were: 5′-TGATCTTCTCTAAAGCATCATGAGAGG-3′ and 5′-TGGGAGATA TCATCACAGCACAAAATGACA-3′, and those used to identify the exon 22–24 deletion breakpoints were: 5′-AGAACTCTGCCAATCCAGAGGTTCCCA-3′ and 5′-CGGTTTTGGAGGACAACTGCAGCTAAA-3′. The resulting breakpoint spanning sequencing data has been submitted to https://grenada.lumc.nl/LOVD2/Usher_montpellier/home.php?select_db=USH2A (patient IDs 1063 and 55555).

In order to search for microhomology and/or small deletions/insertions flanking the breakpoints, the junction sequences were aligned to the genomic sequence using Multalin (http://multalin.toulouse.inra.fr/multalin/). Variants are described according to *USH2A* accession number NM_206933.2 with +1 as A in the translation start codon. Exons are numbered according to NG_009497.1.

Relatedness mapping

The estimation of relatedness locally on the genome was carried out with 'identity by descent' (IBD) mapping based on SNP data from chromosome 1 around *USH2A* (CytoScan files) as published previously.[26] The CytoScan files were sorted and merged. Sites with missing data or annotation were discarded. The alleles were flipped to the plus strand. The data were merged with data from 112 unrelated CEU individuals (Individuals with European ancestry from the CEPH (Centre d'Etude du Polymorphisme Humain) Utah samples) from HapMap3. Non-overlapping sites were removed, leaving 47 7798 sites. Sites with a minor allele frequency below 5% were removed. Linkage disequilibrium (LD) was accommodated by conditioning on one of 50 previous SNPs with the largest amount of LD. The most likely combination of IBD states across the chromosome was estimated using the Viterbi algorithm.

RESULTS

A cohort of 50 Danish unrelated patients with Usher syndrome, 20 USH1 and 30 USH2, was investigated for exon duplications and deletions in *USH2A* and *PCDH15* by MLPA. All patients were investigated by both kits because of the well known clinical overlap between the different subtypes of USH.[27]

No deletions or duplications were identified in *PCDH15*. In contrast, there were abnormal relative peak areas at about 0.5 for several exons of *USH2A* in three USH2 patients, indicating the presence of a heterozygous deletion (Figure 1). According to the MLPA data, two patients had a deletion of exons 4–10 (patients 70353 and 55555) and one patient had a deletion covering exons 22–24 (patient 1063). Patients 55555 and 1063 also had other function-affecting sequence variants in *USH2A*, found by a previous Sanger

sequencing of *USH2A*. Thus, patient 55555 had the previously published variant c.2299delG, p.(Glu767Serfs*21) in heterozygous state.[17] Furthermore, we here identified the variant c.3309C>A, p.(Tyr1103*) in heterozygous state in patient 1063. This variant designated rs397518011 (http://www.ncbi.nlm.nih.gov/snp) has previously been classified as 'affects function' (HGMD Professional 2014.2, August 2014, (http://www.hgmd.org/). To search for a second function-affecting variant in the third patient, 70353, targeted NGS of USH genes led to the identification of the *USH2A* sequence variant c.10684G>T, p.(Glu3562*) in heterozygous state. This variant, also classified as 'affects function', has previously been identified as disease causing when present in a specific context in USH2 patients (HGMD Professional 2, August 2014, http://www.hgmd.org/). The results are summarized in Table 1.

CytoScan analysis of the three samples confirmed the deletions in *USH2A* in all three patients (Figure 1). The deletions in patients 70353, 55555 and 1063 were localized to chr1.hg19: g.216,473,521_216,535,711; chr1.hg19:g.216,473,521_216,536,791; and chr1.hg19:g.216,260,845_216,326,858, respectively.

To identify the exact deletion breakpoints, we performed next-generation mate-pair sequencing on genomic DNA from patients 70353 and 1063 (Supplementary Figure 1). We were able to design PCR primers that amplified across the deletion breakpoints using DNA from the two patients. Subsequent sequencing identified the respective breakpoints (Figure 2). At the protein level, the deletion of *USH2A* exons 22–24 is predicted to be in frame p.(Gly1543_Pro1662del). The deletion of exons 4–10 in patient 70353 partially deleted exon 4 (including the MLPA probe ligation site) by deleting the last 113 bases of exon 4 (and keeping the first 20 bases intact). On the protein level, this deletion is predicted to produce a frameshift from amino acid 224: p.(Ser224Argfs*5).

To test whether the breakpoint in patient 55555 was located at the same position as the breakpoint in patient 70353, we performed PCR on DNA from patient 55555 using the same primers as used for PCR amplification of the breakpoint in patient 70353. Interestingly, the procedure revealed identical breakpoints. We therefore carried out two different inquiries to substantiate whether the deletion had occurred once in a common ancestor or on two independent occasions. In one

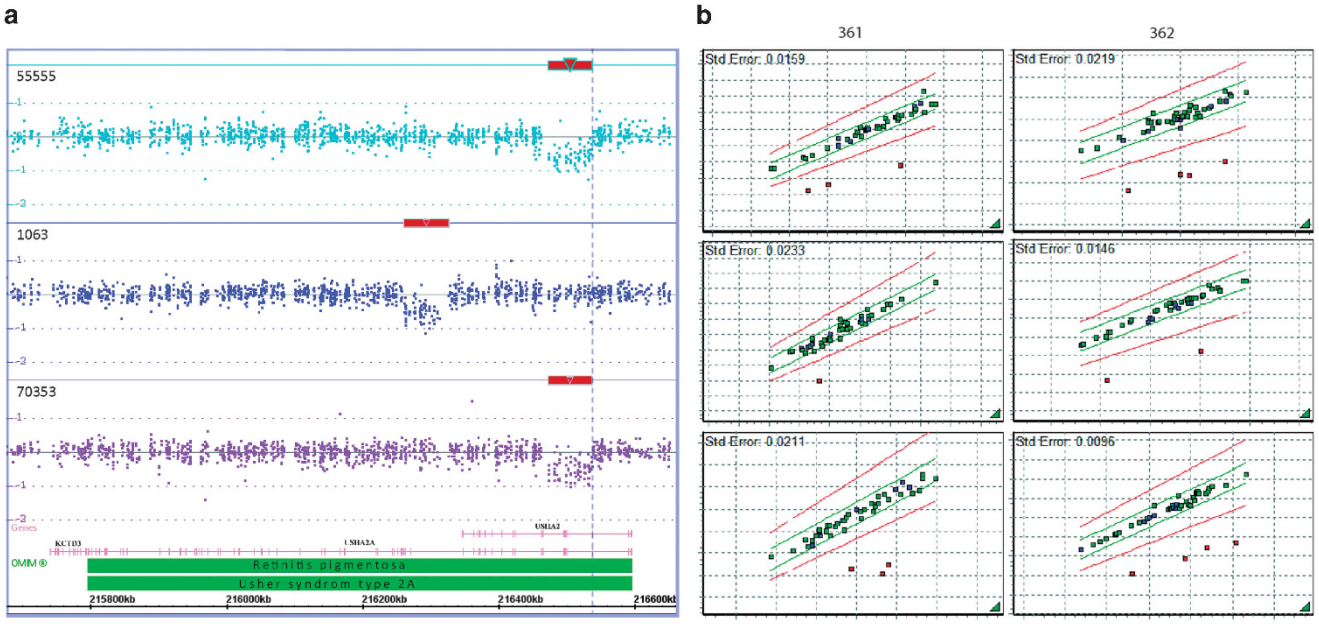

Figure 1 CytoScan HD and MLPA results for patients 70353, 55555 and 1063. (**a**) CytoScan results, where deletions are indicated by a red bar in the *USH2A*/RP region. (**b**) MLPA results using the two probes-mix kits, P361-A1 and P362-A2, respectively, covering *USH2A*. Red dot indicates a deleted probe/exon.

Table 1 Mutations identified in the three USH2 patients and methods used

Patient	*70353 (LOVD: patient data #0004572)*	*55555 (LOVD: patient data #0004571)*	*1063 (LOVD: patient data #0004573)*
USH2A MLPA	Deletion exon 4–10	Deletion exon 4–10	Deletion exon 22–24
CytoScan HD data (hg19)	chr1: g.216473521_216535711del	chr1: g.216473521_216536791del	chr1: g.216260845_216326858del
Mate-pair/breakpoint spanning PCR (hg19)	chr1:g.216464358_216538408del (c.672_1840+1160del)	chr1:g.216464358_216538408del (c.672_1840+1160del)	chr1:g.216259403_216323159del (c.4627+25435_4987+658del)
Targeted NGS analysis[a]	c.10684G>T, p.(Glu3562[a])	ND	ND
USH2A sequencing[a]	ND	c.2299delG, p.(Glu767Serfs[a]21)	c.3309C>A, p.(Tyr1103[a])

Abbreviations: MLPA, multiplex ligation-dependent probe amplification; ND, not done; NGS, next-generation sequencing.
[a]Variants are described according to *USH2A* accession number NM_206933.2 with A in the translation start codon as +1; sequence variants have been submitted to: https://grenada.lumc.nl/LOVD2/Usher_montpellier/home.php?select_db=USH2A.

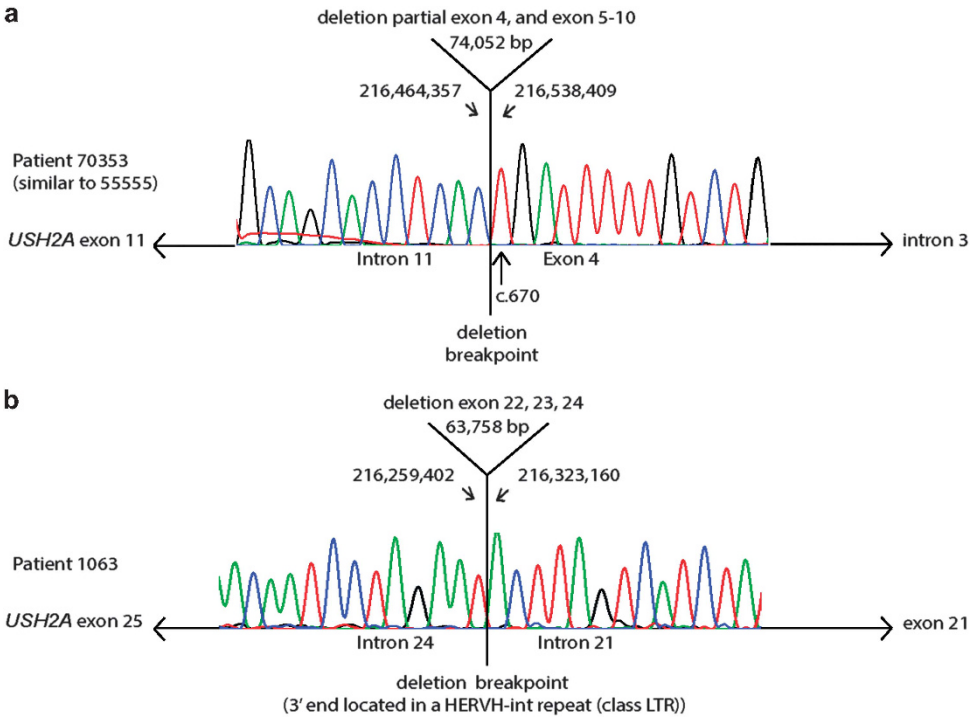

Figure 2 Mapping of two deletion breakpoints in *USH2A*. On the basis of the next-generation mate-pair sequencing results, PCR primers were designed to amplify across the deletion breakpoints from genomic DNA from patients 70353 and 1063. (**a**) Sequence chromatogram showing that patient 70353 (and 55555) have a 74 052-bp deletion (chr1.hg19:g.216464358_216538408del) with breakpoints in exon 4 and in intron 11. (**b**) Sequence chromatogram showing that patient 1063 has a 63 758-bp deletion (chr.hg19:g.216259403_216323159del) with breakpoints in intron 21 and in intron 24.

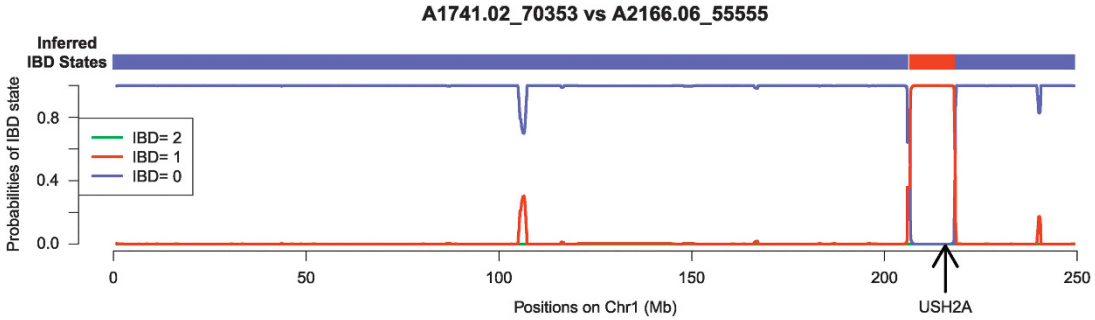

Figure 3 Estimation of relatedness between patient 70353 and patient 55555 based on investigation of SNP data from chromosome 1. Plots of relatedness across chromosome 1 indicate that the region from 206 to 218 Mb on one of the two alleles with very high probability is identical by decent, and thus inherited from a common ancestor. The orange line is the probability for being related for one allele and unrelated for the other (here 100% in *USH2A*). The blue line is the probability for being unrelated (here 0% in *USH2A*). The green line indicating that both alleles exhibit identity by descent (IBD) has 0% probability across the chromosome and is almost not visible. The most likely IBD states across the chromosome are indicated by a bar above the plot, showing that the two individuals are only related at the *USH2A* locus (black arrow).

approach, we used traditional genealogy. For both individuals, we have established their ancestry back to 1850, 4–6 generations, without finding a common ancestor. In a second approach, we used a statistical method to infer relatedness locally across the genome.[26] Individuals who share an inherited (family specific) sequence variant have a high degree of similarity in the region around the variant. Relatedness mapping based on SNPs in the region containing *USH2A* on chromosome 1 revealed a high probability of relatedness in the region located between 206 and 218 Mb (Figure 3), indicating a common ancestor carrying the deletion. As the similarity (relatedness) was low in the rest of the investigated region, the possible common ancestor of patients 70353 and 55555 probably lived many generations back, which agrees with the genealogy. Note, the deletion in *USH2A* is located around chr1.hg19:g.216,464,000_216,538,000 (see Figure 3).

DISUSSION

MLPA revealed two deletions in *USH2A*: one deletion spanning exons 4–10 and another deletion spanning exons 22–24. A deletion of exons 22–24 in *USH2A* has been described previously by Baux *et al.*[28] and deletion breakpoints that differs only by 2 bp (AT) compared with the present case was submitted to https://grenada.lumc.nl/LOVD2/Usher_montpellier/home.php?select_db=USH2A in 2012 (DB-ID USH2A_00095; Patient data: #0000067). In two apparently unrelated patients, 70353 and 55555, we identified a deletion of exons 4–10 with

exactly the same breakpoints (c.672_1840+1160del). Interestingly, we found evidence for the existence of a common ancestor in which the deletion had occurred many generations back, as we were not able to identify a common ancestor by traditional genealogy going back to 1850, yet SNP mapping revealed possible relatedness. A deletion of exons 4–10 has—to our knowledge—not been described previously.

Mate-pair NGS was used as a fast and efficient method for confirmation and directed cloning of the MLPA suggested breakpoints. This method is particularly useful in cases of breakpoints in large introns making long range PCR difficult and in cases where no tissue for RT-PCR studies are available for further studies of the consequences of the deletions at the mRNA level.

At both breakpoints, we observed microhomology of 2 bp (Supplementary Figure 2). We speculate whether the mechanism behind these deletions could be non-homologous end-joining (NHEJ). NHEJ does not require the presence of extensive homology but can be facilitated by the presence of microhomology (1–4 bp).[29] Another mechanism could be microhomology-mediated end-joining (MMEJ), but normally a longer stretch of microhomology (5–25 bp) is required for MMEJ to occur.[30,31]

A total of 435 different sequence variants in *USH2A* have been registered in HGMD Professional (August, 2014). The majority, >50%, consists of missense or nonsense variants, but gross deletions, duplications or complex rearrangements have also been identified and represent about 5% of the variants. In our patients, we found deletions and duplications of the same magnitude, as we identified deletions in *USH2A* in three USH2 patients, corresponding to 10% of the USH2 patients (3/30). However, as patient 70353 and 55555 have inherited the same *USH2A* allele, we only identified a deletion in two different alleles corresponding to 3.33% of the alleles (2/60). No duplications were identified. Steele *et al*.[22] identified also *USH2A* deletions with similar frequency, as deletions were identified in 5% (7/121) unselected USH2 patients.

It has been reported that large deletions or duplications within *PCDH15* are frequent causes for USH1.[32] To date, 71 different *PCDH15* sequence variants are registered in HGMD Professional (August, 2014). Interestingly, partial gene deletions and duplications account for a relatively large fraction, that is, ~17% of the 71 sequence variants identified in *PCDH15*. However, we could not identify any deletion or duplication in *PCDH15* in this study, but recognize that only 20 USH type 1 patients were analyzed. As the P292 kit for *PCDH15* does not include probes for all exons of *PCDH15*, we cannot exclude that deletions or duplications of any of these exons might be present in our cohort of patients. Aller *et al*.[21] identified deletions/duplications in *PCDH15* in 13% of 23 USH1 patients by combining MLPA and CGH procedures. The 23 USH1 patients represent a cohort of selected (remaining) patients, with one or no identified function-affecting sequence variants after screening a larger number of patients by Sanger sequencing for variants in known USH genes. In contrast, our cohort was unselected. Inclusion did not depend on whether the patient had been subjected to molecular genetic tests previously or whether any function-affecting sequence variant was identified, all available patients were included. Our unselected and relatively small USH cohort might, at least partly, explain the absence of *PCDH15* deletions in our patients.

Interestingly, we found a MLPA signal of about 0.5 for *USH2A* exon 12 in one patient and for exon 19 in another patient, indicating a deletion of the exon. However, by sequencing exon 12 and exon 19 in the two patients, we found a function-affecting variant c.2023C>T, p.(Gln675*) in exon 12 and the variant c.4106C>T, p.(Ser1369Leu) (rs201709513) in exon 19, both in heterozygous form, indicating that neither exon 12 nor exon 19 was deleted. Investigation of the location of the MLPA probe in exon 12 and in exon 19 (available from www.mlpa.com) revealed that c.2023C and c.4106C are located in ligation regions, indicating a false-positive MLPA result. Changes in the target sequence have previously been shown to lead to false-positive MLPA results.[33] Great care must therefore be exercised, especially when MLPA results indicate the deletion of a single exon.

MLPA would be a useful technique to test for deletions and duplications in other USH genes as well. Exon deletions or duplications in any of the other genes might probably contribute to USH as well. So far, only very few exon deletions/duplications have been identified in *MYO7A*, *USH1C*, *CDH23*, *CLRN1* and *GPR98*, whereas none have been identified in *USH1G* and *DFNB31* according to HGMD Professional. The absence of commercially available MLPA kits that target these genes is probably the main reason for this.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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