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### ► To cite this version:

Christel Vaché, Thomas Besnard, Catherine Blanchet, David Baux, Lise Larrieu, et al.. Nasal epithelial cells are a reliable source to study splicing variants in Usher syndrome. *Human Mutation*, 2010, 31 (6), pp.734. 10.1002/humu.21255 . hal-00552381

**HAL Id: hal-00552381**

**<https://hal.science/hal-00552381>**

Submitted on 6 Jan 2011

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Journal:	<i>Human Mutation</i>
Manuscript ID:	humu-2009-0577.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	15-Mar-2010
Complete List of Authors:	Vaché, Christel; CHU Montpellier, Laboratoire de Génétique Moléculaire Besnard, Thomas; CHU Montpellier, Laboratoire de Génétique Moléculaire Blanchet, Catherine; CHU Montpellier, Centre de Référence Affections sensorielles rares Baux, David; CHU Montpellier, Laboratoire de Génétique Moléculaire Larrieu, Lise; CHU Montpellier, Laboratoire de Génétique Moléculaire Faugère, Valérie; CHU Montpellier, Laboratoire de Génétique Moléculaire Mondain, Michel; CHU Montpellier, Centre de Référence Affections sensorielles rares Hamel, Christian; CHU Montpellier, Centre de Référence Affections sensorielles rares Malcolm, Sue; Institute of Child Health, Clinical and Molecular Genetics Claustres, Mireille; CHU Montpellier, Laboratoire de Génétique Moléculaire; Inserm, U827; Univ, Montpellier I Roux, Anne-Françoise; CHU Montpellier, Laboratoire de Génétique Moléculaire
Key Words:	Usher syndrome, transcript, unclassified variant, nasal cells, splicing



Humu-2009-0577

Research Article

Supporting Information for this preprint is available from the  
*Human Mutation* editorial office upon request (humu@wiley.com)

## Nasal epithelial cells are a reliable source to study splicing variants in Usher syndrome

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For Peer Review

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**Abstract**

We have shown that nasal ciliated epithelium, which can be easily biopsied under local anaesthetic, provides a good source of RNA transcripts from 8 of the 9 known genes which cause Usher syndrome, namely *MYO7A*, *USH1C*, *CDH23*, *PCDH15*, *USH1G* for Usher type 1 and *USH2A*, *GPR98*, *WHRN* for Usher type 2. Furthermore, the known or predicted effect on mRNA splicing of 8 variants was faithfully reproduced in the biopsied sample as measured by nested RT-PCR. These included changes at the canonical acceptor site, changes within the non-canonical acceptor site and both synonymous and non-synonymous amino acid changes. This shows that mRNA analysis by this method will help in assessing the pathogenic effect of variants, which is a major problem in the molecular diagnosis of Usher syndrome.

**Key words:** Usher syndrome, transcript, unclassified variant, nasal cells, splicing

## Introduction

Usher syndrome (USH) is a clinically and genetically heterogeneous autosomal recessive hereditary disorder characterized by both hearing and vision loss (Saihan et al., 2009). One of the major challenges in the molecular diagnosis of Usher syndrome results from the identification of a large number of sequence variants of unknown clinical significance, so called Unclassified Variants or UVs. To date, five genes causing Usher type 1 have been identified: *MYO7A* (MIM# 276903), *USH1C* (MIM# 605242), *CDH23* (MIM# 605516), *PCDH15* (MIM# 605514) and *USH1G* (MIM# 607696), three genes are known to be implicated in Usher syndrome type 2: *USH2A* (MIM# 608400), *GPR98* (MIM# 602851), *WHRN* (MIM# 607928) and one in Usher type 3: *USH3A* (MIM# 606397) (Saihan et al., 2009). The most frequently mutated USH genes are *USH2A* for Usher type 2 (Aller et al., 2006; Baux et al., 2007; Dreyer et al., 2008; Weston et al., 2000) and *MYO7A* and *CDH23* for Usher type 1 (Oshima et al., 2008; Roux et al., 2006). A large number of isoforms, encoded by alternatively spliced transcripts of the Usher genes, have been described but the clinical relevance of some of them remains imprecise (Ahmed et al., 2008; Bitner-Glindzicz et al., 2000; van Wijk et al., 2004; van Wijk et al., 2006; Verpy et al., 2000).

Mutations in *MYO7A*, *USH2A* and *CDH23* frequently include both synonymous and non-synonymous changes whose significance is uncertain, in addition to intronic variants located at non-canonical positions (Aller et al., 2006; Baux et al., 2007; Oshima et al., 2008; Roux et al., 2006). Various *in silico* analyses have been developed to study the effect on the structure of the protein of these UVs (Baux et al., 2008; Baux et al., 2007), but assessing the potential alteration of pre mRNA splicing is also an essential step for the determination of the pathogenic effect.

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3 Identification of abnormal exon skipping, or activation of a new splice site or pre-existing  
4  
5 pseudo splice site which may result in deletion of exonic bases or retention of intronic bases can  
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7 provide strong evidence for a pathogenic effect of the variant (Baralle and Baralle, 2005).  
8  
9  
10 Spurdle et al., review the programs which are currently available to predict splicing effect of  
11  
12 UVs, and emphasise that, as they all have both strengths and shortcomings, analysis at the  
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14 mRNA level remains essential to establish the clinical relevance and the consequences on splice  
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16 site loss (Spurdle et al., 2008). This mRNA analysis also allows us to assess the presence of  
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18 ectopic splice sites (Arnold et al., 2009). Studies on functional analysis of splicing using reporter  
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20 minigenes have shown their usefulness when patient RNA is not available or to confirm effects  
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22 detected in patients' samples (Tournier et al., 2008) but remain an artificial system with technical  
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24 challenges related to the cloning, the transfection efficiency and the basal level of exon  
25  
26 recognition (Cooper, 2005; Spurdle et al., 2008).  
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31 Recently, we have shown the usefulness of *ex vivo* splicing assays by identifying the splicing  
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33 pattern of 21 sequence alterations, providing an attractive alternative or complementary approach  
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35 to mRNA-based assays from Usher patients (Le Guédard-Mereuze et al., 2010). Only a few  
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37 mRNA analyses from Usher patient tissues have been attempted so far. Cohn et al., measured  
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39 *USH2A* transcripts in biopsies from minor salivary glands in the lip but the drawback to this  
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41 sampling is that it requires an unacceptably invasive biopsy (Cohn et al., 2004). RNA extracted  
42  
43 from peripheral blood was found to be positive for *WHRN* (Ebermann et al., 2007) and *CDH23*  
44  
45 (Becirovic et al., 2008) but not for *MYO7A* (Boulouiz et al., 2007), showing that the study of  
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47 Usher transcripts from RNA extracted from lymphocytes is not reliable for all Usher genes.  
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51  
52 The presence of eight Usher proteins has been demonstrated in nasal ciliated epithelium using  
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54 immunochemistry with fluorescent antibodies (Cohn et al., 2006). The purpose of the present  
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3 work was to determine whether, using a relatively non-invasive biopsy, adequate RNA could be  
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5 obtained from this tissue for the analysis of transcripts for any of the Usher genes. Ideally nasal  
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7 cell and blood sampling can be performed at the same time, prior to the identification of the  
8  
9 causative gene.  
10  
11

12 Primers were designed for this study in order to detect the major known isoforms encoded by the  
13  
14 Usher genes. We show that all the Usher transcripts can be detected from nasal epithelial cells  
15  
16 with the exception of *USH3A*, which in any case contains a restricted number of mutations that  
17  
18 have been well characterised so far (Ben-Yosef et al., 2003; Fields et al., 2002).  
19  
20

21 The second goal of this study was to validate our method by analysing the effect of eight variants  
22  
23 on splicing. The results confirm that nasal epithelial cells are reliable, accessible cells to  
24  
25 discriminate Usher variants with and without effect on the RNA splicing process.  
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## 31 **Materials and Methods**

### 32 *Nasal epithelial cell samples from controls and patients*

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34 Patients were diagnosed with Usher syndrome based on established criteria (reviewed in (Cohen  
35  
36 et al., 2007)). Nasal sampling was performed along with blood sampling in the ENT clinics  
37  
38 during a routine clinical diagnosis appointment. Consent to genetic testing was obtained from  
39  
40 controls, adult probands or parents in the case of minors. Molecular analysis of Usher syndrome  
41  
42 was approved by the local Ethics Committee.  
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48 A local anaesthetic spray (Xylocaïne 5% nébuliseur, Astrazeneca, France) was applied to each  
49  
50 nostril before collection. Nasal specimens were obtained by gentle scraping of the lateral inferior  
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52 turbinates with Rhino-Probe mucosal currettes (ASI Rhino-pro®, Arlington Scientific INC,  
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Arlington Scientific, Springville, UT). Samples were immediately suspended in a cell lysis buffer (Macherey-Nagel, Düren, Germany) and frozen at -30°C until RNA extraction.

#### *RNA analysis*

Total RNA was isolated from nasal cells using Nucleo Spin® RNA II isolation kit (Macherey-Nagel) according to the manufacturer's instructions. RNA quality and quantity were determined with a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Up to 5 ug of RNA could be obtained from sampling of both nostrils. For each sample, 500 ng of total RNA were reverse transcribed using oligodT primers and SuperScript™III transferase (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations.

cDNA was used as template in nested PCR reactions with specific primers (Supp. Table S1 and Supp. Table S2). PCR products were checked on 1.5% agarose gel and visualized by ethidium-bromide staining. For each analysis at least six control samples were amplified.

#### *Sequence analysis*

PCR products were purified by QIAquick PCR purification kit (Qiagen, Hilden, Germany) under the conditions recommended by the manufacturer and sequenced in both directions with BigDye Terminator v1.1 cycle sequencing kit from Applied Biosystems (Warrington, UK) using the internal PCR primers. When necessary, additional primers were used to obtain the whole sequence of interest (Supp. Table S2). Sequence reactions were analysed on a capillary ABI 3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

#### *Splice-site Prediction programs*

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3 The predicted effect of each variant on RNA splicing was studied using three different splice site  
4 analysis programs: 1) Human Splicing Finder (HSF) version 2.4; <http://www.umd.be/HSF/>  
5  
6 (Desmet et al., 2009), 2) Neural Network SPLICE (NNSPLICE)  
7  
8 [http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html) (Reese et al., 1997) and 3) NetGene2  
9  
10 <http://www.cbs.dtu.dk/services/NetGene2/> (Hebsgaard et al., 1996). Exonic and intronic  
11  
12 sequences of the region of interest were submitted according to the requirements of each  
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14 program and default parameter settings were applied in all predictions.  
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### 22 *Nomenclature of variants*

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24 The nomenclature system for the description of changes in DNA (cfs) and RNA follows the  
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26 recommendations of the Human Genome Variations Society (<http://www.hgvs.org> (Horaitis and  
27  
28 Cotton, 2004)). Nucleotide +1 corresponds to the A of the ATG translation initiation codon in  
29  
30 the respective reference sequences (NM 000260.3 for *MYO7A*, NM 206933.2 for *USH2A* and  
31  
32 NM 22124.4 *CDH23*).  
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### 39 **Results**

#### 40 *Presence of Usher transcripts in nasal epithelial cells*

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42 As Usher genes are alternatively spliced and encode different isoforms of the proteins, we have  
43  
44 assessed the presence of the major classes in nasal epithelial cells, by designing primers for  
45  
46 specific amplification (Supp. Table S1). Expression of the five genes implicated in Usher type 1  
47  
48 was studied from at least six different healthy controls. For each gene, products of amplification  
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50 from three controls are presented Figure 1A. *MYO7A*, *CDH23* and *USH1G* transcripts were  
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52 amplified with the corresponding expected sizes (Supp. Table S1). Amplification of *USH1C*  
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3 between exons 13 and 16 revealed the a1 isoform corresponding to the largest class a *USH1C*  
4 isoform (Verpy et al., 2000). When amplification was performed between exons 13-19 (also  
5 known as exon D), several products were observed, and correspond to alternative forms of class  
6 c *USH1C* isoforms (Verpy et al., 2000). The *PCDH15* CD3 isoform was detected using primers  
7 designed to specifically amplify the region encoding the cytoplasmic domain 3 (exons 32-36)  
8 (Ahmed et al., 2008).  
9

10  
11 Expression of the three genes implicated in Usher type 2 was analysed similarly (Figure 1B).  
12 Expected products were observed for both *USH2A* isoforms. PCR was carried out between exons  
13 19-21, and 48-52 to discriminate *USH2Aa* (short isoform) and *USH2Ab* (long isoform). The  
14 *GPR98* transcript was also detected by amplification of the region comprising exons 49-51. For  
15 *WHRN*, the two classes of isoforms could also be distinguished using primers designed by van  
16 Wijk. et al., (van Wijk et al., 2006).  
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### 34 ***Effect of variants on splicing***

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36 Variants were first identified at the DNA level in 6 patients using the molecular diagnostic  
37 procedure consisting of exhaustive sequencing of the exons and flanking intronic sequences of  
38 the Usher genes. Genotypes of the patients are presented Table 1.  
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43 Transcript analyses revealed different effects on splicing which are summarized Table 2.  
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### 48 **Splicing effect of *USH1* variants**

#### 49 ***CDH23* c.3580-1G>T (c.IVS29-1G>T)**

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51 In patient 1, primers were designed to amplify a 234 bp fragment between exons 29 and 31  
52 (Supp. Table S2). cDNA analysis from RT-PCR (Fig. 2-1) revealed the presence of two  
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3 transcripts in the patient, a normal transcript (234 bp) and an aberrant transcript (98 bp)  
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5 corresponding to skipping of exon 30 and predicting the creation of a premature termination  
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7 codon (PTC) in exon 31.  
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### 10 11 12 ***CDH23 c.6050-9G>A (c.IVS45-9G>A)***

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14  
15 The c.6050-9G>A variant was analysed in patient 2 and his mother (Fig. 2-2). Both carried the  
16  
17 complex allele c.[6050-9G>A;6130G>A] (Table 1). Examination of the RT-PCR products in the  
18  
19 patient (Fig. 2-2) revealed a fragment corresponding to the expected size (333 bp) and a slightly  
20  
21 bigger one (340 bp). Sequencing of the 333 bp amplicon showed the presence of both alleles i.e.  
22  
23 heterozygosity for c.6130G>A (allele 1) and c. 5985C>A (allele 2) with normal splicing. The  
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25 340 bp fragment corresponded exclusively to the variant transcript or allele 1 and included the  
26  
27 last 7 nucleotides of intron 45 predicting the creation of a PTC in exon 46.  
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### 34 35 ***MYO7A c.3719G>A (p.Arg1240Gln) and c.3979G>A (p.Glu1327Lys)***

36  
37 These two variants have been identified in patient 3 in a compound heterozygous state (Table 1).  
38  
39 Primers were designed to amplify the two variants c.3719G>A and c.3979G>A (exons 27-33) in  
40  
41 a single RT-PCR (Fig. 2-3). No difference in size was observed between the patient and the  
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43 control amplification. Sequencing showed the presence of the 2 alleles with heterozygosity for  
44  
45 both variants (Fig. 2-3). No abnormal splicing was observed.  
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### 51 ***Splicing effect of USH2 variants***

### 52 53 ***USH2A c.2168-1G>C (c.IVS12-1G>C)***

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3 Examination of the RT-PCR product of patient 4 (Table 1) revealed no additional fragment (Fig.  
4 3-1). However, sequencing demonstrated the presence of a normal transcript and an aberrantly  
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6 spliced product lacking the first 7 nucleotides of exon 13 leading to a PTC in exon 14.  
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12 ***USH2A c.7595-8C>G (c.IVS40-8C>G )***  
13

14  
15 Analysis of the RT-PCR amplification in patient 5 (Table 1) did not show additional fragments  
16  
17 between patient and control (Fig. 3-2). Again, sequencing of the products revealed the presence  
18  
19 of two distinct transcripts, a normal transcript and an abnormal transcript that included the last 7  
20  
21 nucleotides of intron 40 resulting in a PTC in exon 42.  
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27 ***USH2A c.4576-4579dupGGGT***  
28

29 In addition to the c.7595-8C>G anomaly, patient 5 carried the c.4576-4579dupGGGT (Table 1).  
30  
31 Agarose analysis of the RT-PCR product in the region of the duplication did not detect multiple  
32  
33 fragments. However, sequencing revealed clearly the presence of two distinct transcripts: a  
34  
35 normal splice transcript and a product lacking the last 50 nucleotides of exon 21 resulting in a  
36  
37 PTC in exon 22 (Fig. 3-3).  
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44 ***USH2A c.949C>A (p.Arg317Arg)***  
45

46 The c.949C>A variant was identified in patient 6 in trans to the c.8167C>T mutation (Table 1).  
47  
48 Two products of 598 and 405 bp were detected. Sequencing showed the presence of three  
49  
50 transcripts, corresponding to the normal allele, the mutant allele lacking the last 193 bases of  
51  
52 exon 6 leading to a PTC in exon 7 and a minor product corresponding to the mutant allele with  
53  
54 normal splicing (Fig. 3-4).  
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### *In silico analysis*

All analysed variants were run against various *in silico* predictors, and results are shown in Supp. Table S3. Comparison of predictions and experimental results (Table 3) showed that variants with demonstrated effect on splicing were always predicted by at least two programs. On the other hand, the precise effect of c.3580-1G>T on transcripts, *i.e.* the use of an in frame cryptic site, was not predicted. Only HSF predicted the creation of the splice site used in the presence of the c.7595-8C>G variant. In the two *MYO7A* variants, which did not experimentally alter splicing, no false-positive prediction was observed.

### **Discussion**

As Usher proteins are expressed in different tissues containing ciliary processes or microvillar structures including human ciliated nasal epithelium (Cohn et al., 2004; Cohn et al., 2006), we hypothesised these cells could be used as a source to analyse Usher transcripts in a non-invasive way.

### *Usher transcripts*

We demonstrated that *USH1* and *USH2* transcripts could be amplified from nasal epithelial cells. The presence of several isoforms could be demonstrated for *USH2A*, *WHRN* and *USH1C*. Class b *USH1C* isoforms could not be detected in accordance with the fact that this class seem to be

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3 restricted to inner ear (Verpy et al., 2000) and a few other tissues (retina and brain) (Reiners et  
4  
5 al., 2003).

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8 Despite the combination of numerous primers located in the different exons of *USH3A*,  
9  
10 transcripts could not be detected in nasal cells. These results are surprising considering the broad  
11  
12 expression pattern of this gene (Fields et al., 2002) and the presence of Clarin-1 in the nasal  
13  
14 epithelium (Cohn et al., 2006). As only a few patients have been diagnosed with *USH3A*  
15  
16 mutations in our cohort (unpublished) and considering the overall restricted number of mutations  
17  
18 identified in this gene, the failure to detect *USH3A* expression in nasal cells should have limited  
19  
20 consequences in a diagnostic setting.  
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23

### 24 25 26 27 *Alteration of splicing*

28  
29 To validate the use of nasal cells for the analysis of aberrant transcripts, we analysed 4 intronic  
30  
31 and 4 exonic variants, mostly predicted to have an impact on splicing. All potential mutations  
32  
33 were analysed using three prediction programs as described in Materials and Methods. The  
34  
35 programs had mixed results in predicting the observed changes. In part, this is likely to be  
36  
37 because of tissue specific variations in splice site efficiency.  
38  
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40  
41 Based on their location at the consensus canonic splice sites, the two *USH2A* c.2168-1G>C and  
42  
43 *CDH23* c.3580-1G>T variants were strongly predicted to affect splicing (Baux et al., 2007;  
44  
45 Blanchet et al., 2007). For these variants we demonstrated the presence of the two predicted  
46  
47 transcripts (*USH2A* r.2168\_2174del and *CDH23* r.3580\_3715del). As there is no SNP located in  
48  
49 the amplified fragment designed to study the *USH2A* c.2168-1G>C variation, the proportion of  
50  
51 the 2 alleles could not be ascertained. Therefore the possibility that allele 1 (*USH2A* c.2168-  
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53 1G>C) also generates some normal transcript could not be excluded. However, for the *CDH23*  
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3 c.3580-1G>T variant, the use of a set of primers encompassing exons 26 to 31 (and allowing  
4 discrimination between allele 1 and 2; Fig. 2-1), confirmed that the allele carrying *CDH23*  
5 c.3580-1G>T was leading only to abnormal splicing, *i.e.* skip of exon 30. In addition, under  
6 these experimental conditions, the use of an in frame cryptic acceptor splice site, located in exon  
7 30, leading to the loss of the first 51 nucleotides of this exon was also observed in allele 2 (data  
8 not shown).  
9

10  
11 The splicing effect of *CDH23* c.6050-9G>A has been previously shown by exon trapping (von  
12 Brederlow et al., 2002) which characterized the use of a new acceptor site leading to the insertion  
13 of 7 intronic nucleotides in the transcript. This deleterious effect is confirmed by our method.  
14 Nasal epithelium studies additionally detect residual normal splicing in the allele carrying the  
15 mutation (see below).  
16

17  
18 For the *USH2A* c.7595-8C>G variant, a minigene analysis has shown that this variation was  
19 responsible for a total misplicing leading to the aberrant r.7595-7\_7595-1ins transcript (Le  
20 Guédard-Méreuze et al., 2010). In nasal cells, the presence of this aberrant transcript was also  
21 observed but without being able to discriminate between the alleles confirmation of a 100%  
22 splicing defect, although very likely, is not possible.  
23

24  
25 Four exonic variants were tested and two of them were shown to have effects on splicing.  
26 *USH2A* c.949C>A predicts a synonymous change at Arg317. We considered this variant as  
27 pathogenic after using our normal criteria (Baux et al., 2007) *i.e.* absence in 110 control alleles,  
28 in trans to a deleterious mutation in 3 different patients, no other likely pathogenic change after  
29 exhaustive sequencing of the gene and prediction analyses. In this study we definitively show  
30 that c.949C>A alters splicing by activating a cryptic donor site leading to loss of 193 nucleotides  
31 and creation of a PTC and is likely to be deleterious. Contrary to results obtained with minigene  
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3 analysis (data not shown), the splicing defect observed in nasal cells was not total, as some  
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5 residual mutant transcript with normal splicing was detected (see below) showing the merit of  
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7 determining splice variants directly. This mutation has been previously described: Penning et al.,  
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9 suggested a splicing defect based on prediction (Pennings et al., 2004), Dreyer et al., classified it  
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11 as non pathogenic based on lack of information (Dreyer et al., 2008).  
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18 *USH2A* c.4576-4579dupGGGT is a frameshift mutation and would in any case be considered to  
19  
20 be deleterious as this would create a premature stop codon lying in exon 21 at position 1530 of  
21  
22 the encoded protein. But the HSF and NetGene2 programs predict an increase in the strength of a  
23  
24 donor cryptic splice site in exon 21 (Supp. Table 3), which would result in the loss of the last 50  
25  
26 nucleotides and a premature stop codon in exon 22, so we decided to consider this variant as a  
27  
28 potential splicing mutation. The finding of altered splicing in all detectable transcripts  
29  
30 emphasises the importance of both *in silico* and functional analysis for molecular diagnosis.  
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37 Two *MYO7A* missense variants did not lead to abnormal splicing in the patient's transcripts.  
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39 Both missense variants are predicted to have an adverse effect on protein function.  
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41 p.Arg1240Gln is the most common mutation observed in USH1 patients in Europe (Baux et al.,  
42  
43 2008) and accounts for 6.4% of mutated *MYO7A* alleles in our cohort (unpublished results). It  
44  
45 alters a highly conserved charged residue in a highly conserved region of a domain (MyTH4-1,  
46  
47 data not shown), which is known to interact with the central domain of SANS (Adato et al.,  
48  
49 2005). p.Glu1327Lys was initially reported by Najera et al., (Najera et al., 2002). This variant,  
50  
51 located in the FERM 1 domain, is classified as likely to be pathogenic (unpublished results)  
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53 based on perfect ortholog conservation. Although the effect on the structure is not known  
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3 p.Glu1327Lys could also be a residue involved in protein-protein interactions (again with the  
4 central domain of SANS) (Adato et al., 2005). None of these arguments in themselves precludes  
5 there being an effect of splicing but the finding of only normal transcripts suggests that they are  
6 truly acting at the protein level.  
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### 12 13 14 15 ***Residual normal splicing of mutant alleles***

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17 Two of the variants *CDH23* c.6050-9G>A and *USH2A* c.949C>A do not lead to a 100%  
18 abnormal pattern and estimation of the relative proportion of each transcript was not possible in  
19 either of them. Variable proportions of mutant/normal transcripts have been shown to be tissue  
20 dependent and related in some cases to histopathologic expression of the disease (Chiba-Falek et  
21 al., 1999). Usher gene splicing is likely to undergo different regulation in nasal epithelial cells  
22 and cochlea or retina possibly leading to variable ratios of mutant/normal transcripts in these  
23 tissues. In any case, clinical signs of the two patients carrying these *CDH23* and *USH2A* variants  
24 were typical of Usher type I and II, respectively.  
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### 39 ***Nonsense Mediated Decay***

40 mRNA transcripts containing premature termination codons (PTCs) are routinely subjected to  
41 nonsense mediated decay (NMD) (Wagner and Lykke-Andersen, 2002). Indeed loss of  
42 heterozygosity revealed by NMD has been used as a method to distinguish disease causing loci  
43 in tuberous sclerosis (Jeganathan et al., 2002). Although several of the mutations reported in this  
44 paper are predicted to result in PTCs, for example *CDH23* c.3580-1G>T and *CDH23*  
45 p.Gln1123X, both alleles were detected after RT-PCR showing that nonsense mediated decay is  
46 not operating for these transcripts in this tissue. If there were NMD resulting in detection of only  
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3 the trans allele it could give a misleading result. However, the primers are always designed to  
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5 check for this possibility either by including a region of heterozygosity within the test region or  
6  
7 establishing heterozygosity at the site of the mutation on the other chromosome.  
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12 Use of nasal cells was shown to be very effective as a source of RNA from nearly all Usher  
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14 genes, although the relatively small amount of RNA obtained necessitated the use of nested PCR.  
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16 Sampling can readily be set up with training in local clinics. It provides a useful alternative or  
17  
18 complement in Usher syndrome to *ex vivo* methods such as splicing reporter minigenes (Le  
19  
20 Guédard-Mereuze et al., 2010). Nasal cells represent the simplest available tissue to study  
21  
22 splicing defects of the Usher genes. Although splicing factors (hnRNP proteins or even core  
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24 components of the spliceosome) may differ between cell types and may influence splicing, the  
25  
26 results presented here all suggest that clinically relevant and valuable results will be obtained.  
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28 Because Usher syndrome can be classified as a retinal ciliopathy, the approach presented here  
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30 could be useful in analysing splicing defects in other genes involved in this group of disorders.  
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#### 41 Acknowledgments

42  
43 TB was supported by a grant from “Ministère de la Recherche: PHRC National 2004”.  
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### **Figure legends**

#### **Figure 1.** Expression of Usher genes in nasal epithelial cells

Agarose gels showing the RT-PCR products obtained for Usher type 1 genes (**A**) and Usher type 2 genes (**B**). For each isoform, three controls are shown.

#### **Figure 2.** Molecular characterization of USH1 variants

**2-1:** *CDH23* c.3580-1G>T; **2-2:** *CDH23* c.6050-9G>A; **2-3:** *MYO7A* c.3719G>A and c.3979G>A. Left- representation of the genomic sequence surrounding the variant(s) of interest. Black arrows indicate the position of the primers used for RT-PCR and sequencing. Right- Agarose gel and sequencing of the RT-PCR products obtained from the patient (P). A control sample (C) was run along on the gel. \*: premature stop codon (PTC). Mo: Mother. For each variant, alleles 1 and 2 are detailed in Table 1.

#### **Figure 3.** Molecular characterization of USH2 variants

**3-1:** *USH2A* c.2168-1G>C; **3-2:** *USH2A* c.7595-8C>G; **3-3:** *USH2A* c.4576-4579dupGGGT; **3-4:** *USH2A* c.949C>A. Left- representation of the genomic sequence surrounding the variant(s) of interest. Black arrows indicate the position of the primers used for RT-PCR and sequencing. Right- Agarose gel and sequencing of the RT-PCR products obtained from the patient (P). A control sample (C) was run along on the gel. \*: premature stop codon (PTC). For each variant, alleles 1 and 2 are detailed in Table 1.

**Table 1. Genotypes of the six Usher patients presented in this study**

Patient	Gene	Exon/intron	Allele 1	Allele 2	Exon/intron
1*	<i>CDH23</i>	exon 27	c.3367C>T (p.Gln1123X)	<b>c.3580-1G&gt;T</b>	intron 29
2	<i>CDH23</i>	intron 45 and exon 46	<b>c.[6050-9G&gt;A; c.6130G&gt;A<sup>a</sup>]</b>	c.5985C>A (p.Tyr1995X)	exon 45
3	<i>MYO7A</i>	exon 29	<b>c.3719G&gt;A (p.Arg1240Gln)</b>	<b>c.3979G&gt;A (p.Glu1327Lys)</b>	exon 31
4**	<i>USH2A</i>	intron 12	<b>c.2168-1G&gt;C</b>	c.1000C>T (p.Arg334Trp)	exon 6
5	<i>USH2A</i>	intron 40	<b>c.7595-8C&gt;G</b>	<b>c.4576-4579dupGGGT</b>	exon 21
6	<i>USH2A</i>	exon 41	c.8167C>T (p.Arg2723X)	<b>c.949C&gt;A</b>	exon 6

Variants tested at RNA level are indicated in bold.

\*: Patient already reported in Blanchet et al., 2007; \*\*: Patient already reported in Baux et al., 2007. <sup>a</sup>: rs10466026:G>A.

**Table 2. Effects of Usher variants on splicing: analysis at RNA level**

Gene	Variant	Effect on RNA level	Variant name (RNA level)
<i>CDH23</i>	c.3580-1G>T	Transcript with a skip of exon 30 + transcript with a deletion of the 51 first nucleotides of exon 30	r.[3580_3715del, 3580_3630del]
	c.6050-9G>A	Normal transcript + transcript with an insertion of 7 intronic nucleotides	r.[=, 6050-7_6050-1ins]
<i>MYO7A</i>	c.3719G>A	No effect on splicing	r.3719G>A
	c.3979G>A	No effect on splicing	r.3979G>A
<i>USH2A</i>	c.2168-1G>C	Deletion of the first 7 nucleotides of exon 13	r.2168_2174del
	c.7595-8C>G	Insertion of 7 intronic nucleotides	r.7595-7_7595-1ins
	c.4576-4579dupGGGT	Deletion of the last 50 nucleotides of exon 21	r.4578_4627del
	c.949C>A	Normal splicing + deletion of the 193 last nucleotides of exon 6	r.[949C>A, 951_1143del]

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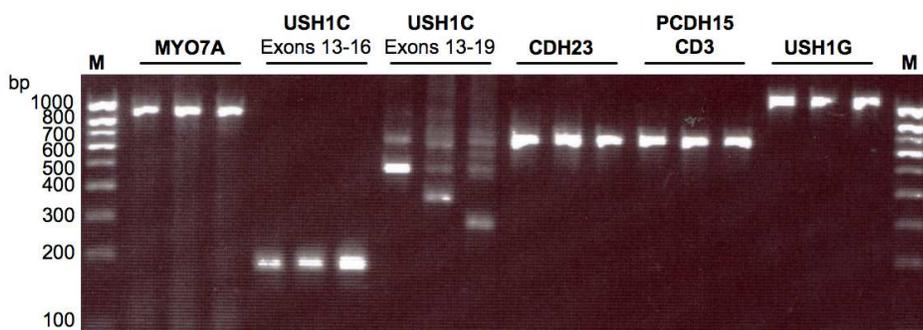
**Table 3. Comparisons of *in silico* predictions with experimental results obtained at RNA level**

Usher gene	Variant	splice site type	HSF		Fruit Fly Score	NetGene2 Confidence
			HSF matrice	Max Ent Score		
CDH23	c.3580-1G>T	acceptor	+ abolition of the natural splice site - use of an in frame cryptic site			
	c.6050-9G>A	acceptor	+ creation of a new splice site	- recognition of splice site (control and mutant)	+ creation of a new splice site	
MYO7A	c.3719G>A	acceptor	+ no effect on splicing	- recognition of splice site (control and mutant)	+ no effect on splicing	
	c.3979G>A	acceptor	+ no effect on splicing			
USH2A	c.2168-1G>C	acceptor	+ abolition of the natural splice site			
			+ use of a cryptic site			- use of a cryptic site
	c.7595-8C>G		+ decrease of the strength of the natural splice site			
			+ creation of a new splice site	- creation of a new splice site		
	c.4576-4579dupGGGT	donor	+ increase of the strength of a cryptic site	- no effect on splicing	+ increase of the strength of a cryptic site	
c.949C>A	donor	+ increase of the strength of a cryptic site (< to the strength of the natural splice site)				

+: prediction was in accordance with experimental results, - : effect on splicing was not predicted.

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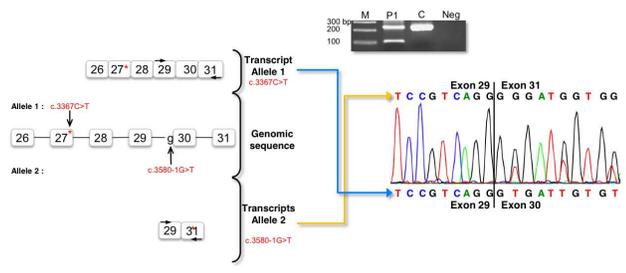


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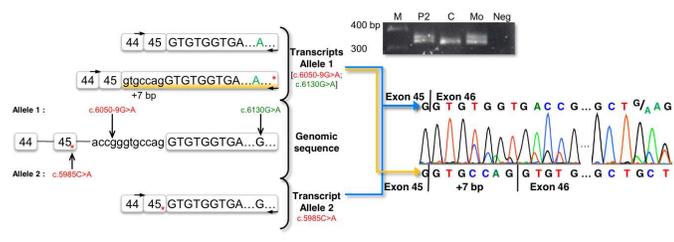
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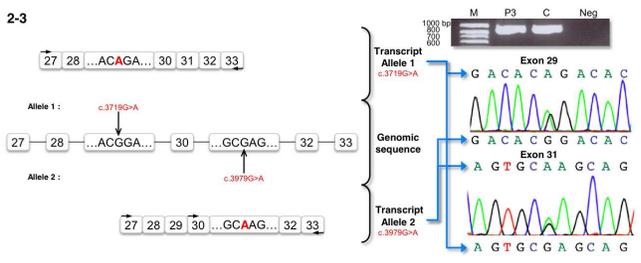
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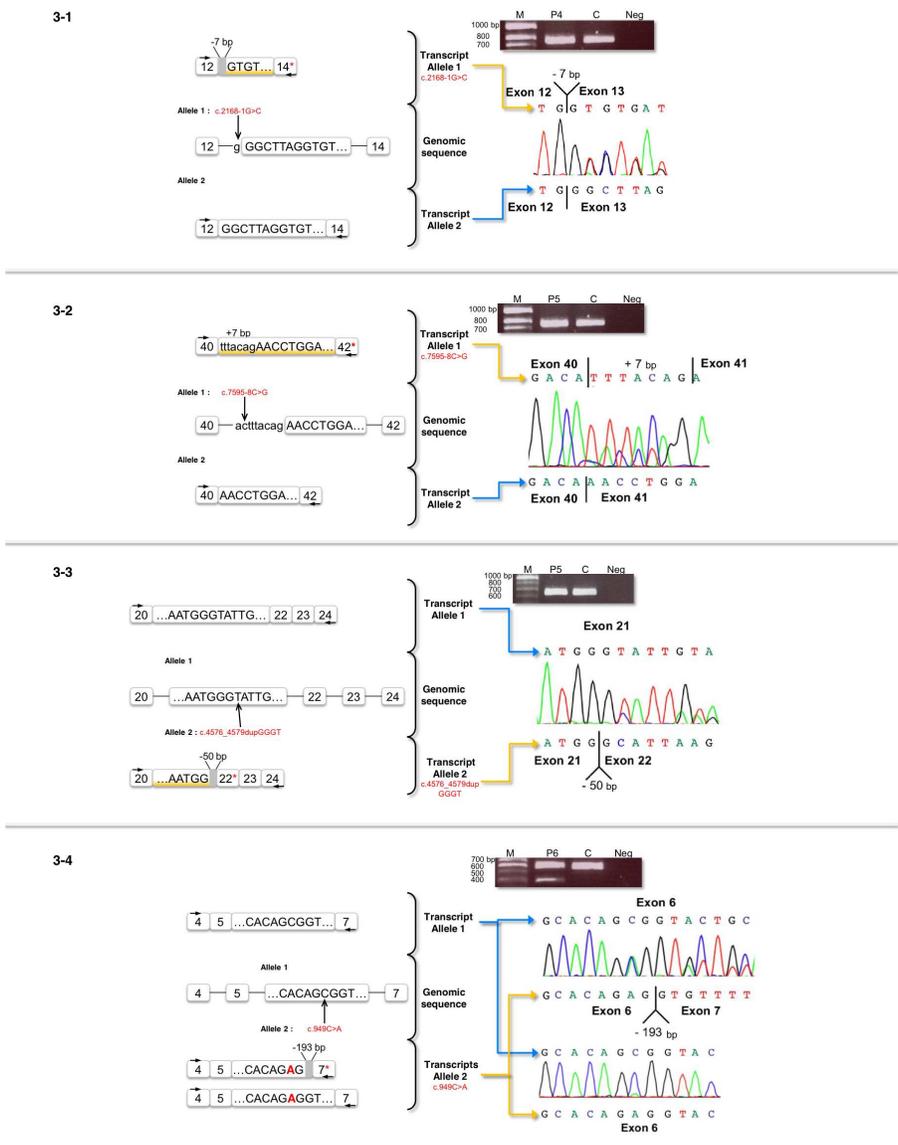


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**Supp. Table S1. Primers used in RT-PCR and sequencing for the analysis of Usher type 1 and 2 transcripts**

Transcript	Accession nb	Forward primer (5'-3')	Reverse primer (5'-3')	Amplified exons	Expected product size (bp)
<b>USHER type 1</b>					
<i>MYO7A</i>	NM 000260.1	CAGAGGAGGTGACCAAGAGG GAAGCTGCACTTCATCATCG	AGGTCACGAATGTCTCAGC CTTCTGGGCATCAGTTCTCC	27-33	904
<i>USH1C</i>	NM 005709.1	GAAAAGAAATTGCCCAGAAGG CAGCAGAGGAAAATGAGAGATACC	AAATCCTGCTCTCCCTGCTC CTCGAGCTCAGGTTCCACTC	13-(15/16)	194
	NM153676.2	GAAAAGAAATTGCCCAGAAGG CAGCAGAGGAAAATGAGAGATACC	GGTTGCTCAGTGCTTCTTCC ACATGTCCAAAGGGATGTCTG	13-19(D)	
<i>CDH23</i>	NM 22124.4	GTTGTGCTAGAGGACATCAACG TCTGGCCATCCACTACTTCC	CACTCAGGTTCTCCGGTAGG ACAATGGCCTTGAGCTTCC	59-64	653
<i>PCDH15</i> Isoform CD3	EU718482**	CTCCAGAGGCAGTGACCAGC CTGAGTGTACAAAGACTGCACG	CGATTAATTGATAACAATGTG GGCTTACCGCTGTATTGTCAG	32-36	647
<i>USH1G</i>	NM173477.2	CATGCCTCAGCCCTAATACC CCGAAAGGAGCTGAATGC	TCCCCAAGTCTACATGTCC TCAAGACCCCTCAGAACTGG	1-3	1551
<b>USHER type 2</b>					
<i>USH2A</i> Short isoform (a)	NM 007123.5	CCTGGGTCTCAGAAAGAACG CAGTCTTCCCCTCTCTTCG	ATAGCATGAGGGCATTTTGC TCAGGCAAAAGCTATCAAAGG	19-21	662
		Long isoform (b)	NM 206933.2	GATCAGAGTGATGAGCTCTGC CCTGAATCTATCTGTGGAC	GAGGATCCTGCACTCTTTGG
<i>GPR98</i>	NM 032119.3	GCATCAAAAACCTCCCTGTCC GCTAATGCCAGGCTAAACTCC	GAATGGCTGGAAATGTAGGC TGAATGAGCTCCCACTAGAGC	49-51	444
<i>WHRN</i> Short isoform	AK022854.1	CACGATGCATGGTTCTCTTG* GCTCAAATGCACAACCTGGAG	TGTTGAGCAGCTTGAACAGG* CCAGGTAGTAGGCCATGGTG	5-6	216
		Long isoform	NM 015404.2	TGCTCTTCGACCAATACACG* TGCTCTTCGACCAATACACG*	GCTTCTTGAGCCCTTCAG

\*: from van Wijk et al., (2006); \*\*: from Ahmed et al., (2008).

**Supp. Table S2. Primers used in RT-PCR and sequencing for the analysis of Usher patients**

Variant	Forward primer	Reverse primer	Amplified exons	Expected product size (bp)
<b>USHER type 1</b>				
<i>CDH23</i> c.3580-1G>T  c.6050-9G>A	AACTTCCGGATCCATGTCAG	CGGTGATGATAAGCCCTGTGC		
	CATCCCACGTGCTGATAGTG	ACAGGATGCGGTAGTTCACC	29-31	234
	CGCATAGCCAGGAGGGATTATG	GCACGCTCACTGTCTGGATGG		
	AACTCTCCCGCTGGCACCC	GTTCTCTAGCAGATGGAC	44/45-46	333
	GATGCAGACCAAGACATCTACG			
<i>MYO7A</i> c.3719G>A/c.3979G>A	CAGAGGAGGTGACCAAGAGG	AGGTCACGAATGTCCTCAGC		
	GAAGCTGCACTTCATCATCG	CTTCTGGGCATCAGTTCTCC	27-33	904
	GTGACATTCATGGATGGGACC			
<b>USHER type 2</b>				
<i>USH2A</i> c.2168-1G>C	CTGTAACTGCAATACCTCTGG	CAAACACACTGACCAGTCAGG		
	GATGGAGATATTACCTGTCACC	GATTAAGTGCACCAGTTGTATGG	12-14	775
c.7595-8C>G	TTTCCAATCCTTCTGCATCG	CTCATATCTCAAATTAGGTCC		
	TCCATTCATGACCGCAGAGG	CACTGCGGAAGTCACATTGG	40-42	754
c.4576_4579dupGGGT	GTTGGTTGTGTGACCAGTGC	CCCAGGAAGTGTGTACAGC*		
	GTGACCAGTGCTTCGGGA	CAGTACTACCATTGAGGATGG	20-24	559
c.949C>A	CAATGGCGTGGAGAAGGATC	GGAGTAAAATTGGAAAGCTG		
	CTCTAAGTGGTTCAATTACAG	GAAGACAGTTGACAGAATCAG	4-7	598
		CAACTGAAATAGTCACTCC		

\*: primer from van Wijk et al., (2004).

Supp. Table S3. Effect of Usher variants on splicing by *in silico* analysis

Usher gene	Variant	splice site type	HSF						Fruit Fly Score			NetGene2 Confidence		
			HSF matrice			Max Ent Score			Natural	Wild Type	Mutant	Natural	Wild Type	Mutant
			Natural	Wild Type	Mutant	Natural	Wild Type	Mutant						
CDH23	c.3580-1G>T	acceptor	88.76	88.76	59.82	8.92	8.92	0.32	0.91	0.91	nr	0.96	0.96	nr
	c.6050-9G>A	acceptor	81.56	54.41	83.36	6.34	(-) 4.4	3.54	nr	nr	nr	0.18	nr	0.25
MYO7A	c.3719G>A	acceptor	87.19	46.69	75.63	1.86	(-) 6.56	1.39	nr	nr	nr	0.2	nr	nr
	c.3979G>A	acceptor	91.68	nr	nr	3.16	nr	nr	0.99	nr	nr	1	nr	nr
USH2A	c.2168-1G>C	acceptor	88.04	88.04	59.09	8.95	8.95	0.89	0.97	0.97	nr	0.97	0.97	nr
				78.9	81.44		1.8	7.11		nr	0.71			
	c.7595-8C>G	acceptor	91.26	91.26	88.72	7.95	7.95	1.31	0.93	0.93	0.86	0.48	0.48	0.27
				47.5	76.45		0.1	8.16						
	c.4576-4579dupGGGT	donnor	95.25	70.11	83.66	10.65	2.85	3.82	1	nr	nr	0.63	nr	0.89
c.949C>A	donnor	85.75	70.44	75.19	9.43	4.48	7.67	0.94	nr	0.85	0.91	nr	0.54	

nr : not recognized.