Complete exon sequencing of all known Usher syndrome genes greatly improves molecular diagnosis

Crystel Bonnet1,2†, M’hamed Grati1,2,30†, Sandrine Marlin1, Jacqueline Levilliers2, Jean-Pierre Hardelin2, Marine Parodi3, Magali Niasme-Grare3, Diana Zelenika4, Marc Délépine5, Delphine Feldmann1,3, Laurence Jonard1,3, Aziz El-Amraoui2, Dominique Weil2, Bruno Delobel5, Christophe Vincent6, Hélène Dollfus7, Marie-Madeleine Eliot8, Albert David9, Catherine Calais10, Jacqueline Vigneron11, Bettina Montaut-Verient12, Dominique Bonneau13, Jacques Dubin14, Christel Thauvin15, Alain Duvillard16, Christine Francannet17, Thierry Mom18, Didier Lacombe19, François Duriez20, Valérie Drouin-Garraud21, Marie-Françoise Thuillier-Obstoy22, Sabine Sigaudy23, Anne-Marie Frances24, Patrick Collignon24, Georges Challe25, Rémy Couderc1, Rémy Couderc1, Mark Lathrop4, José-Alain Sahel26, Jean Weissman27, Christine Petit2,28*, and Françoise Denoyelle2,29*

Abstract

Background: Usher syndrome (USH) combines sensorineural deafness with blindness. It is inherited in an autosomal recessive mode. Early diagnosis is critical for adapted educational and patient management choices, and for genetic counseling. To date, nine causative genes have been identified for the three clinical subtypes (USH1, USH2 and USH3). Current diagnostic strategies make use of a genotyping microarray that is based on the previously reported mutations. The purpose of this study was to design a more accurate molecular diagnosis tool.

Methods: We sequenced the 366 coding exons and flanking regions of the nine known USH genes, in 54 USH patients (27 USH1, 21 USH2 and 6 USH3).

Results: Biallelic mutations were detected in 39 patients (72%) and monoallelic mutations in an additional 10 patients (18.5%). In addition to biallelic mutations in one of the USH genes, presumably pathogenic mutations in another USH gene were detected in seven patients (13%), and another patient carried monoallelic mutations in three different USH genes. Notably, none of the USH3 patients carried detectable mutations in the only known USH3 gene, whereas they all carried mutations in USH2 genes. Most importantly, the currently used microarray would have detected only 30 of the 81 different mutations that we found, of which 39 (48%) were novel.

Conclusions: Based on these results, complete exon sequencing of the currently known USH genes stands as a definite improvement for molecular diagnosis of this disease, which is of utmost importance in the perspective of gene therapy.

Background

Usher syndrome (USH, MIM 276900, MIM 276905, MIM 605472) combines sensorineural hearing impairment with retinitis pigmentosa [1]. In addition, vestibular dysfunction can be observed in some patients. USH occurs in ~1/20,000 individuals, and represents 50% of all monogenic deaf-blindness cases. Three clinical subtypes can be distinguished. USH type I (USH1) is characterized by severe to profound congenital hearing impairment, prepubertal onset of retinitis pigmentosa, and vestibular areflexia. USH type II (USH2) combines congenital moderate to severe hearing impairment, onset of retinitis pigmentosa in the first or second decade of life, and absence of vestibular dysfunction.
Finally, USH type III (USH3) patients present with congenital or early onset progressive hearing impairment, variable age of onset and severity of retinitis pigmentosa, and variable vestibular dysfunction. USH is inherited in the autosomal recessive mode, and is genetically heterogeneous. To date, nine causative genes have been identified. Mutations in MYO7A [2], USH1C [3,4], CDH23 [5,6], PCDH15 [7,8] and USH1G [9] cause USH1, mutations in USH2A [10], VLGR1 [11] and WHRN [12] cause USH2, and mutations in USH3A [13] cause USH3. Mutations in MYO7A [14-16], USH1C [17,18], CDH23 [6], PCDH15 [17] and WHRN [19] have also been reported in patients affected by hearing impairment only, while USH2A is also involved in isolated retinitis pigmentosa [20].

The USH1 genes encode the actin-based motor protein myosin VIIa (USH1B), two Ca²⁺-dependent transmembrane adhesion proteins, cadherin-23 (USH1D) and protocadherin-15 (USH1F), the PDZ domain-containing submembrane protein harmonin (USH1C), and the scaffold protein sans that contains ankyrin repeats and a sterile alpha motif domain (USH1G). The USH2 genes encode two large transmembrane proteins, usherin (USH2A) and VLGR1 (very large G protein-coupled receptor, USH2C), and the PDZ domain-containing submembrane protein whirlin (USH2D). Finally, USH3A encodes the four-transmembrane-domain protein clarin-1. Each USH1 gene encodes several protein isoforms, except MYO7A and USH1G.

Absence of an early diagnosis of USH is devastating. In USH1 patients, sign language becomes a less and less efficient mode of communication as the visual defect progresses, and ultimately, the patients may become unable to communicate except by tactile exchanges. As a result of an early diagnosis of USH1, early bilateral cochlear implantation allowing the development of an oral mode of communication and early physical therapy for vestibular disorders are strongly recommended. The early diagnosis is also critical for genetic counseling, educational orientation and therapeutic management, which may include retinal gene therapy in the future [21,22]. So far, a comprehensive molecular diagnosis of USH has been hampered both by the genetic heterogeneity of the disease and the large number of exons for six out of the nine known USH genes. The five USH1, three USH2, and one USH3 genes are collectively composed of 183, 173, and five coding exons, respectively [23].

Cremers and collaborators have developed a genotyping microarray for USH, based on the arrayed primer extension (APEX) method. This approach, in a first version, included the analysis of 298 USH-associated sequence variants located in eight genes: MYO7A, USH1C, CDH23, PCDH15, USH1G, USH2A, VLGR1 and USH3A [24]. The mutations detected by the array subsequently increased, and currently include 612 previously identified disease-associated variants in the nine known USH genes [25]. The selected variants were prevalent in the following European countries: Belgium, Denmark, UK, Germany, Italy, Spain, Switzerland and Netherlands, and in the USA. The authors could prove that the chip, with >98% accuracy, is an adaptable and affordable mutation screening tool. However, the efficiency of the chip was both dependent on the USH subtype examined and the studied population, ranging from 30% in the USA to 80% in Denmark in USH1 cases [24]. Recently, Jaijo et al., using an intermediate genotyping microarray (429 reported mutations), found mutations in only 34% of the patients tested [26], which is indicative of a large number of private mutations. Therefore, improvement of the molecular diagnosis is needed.

Alternative strategies include direct sequencing of USH gene coding exons [27-30]. To determine the most efficient strategy, some critical information is, however, still lacking. Is the clinically diagnosed USH subtype a reliable indication of the causative gene? What is the frequency of digenic/oligogenic inheritance in this disease? Such a mode of inheritance is suggested by the colocalization and direct in vitro interactions of the USH1 proteins [31-39], and of the USH2 proteins [40,41]. In a few USH1 patients, digenic inheritance involving PCDH15 and CDH23 has indeed been reported [42]. To address these issues, we undertook a large-scale mutation screening of all currently known USH genes in a cohort of 54 USH patients.

Subjects and Methods
Subjects
Fifty-four unrelated Caucasian patients including five patients originating from Maghreb were included in the study. Most patients were referred to Armand-Trousseau Children’s Hospital in Paris, and other patients were referred to genetic consultations throughout France. All patients were tested by audiograms and electroretinogram. Auditory function was assessed by oto- scopy, tympanometry, standard pure tone audiometry, and recording of auditory brainstem responses and otoacoustic emissions. The cochlear origin of the hearing impairment was confirmed by auditory brainstem responses, and by the absence of otoacoustic emissions. USH was diagnosed on the basis of simultaneous occurrence of sensorineural deafness and retinal degeneration. Scrutiny of the time of onset, evolution and severity of the hearing impairment, and quality of vestibular responses enabled to assign the patients to one of the three clinical types of the disease [43]. Patients were considered as USH3 when their hearing impairment had been detected in adulthood and showed clear progressiveness. For these patients, vestibular function
determined by caloric tests was normal. Parents of most of the patients were available for the study, and had normal hearing. This study was approved by the local ethics committee, and written consent for genetic testing was obtained from adult probands or parents of minor patients.

**PCR amplification and sequencing**

Genomic DNA was extracted from peripheral blood using standard procedures. The coding exons and flanking intronic sequences of all nine USH genes were amplified and sequenced using forward and reverse primers (primer sequences and conditions available upon request). We also searched for the previously reported 684 kb deletion in *PCDH15* using the reported primers [44]. Sequences were run on ABI 3100 DNA analyzer, and assembled using ABI Prism Seqscape 2.1 to Genbank reference sequences [45].

**Control DNAs**

The genomic DNAs from 153 unaffected Caucasian control individuals were sequenced (306 control alleles). For the mutations possibly involved in oligogenic inheritance, DNAs from 333 healthy unrelated Caucasian individuals were used as controls. For the mutations present in patients originating from Maghreb, the DNAs from 95 Moroccan and 91 Algerian healthy unrelated individuals were used as controls.

**In silico analysis of sequence variants**

The SIFT (Sorting Intolerant from Tolerant) [46] and Polyphen [47] software programs were used to predict the influence of any amino acid substitution on the protein structure and function. NetGene2 [48] and “Splice site prediction by neural network” [49] interfaces were used to predict the influence of nucleic acid substitutions on splice donor and acceptor sites. Presence of Exonic Splicing Enhancers (ESE) was detected using ESE Finder [50].

**Segregation analysis**

Segregation of all sequence variants identified in the patients was studied by sequencing the corresponding DNA fragments in the parents and other relatives. In all patients carrying two distinct mutations in a given USH gene, biallelic transmission was confirmed by the segregation analysis.

**Mutation nomenclature**

The mutation nomenclature complies with the mutation nomenclature correction tool Mutalyzer [51] according to the HGVS Guidelines & Recommendations [52]. The +1 position in mutation numbering corresponds to the A of the ATG initiation codon.

**Protein Accession numbers**

MYO7A, [Swiss-Prot:Q13402]; USH1C, [Swiss-Prot: Q7RTU8]; CDH23, [Swiss-Prot:Q9H251]; PCDH15-CD1, [Swiss-Prot:Q96QU1]; PCDH15-CD2, [NCBI-RefSeq: NP_001136241.1]; PCDH15-CD3, [Swiss-Prot:C9J4F3]; USH1G, [Swiss-Prot:Q495M9]; USH2A, [Swiss-Prot:075445]; VLGR1, [Swiss-Prot:Q8WXX9]; WHRN, [Swiss-Prot:Q9P202]; USH3A, [Swiss-Prot:P58418] and [Swiss-Prot:P58418-1] for “a” and “c” variants, respectively.

**Results**

**Mutation analysis: high prevalence of novel mutations**

We analyzed the nine USH genes in a cohort of 54 French patients, of whom 27 were affected by USH1, 21 by USH2, and six by USH3. From the patient and parent questionnaires, consanguinity was established for nine families (see Table 1). Sequencing of the coding and non coding exons of all currently known USH genes was carried out in every patient. Screening for predicted causative missense and splice site mutations was performed using prediction software programs. Amino acid substitutions were considered likely to be pathogenic missense mutations when predicted possibly or probably deleterious by Polyphen software and not-tolerated by the SIFT program. Nucleotide variations were considered likely to be splice site mutations when predicted highly confident donor or acceptor site mutations by Netgene2 and “Splice site prediction by neural network” programs. These sequence variants were ultimately classified as presumably pathogenic mutations only if the affected amino acid residues were evolutionarily conserved (Additional file 1 Figures S1 to S3) and/or these variants were not found in the control individuals (see Subjects and Methods).

A total of 81 distinct, presumably pathogenic mutations were detected, specifically, 16 nonsense mutations, five nucleotide duplications, 17 frame-shifting deletions, seven splicing defect-causing mutations, 34 missense mutations, and one isocoding variation. Thirty-nine (48%) of these mutations, i.e. 27% to 100% of the mutations found in each USH gene, had not been previously reported (Tables 2, 3 and 4, Figure 1). In addition, 103 amino acid substitutions were classified as presumably nonpathogenic sequence variants, including 33 new variants and six variants that had previously been reported as pathogenic mutations (Table 5). Numerous, presumably neutral, isocoding and intronic variants were also observed (listed in Additional file 2, Table S1).

Twenty-six pathogenic or presumably pathogenic mutations in MYO7A were found in 19 patients, specifically, eight nonsense mutations, one nucleotide duplication, five nucleotide deletions, four splice site mutations, and eight missense mutations. Seven of these mutations had not been previously reported, including two
| Genes   | MYO7A | USH1C | CDH23 | PCDH15 | USH1G | USH2A | VLGR1 | WHRN | USH3A |
|---------|-------|-------|-------|--------|-------|-------|-------|-------|-------|
| Patient | USH   | type  |       |        |       |       |       |       |       |
| U37     | I      | [p.R666X] + [p.E1917X] |       |       |       |       |       |       |       |
| U57     | I      | [p.C1198X] + [p.R1240Q] |       |       |       |       |       |       |       |
| P0485   | I      | [p.Q1798X] + [p.E1917X] |       |       |       |       |       |       |       |
| U14     | C      | [p.R972X] + [p.R972X] |       |       |       |       |       |       |       |
| U9      | C      | [p.K164X] + [p.K164X] |       |       |       |       |       |       |       |
| U36     | I      | [p.R2024X] + [p.G519D] | [p.R1060W] |       |       |       |       |       |       |
| U20     | I      | [p.R669X] + [p.R1883Q] |       |       |       |       |       |       |       |
| P0505   | I      | [p.Q1798X] + [p.A2009fsX32] |       |       |       |       |       |       |       |
| S1556   | C      | [p.H133fsX7] + [p.H133fsX7] |       |       |       |       |       |       |       |
| S1295   | C      | [p.Y1302fsX97] + [p.Y1302fsX97] | [p.G1301V] | [p.Q5459H] |       |       |       |       |       |
| P0504   | I      | [p.D75fsX31] + [p.R1240Q] |       |       |       |       |       |       |       |
| U45     | I      | [p.D75fsX31] + [p.T165M] |       |       |       |       |       |       |       |
| P0411   | C      | [c.2283-1G>T] + [c.2283-1G>T] | [p.D4707Y] |       |       |       |       |       |       |
| P0070   | I      | [p.G163R] + [p.A198T] |       |       |       |       |       |       |       |
| P0052   | I      | [c.1690+1G>A] + [p.F1963del] |       |       |       |       |       |       |       |
| U3      | I      | [p.L2186P] | [p.L16V] | [p.C3307W] |       |       |       |       |       |
| DID     | I      | [p.R80fsX69] + [p.R80fsX69] | [p.R3043W] |       |       |       |       |       |       |
| U47     | I      | [p.R80fsX69] + [p.R103H] |       |       |       |       |       |       |       |
| P0469   | I      | [p.E2135fsX3] + [c.6050-9G>A] |       |       |       |       |       |       |       |
| S1212   | I      | [p.R1279P] + [p.D2639G] |       |       |       |       |       |       |       |
| U38     | I      | [p.R991X] | [p.D2639G] | [p.W38X] |       |       |       |       |       |
| S1530   | I      | [p.R1273S] |       |       |       |       |       |       |       |
| P0257   | I      |       |       |       |       |       |       |       |       |
| Genotype | [p.A457V] + [p.K269del] |
|----------|-------------------------|
| U6       | [p.E767fsX21] + [p.E767fsX21] |
| U24      | [p.P1220L] |
| U48      | [p.W3955X] + [p.R2509fsX19] |
| P0483    | [p.E1492X] + [p.T3571M] |
| P0418    | [p.K268R] |
| U56      | [p.T2991fsX61] + [p.T2991fsX61] |
| U42      | [p.E767fsX21] + [p.Y4128fsX24] |
| P0449    | [p.E767fsX21] + [p.C575Y] |
| P0493    | [p.H308fsX16] + [p.T4809M] |
| P0432    | [p.R1189W] |
| U51      | [p.V218E] + [p.R317R] |
| P0511    | [p.T3571M] + [p.T3520] |
| U49      | [p.E4321X] + [p.Q753fsX8] |
| P0473    | [p.P522fsX8] + [p.S11R] |
| US8      | [p.F112fsX29] + [p.H3399P] |
| P0463    | [p.E4186fsX17] |
| U10      |  |
| U53      | [p.P246fsX13] + [p.P246fsX13] |
| U19      | [p.H755Y] |
| P0426    |  |
| U21      | [p.Y1730fsX6] + [c.10586-1G>C] |
nonsense mutations (p.K164X, p.C1198X), a nucleotide duplication (c.397dupC; p.H133fsX7), a nucleotide deletion c.3904delT (p.Y1302fsX97), a nucleotide substitution (c.1690+1G>A) predicted to alter the splice donor site of intron 14, and two missense mutations (p.K268R and p.P1220L) that change amino acid residues located in the motor head and the first MyTH4 domain of the myosin VIIa tail, respectively (Tables 2, 3 and Figure 1).

Three distinct pathogenic or presumably pathogenic mutations in USH1C were detected in three patients, specifically, a nucleotide duplication (c.238_239dupC; p.R80fsX69) already reported in several patients [3,4,27,53], a known missense mutation (p.R103H) affecting an amino acid residue located in the PDZ1 domain of the protein [27], and a novel missense mutation (p.R357W), predicted to affect the first coiled-coil domain of the protein. These mutations are expected to affect the three classes of harmonin isoforms (Tables 2, 3, Figure 1) [4].

Eight pathogenic or presumably pathogenic mutations in CDH23 were found in six patients, specifically, a previously reported mutation that affects splicing (c.6050-9G>A) [54], a novel nucleotide deletion (c.6404_6405delAG; p.E2135fsX21), and six missense mutations [55,56], four of which (p.R1189W, p.R1379P, p.D2639G, and p.P1220L) had not been previously reported. They affect amino acid residues located in the 11th, 13th and 25th cadherin repeat and the extracellular region adjacent to the transmembrane domain (3065-3085), respectively (Tables 2, 3 Figure 1). Intriguingly, the p.R1060W mutation, which affects a residue in the 10th cadherin repeat that belongs to a canonical motif (DRE) predicted to bind Ca2+ [57], has previously been reported in an isolated form of deafness, DFNB12 (cited in Astuto et al. [55]).

Two pathogenic or presumably pathogenic mutations in PCDH15, specifically, a nonsense mutation (p.R991X) [27] and a novel missense mutation (p.R1273S), were found in two patients. The missense mutation affects an amino acid residue located immediately after the 11th cadherin repeat (Tables 2, 3, Figure 1). The large genomic rearrangement in PCDH15 previously reported by Le Guedard et al. [44] was not detected in this group of patients.

Three pathogenic or presumably pathogenic mutations in USH1G were found in three patients, specifically, an already reported nonsense mutation (p.W38X) [58], a novel nucleotide duplication (c.84dupC; p.D29fsX29), and a novel sequence variant (c.46C>G; p.L16V). This variant was absent from the control DNAs (0/666 alleles) and, according to the prediction software programs (NetGene2 and ESE finder), should create a splice donor site resulting in a premature stop codon at codon position 17 (Tables 2, 3, Figure 1).

Twenty-five pathogenic or presumably pathogenic mutations in USH2A were found in 17 patients including three USH3 patients, specifically, five nonsense mutations, one nucleotide duplication, six nucleotide deletions [59], two splice site mutations, 10 missense mutations, and one isocoding variation possibly creating a splice donor site (Tables 2, 3). All these mutations affect the extracellular region of usherin (Figure 1). Nine mutations had not been previously reported, specifically, five frame-shifting deletions (c.4030_4037del ATGGCTGG/p.M1344fsX42, c.5189_5199delATATGTTTCAT/p.Y1730fsX6, c.7522delT/p.R2509fsX19, c.8970_8971delCA/p.T2991fsX61, and c.12381_12382delCT/p.Y4128fsX24), one splice acceptor site mutation (c.10586-1G>C) that is expected to result in exon 54 skipping and premature termination of the protein, and three missense mutations (p.C575Y, p.G1301V, p.C3307W) that affect amino acid residues located in the 14th fibronectin type III domain and the trideca-cysteine domain (residue 3192 to 3371) between the 18th and the 19th fibronectin type III domains (Figure 1). Notably, the isocoding mutation (c.949C>A; p.R317R) has been predicted to be pathogenic by Penning [60] and considered as nonpathogenic by Dreyer [28]. Segregation analysis in our family was compatible with a pathogenic effect of this mutation (Additional file 1 Figure S4).

Eleven pathogenic or presumably pathogenic mutations in VLGR1 were detected in eight patients including two USH3 patients. All were novel mutations, specifically, a nonsense mutation (p.E4321X), a nucleotide duplication (c.1563dupT; p.D29fsX29), four nucleotide deletions (c.333_334delTT/p.R112fsX29, c.2258_2270del
Table 2 Pathogenic DNA variants

| Gene    | Nucleotide change | Exon | Amino acid change         | Frequency in USH alleles (×/108) | Frequency in control alleles | Patient origin & reference |
|---------|-------------------|------|---------------------------|----------------------------------|-----------------------------|---------------------------|
| **MYO7A** |                   |      |                           |                                  |                             |                           |
| 223delG |                   | 4    | D75fsX31                  | 2                                |                             | Australia, Italy, France [78] |
| 397dupC |                   | 5    | H133fsX7                  | 2                                |                             | This study                |
| 490A>T  |                   | 6    | K164X                     | 2                                |                             | This study                |
| 592G>A  |                   | 6    | A198T + splice defect     | 1                                | 0/306                       | Algeria [27]              |
| 1556G>A |                   | 14   | GS19D/splice defect       | 1                                | 0/306                       | USA, France [63]          |
| 1690+1G>A|                  | 14   | Splice defect             | 1                                |                             | This study                |
| 1996C>T |                   | 17   | R666X                     | 1                                |                             | Great Britain, Denmark [62] |
| 2005C>T |                   | 17   | R669X                     | 1                                |                             | USA [24]                  |
| 2283-1G>T|                  | 20   | Splice defect             | 2                                |                             | Algeria [27]              |
| 2914C>T |                   | 24   | R972X                     | 2                                |                             | Pakistan [79]             |
| 3594C>A |                   | 28   | C1198X                    | 1                                |                             | This study                |
| 3904delT|                   | 30   | Y1302fsX97                | 2                                |                             | This study                |
| 5392C>T |                   | 39   | Q1798X                    | 2                                |                             | Denmark, German, Great Britain/France [62] |
| 5749G>T |                   | 42   | E1917X                    | 2                                |                             | unknown [80]              |
| 6025delG|                   | 44   | A2009fsX32                | 1                                |                             | Spain [63]                |
| 6070C>T |                   | 45   | R2024X                    | 1                                |                             | unknown [80]              |
| **USH1C** |                   |      |                           |                                  |                             |                           |
| 238_239dupC |             | 3    | R80fsX69                  | 3                                |                             | Pakistan, Europe, Guinea [4] |
| **CDH23** |                   |      |                           |                                  |                             |                           |
| 6050-9G>A|                  | 46   | Splice defect             | 1                                |                             | Germany [54]              |
| 6404_6405delAG |           | 47   | E2135fsX31                | 1                                |                             | This study                |
| **PCDH15** |                   |      |                           |                                  |                             |                           |
| 2971C>T |                   | 22   | R991X                     | 2                                |                             | France [27]               |
| **USH1G** |                   |      |                           |                                  |                             |                           |
| 84dupC  |                   | 1    | D29fsX29                  | 2                                |                             | This study                |
| 113G>A  |                   | 1    | W38X                      | 1                                |                             | USA [58]                  |
| **USH2A** |                   |      |                           |                                  |                             |                           |
| 920_923dupGCCA |           | 6    | H308fsX16                 | 1                                |                             | Denmark [81]              |
| 2299delG |                   | 13   | E767fsX21                 | 4                                |                             | Europe, USA, Africa, China [10] |
| 3920C>G  |                   | 18   | S1307X                    | 1                                |                             | France [82]               |
| 4030_4037delATGGCTGG |   | 18   | M1344fsX42                | 1                                |                             | This study                |
| 4474G>T  |                   | 21   | E1492X                    | 1                                |                             | Spain [83]                |
| 5189_5199delATATGTTTTCAT | | 26   | Y1730fsX6                 | 1                                |                             | This study                |
| 5776+1G>A |                  | 28   | Splice defect             | 1                                |                             | Norway [28]               |
| 7522delT |                   | 40   | R2509fsX19                | 1                                |                             | This study                |
| 8970_8971delCA |          | 45   | T2991fsX61                | 2                                |                             | This study                |
| 10586-1G>C |                  | 54   | Splice defect             | 1                                |                             | This study                |
| 10684G>T |                   | 54   | E3562X                    | 1                                |                             | Denmark, Norway [28]      |
| 11864G>A |                   | 61   | W3955X                    | 1                                |                             | Netherlands [84]          |
| 12381_12382delCT |         | 63   | Y4128fsX24                | 1                                |                             | This study                |
AAGTGCTGAAATC/p.Q753fsX8, c.12552_12553delGG/p.E4186fsX17), and five missense mutations (p.D1944N, p.H3399P, p.D4707Y, p.N4885S, p.Q5459H) that all affect amino acid residues located in the large extracellular region of the protein, between the 13th and 14th β-Calx domains, in the 4th Epilepsy Associated Repeat domain, in the 32nd β-Calx domain, between the 32nd and 33rd β-Calx domains, and in the 35th β-Calx domain, respectively (Tables 2, 3, Figure 1).

Three pathogenic or presumably pathogenic mutations in WHRN were detected in three patients including one USH3 patient, specifically, a novel deletion (c.737delC; p.P246fsX13), and two novel missense mutations (p.S11R and p.R379W) that affect amino acid residues located in the N-terminal Ala/Gly/Ser-rich stretch (aa 9-31) and immediately after the PDZ2 domain, respectively (Tables 2, 3, Figure 1).

Three pathogenic or presumably pathogenic mutations in WHRN were detected in three patients including one USH3 patient, specifically, a novel deletion (c.737delC; p.P246fsX13), and two novel missense mutations (p.S11R and p.R379W) that affect amino acid residues located in the N-terminal Ala/Gly/Ser-rich stretch (aa 9-31) and immediately after the PDZ2 domain, respectively (Tables 2, 3, Figure 1). Notably, these missense mutations only affect the longer whirlin isoform [19], which is a component of the ankle link molecular complex together with VLGR1 and usherin [40,41].

No mutations in USH3A were detected in our series of USH patients.

Transmission modes: evidence for digenic/oligogenic inheritance in some patients

We found mutations in 49 out of 54 (91%) USH patients, specifically, in 24 out of 27 (89%) USH1 patients, 19 out of 21 (90%) USH2 patients, and all six (100%) USH3 patients (see Table 1). Mutations in MYO7A, USH1C, CDH23, PCDH15, and USH1G, were found in 55%, 7%, 7%, 7%, and 4% of the USH1 cases, respectively. Mutations were detected on both alleles in 15 USH2 patients (including a consanguineous family), and on one allele in the remaining four USH2 patients. Finally, as regards the USH3 patients, biallelic mutations in USH2A and monoallelic mutations in VLGR1 or WHRN were found in three patients, two patients, and one patient, respectively.

One USH1 and two USH2 patients were heterozygotes for mutations in two or three USH genes, suggesting a possible digenic/oligogenic inheritance of the syndrome. In the USH2 patients, however, segregation analysis did not support digenic inheritance. Patient P0418 carries a nonsense mutation in USH2A (p.S5030X) and a missense mutation in MYO7A (p.K268R), but his brother, who is also clinically affected, does not carry the MYO7A mutation. Patient P0432 has a c.4030_4037delATGGCTGG (p.M1344fsX42) mutation in USH2A and a missense mutation in CDH23 (p.R1189W), but his father, who has neither deafness nor retinitis pigmentosa, also carries these two mutations, and his clinically affected sister does not carry the mutation in CDH23. In the USH1 patient, we found three presumably pathogenic mutations in MYO7A (c.6657T>C), USH1G (c.46C>G; p.L16V) and USH2A (c.9921T>G). Her father carries the mutations in MYO7A and USH2A without displaying symptoms of the disease, whilst her unaffected mother carries the mutation in USH1G. The mutations in MYO7A, USH1G and USH2A were not found in 666 control alleles. Of the four siblings, the affected girl is the only one who carries the mutations in MYO7A and USH1G, and, all the more, the mutations in the three genes (Figure 2). Therefore, a combination of monoallelic mutations in three USH genes may be responsible for the disease in this patient.
Table 3 Presumably pathogenic DNA variants

| Gene   | Nucleotide change | Exon | Amino acid change | Protein domain       | Frequency in USH alleles (x/108) | Frequency in control alleles | Patient origin & reference |
|--------|-------------------|------|-------------------|----------------------|---------------------------------|-----------------------------|---------------------------|
| MYO7A  | 487G>C            | 6    | G163R             | Motor head           | 1                               | 0/306                       | Algeria [27]              |
|        | 494C>T            | 6    | T165M             | Motor head           | 1                               | 0/306                       | Great Britain, France [58]|
|        | 803A>G            | 8    | K268R             | Motor head           | 1                               | 0/306                       | This study                |
|        | 805_807delAAAG    | 8    | K269del           | Motor head           | 1                               | 0/306                       | Italy, France [63]        |
|        | 1370C>T           | 13   | A457V             | Motor head           | 1                               | 0/306                       | Ireland, France [63]      |
|        | 3659C>T           | 29   | P1220L            | MyTH4 (1)            | 1                               | 0/666                       | This study                |
|        | 3719G>A           | 29   | R1240Q            | MyTH4 (1)            | 2                               | 0/306                       | Denmark, Great Britain/France [62] |
|        | 5648G>A           | 41   | R1883Q            | MyTH4 (2)            | 1                               | 0/306                       | USA [58]                  |
|        | 5887_5890delTTTC   | 43   | F1963del          | FERM (2)             | 1                               | 0/666                       | Europe, USA [24]          |
|        | 6657T>C           | 48   | L2186P            | FERM (2)             | 1                               | 0/666                       | France [85]               |
| USH1C  | 308G>A            | 4    | R103H             | PDZ1                 | 1                               | 0/306                       | France [27]               |
|        | 1069C>T           | 13   | R357W             | Coiled-coil          | 1                               | 0/498                       | This study                |
| CDH23  | 2263C>T           | 20   | H755Y             | cd7                  | 1                               | 0/306                       | USA [56]                  |
|        | 3178C>T           | 26   | R1060W            | cd10                 | 1                               | 0/626                       | Europe [53]               |
|        | 3565C>T           | 29   | R1189W            | cd11                 | 1                               | 0/306                       | This study                |
|        | 4136G>C           | 33   | R1379P            | cd13                 | 1                               | 0/306                       | This study                |
|        | 7916A>G           | 55   | D2639G            | cd25                 | 1                               | 0/306                       | This study                |
|        | 9127C>T           | 62   | R3043W            | adjacent to TM (extracellular) | 1                           | 0/490                       | This study                |
| PCDH15 | 3817C>A           | 29   | R1273S            | cd11                 | 1                               | 0/306                       | This study                |
| USH1G  | 46C>G             | 1    | L16V              | 1                    | 0/666                          | This study                  |
| USH2A  | 653T>A            | 4    | V218E             | Nter laminin         | 1                               | 0/306                       | Great Britain [86]        |
|        | 908G>A            | 6    | R303H             | Nter laminin         | 1                               | 0/306                       | USA [87]                  |
|        | 949C>A            | 6    | R317R             | Nter laminin         | 1                               | 0/306                       | Netherlands [60]          |
|        | 1055C>T           | 6    | T352I             | Nter laminin         | 1                               | 0/306                       | Norway [28]               |
|        | 1606T>C           | 9    | C536R             | 1st laminin EGF-like | 1                               | 0/306                       | Denmark [81]              |
|        | 1724G>A           | 10   | C575Y             | 2nd laminin EGF-like | 1                               | 0/306                       | This study                |
|        | 3902G>T           | 18   | G1301V            | 14th FnIII           | 1                               | 0/484                       | This study                |
|        | 8254G>A           | 42   | G2752R            | 3rd laminin EGF-like | 1                               | 0/306                       | Japan [88]                |
|        | 9921T>G           | 50   | C3307W            | 18th-19th FnIII      | 1                               | 0/482                       | This study                |
|        | 10712C>T          | 54   | T3571M            | 20th FnIII           | 2                               | 0/306                       | Spain [89]                |
|        | 14426C>T          | 66   | T4809I            | 33rd FnIII           | 1                               | 0/306                       | Canada [90]               |
| VLGRI  | 5830G>A           | 28   | D1944N            | 13th -14th β-Calx    | 1                               | 0/306                       | This study                |
|        | 10196A>C          | 49   | H3399P            | 4th EAR              | 1                               | 0/306                       | This study                |
Seven patients out of 54 (13%) carried two presumably pathogenic mutations in an USH gene, plus one or two additional mutations in another USH gene. Taking into account only the 39 patients for whom biallelic mutations have been identified, 18% (7 out of 39) carry additional mutations. Specifically, five USH1 patients carried biallelic mutations in an USH1 gene plus one or two additional mutations in another USH1 (three patients) or USH2 (two patients) gene, and two USH2 patients carried biallelic mutations in USH2 genes plus one additional, presumably pathogenic mutation in an USH1 or an USH2 gene (Table 1). Parents and siblings available in six out of seven families indeed showed that the two mutations present in the same gene originated from one parent each (Figure 3). The mutations found in the genes that were mutated on both alleles in the patients consist of two nonsense mutations, five nucleotide deletions, one splice site mutation, and three missense mutations. The eight additional mutations found in these patients were amino acid substitutions that were predicted “probably damaging” and “not tolerated” by Polyphen and SIFT program, respectively. One of these mutations, p.R1060W in CDH23, has already been reported in USH patients [55].

Discussion

The major goal of the study was to design a powerful and reliable strategy for molecular diagnosis of USH. For that purpose, some essential, so far missing information was gathered by: i) comparing the strategy for mutation detection currently in use with the here developed USH exome sequencing (including splice sites), ii) determination of whether the phenotype can restrict the mutation screening to the USH genes corresponding to the clinical subtype in a given patient, and iii) defining the possible existence of digenic/oligogenic inheritance of the disease in some patients.

We found mutations in eight of the currently known nine USH genes, in 49 out of 54 (91%) patients (Table 1). Two or more mutations were identified in 41 patients, including 39 patients (72%) with biallelic mutations, and one mutation was found in the remaining seven patients (13%), that is a total of 81 different mutations. Current diagnostic strategies use a genotyping microarray based on the arrayed primer extension method [24]. Were the international USH genotyping microarray used to identify the mutations, only 30 out of the 81 mutations (37%) would have been possibly detected because of the high prevalence of novel mutations, whatever the USH clinical type. Only 9 mutations previously reported as recurrent were detected in our series of patients (i.e. 11% of the mutations), specifically, c.1996C→T, c.223delG, c.1556G>A, c.494C→T, c.3719G>A and c.5749G>T in MYO7A, c.238_239dupC in USH1C, and c.2299delG and c.10712C>T in USH2A. Therefore, in the process of designing any strategy for USH molecular diagnosis, taking into account the high prevalence of novel mutations appears to be of major importance.

Previous mutation research studies performed in patients referred to medical genetic clinics showed high proportions of mutations for MYO7A, CDH23 and PCDH15 in USH1 patients [27], specifically, 29%-55% for MYO7A [61-64], 19%-35% for CDH23 [58], 11%-15% for PCDH15 [65], and for USH2A in USH2 patients [28,60,66], whereas the implication of VLGR1 and WHRN in the latter was minor [11,12]. The present analysis confirms these results by showing a major implication of MYO7A in USH1 (55% of the cases), and of USH2A in USH2 (62% of the cases).

Surprisingly, mutations were found in genes that did not fit the clinically diagnosed USH type. None of the six patients diagnosed as USH3 on the basis of the post-lingual onset and progressive nature of the deafness, and the absence of vestibular dysfunction (see Subjects and methods) carried a mutation in USH3A. Yet, mutations in USH2 genes were present in all of them, and with a gene distribution similar to that observed in USH2 patients. This finding, which concerns six out of 24 patients carrying mutations in USH2 genes, calls for a revision of the USH2 clinical features. Along the same line, one patient diagnosed as USH2, because he did not have a vestibular dysfunction, carried biallelic missense mutations in an USH1 gene, MYO7A. The two
mutations (p.A457V and p.K269del) affect amino acid residues located in the motor head of myosin VIIa, and have previously been reported in USH1 patients [63]. They may preserve a residual activity of the protein, thus causing less severe hearing, balance and visual impairments. Alternatively, one of these mutations or both might be deleterious for the myosin VIIa activity associated with the ankle-link protein complex that underlies the USH2 phenotype [40], but not with the transient hair bundle lateral-link and tip-link molecular complexes that are involved in USH1 pathogenesis. These phenotype/genotype discrepancies further argue in favor of a comprehensive mutation screening.

Table 4 Distribution of the pathogenic and presumably pathogenic mutations

| Pathogenic and presumably pathogenic mutations (Novel mutations) |
|---------------------------------------------------------------|
| MYO7A 26 (7) |
| USH1C 3 (1) |
| CDH23 8 (5) |
| PCDH15 2 (1) |
| USH1G 3 (2) |
| USH2A 25 (9) |
| VLGR1 11 (11) |
| WHRN 3 (3) |
| USH3A 0 |

Figure 1 Schematic representation of USH1 and USH2 proteins and localization of the novel, presumably pathogenic mutations. The long isoform of each USH protein is shown. *Splice site mutations. Abbreviations: IQ motifs, isoleucine-glutamine motifs; SAH, stable single α-helix; MyTH4, myosin tail homology 4; FERM, band 4.1-ezrin-radixin-moesin; PDZ, PSD95, discs large, ZO-1; PST, proline-serine-threonine-rich region; EC, extracellular cadherin; TM, transmembrane domain; Ank, ankyrin domains; cent, central region; SAM, sterile alpha motif; LamG, laminin G; LamG/TspN/PTX, N-terminal thrombospondin/pentaxin/laminin G-like domain; LamN/T, laminin N-terminal; EGF, Lam, laminin-type EGF-like; FnIII, fibronectin type III; VLGR1, very large G protein-coupled receptor; Ca2+, Ca2+-binding calcium exchanger β; EAR, Epilepsy Associated Repeats; Ala/Gly/Ser rich, alanine, glycine, and serine rich region; Pro rich, proline rich region.
Table 5 Presumably neutral missense variants

| Gene | Nucleotide change | Exon | Amino acid change | Frequency in USH alleles (×/108) | Frequency in control alleles | References |
|------|-------------------|------|-------------------|----------------------------------|------------------------------|------------|
| USH1C |                   |      |                   |                                  |                              |            |
|      | 2192G>A           | 21   | R731Q             | 1                                |                              | This study |
|      | 2457G>C           | 24   | E819D             | >10                              |                              | [92]       |
| CDH23 |                   |      |                   |                                  |                              |            |
|      | 7C>T              | 1    | R3C               | >10                              |                              | [55]       |
|      | 1469G>C           | 14   | G490A             | >10                              |                              | [55]       |
|      | 1487G>A           | 14   | S496N             | >10                              |                              | [55]       |
|      | 3625A>G           | 30   | T1209A*           | 1                                | 5/486                        | [55]       |
|      | 3664G>A           | 30   | A1222T            | 4                                |                              | [55]       |
|      | 4051G>A           | 31   | D1351N            | >10                              |                              | [55]       |
|      | 4310G>A           | 34   | R1437Q            | 6                                |                              | [55]       |
|      | 4723A>G           | 37   | T1575A            | >10                              |                              | [55]       |
|      | 4858G>A           | 38   | V1620M            | 1                                | 2/306                        | [55]       |
|      | 5023G>A           | 38   | V1675I            | >10                              |                              | [55]       |
|      | 5411G>A           | 41   | R1804Q            | >10                              |                              | [55]       |
|      | 5418C>G           | 41   | D1806E            | 2                                |                              | [93]       |
|      | 5692G>A           | 42   | A1898T            | 1                                | 0/306                        | This study |
|      | 5996C>G           | 45   | T1999S            | >10                              |                              | [55]       |
|      | 6137G>A           | 46   | E2044K            | >10                              |                              | [55]       |
|      | 6197G>A           | 46   | R2066Q            | 1                                | 0/306                        | [55]       |
|      | 6329C>T           | 47   | A2110V            | 1                                |                              | This study |
|      | 6596T>A           | 47   | I2199N            | 1                                | 0/306                        | This study |
|      | 6809G>A           | 48   | R2270H            | 1                                |                              | This study |
|      | 6847G>A           | 49   | V2283I            | 6                                |                              | [55]       |
|      | 6869C>T           | 49   | T2290M            | 1                                | 0/306                        | This study |
|      | 7073G>A           | 50   | R2358Q            | >10                              |                              | [55]       |
|      | 7139C>T           | 50   | P2380L            | >10                              |                              | [55]       |
|      | 7762G>C           | 54   | E2588Q            | 1                                | 1/306                        | [55]       |
|      | 9049G>A           | 61   | D3017N            | 1                                |                              | This study |
|      | 9373T>C           | 65   | F3125L            | 1                                | 7/306                        | [56]       |
|      | 9949G>A           | 69   | A3317T            | 1                                | 1/306                        | This study |
| PCDH15 |                 |      |                   |                                  |                              |            |
|      | 5ST>G             | 2    | S19A              | >10                              |                              | [94]       |
|      | 1039C>T           | 10   | L347F             | 1                                | 3/666                        | This study |
|      | 1138G>A           | 11   | G380S             | >10                              |                              | This study |
|      | 1304A>C           | 11   | D435A             | >10                              |                              | AL834134   |
|      | 1910A>G           | 15   | N637S             | 2                                |                              | [92]       |
|      | 2786G>A           | 21   | R929Q             | >10                              |                              | AL834134   |
|      | 4850A>G           | 34§  | N1617S            | 2                                |                              | This study |
|      | 4853A>C           | 36§  | E1618A            | >10                              |                              | This study |
Table 5 Presumably neutral missense variants (Continued)

| Gene   | Chromosome | Position | Variant | Allele 1 | Allele 2 | Effect | Association     |
|--------|------------|----------|---------|----------|----------|--------|-----------------|
| USH2A  |            |          |         |          |          |        |                 |
|        |            |          | 4982A>C | 37       | A>C      | >10    | This study      |
|        |            |          | 1434G>C | 8        | E>C      |        | [95]            |
|        |            |          | 1663C>G | 10       | S>S      | 1      | 0/306 [96]      |
|        |            |          | 1931A>T | 11       | D>D      | >10    |                 |
|        |            |          | 4457G>A | 21       | R>R      | >10    | AF055580        |
|        |            |          | 4994T>C | 25       | I>I      | >10    | [89]            |
|        |            |          | 6317T>C | 32       | I>I      | >10    | [89]            |
|        |            |          | 6506T>C | 34       | I>I      | >10    | [89]            |
|        |            |          | 6713A>C | 35       | E>E      | 6      | 5/306 [89]      |
|        |            |          | 6875G>A | 36       | R>R      | 4      | [28]            |
|        |            |          | 8624G>A | 43       | R>R      | 4      | [89]            |
|        |            |          | 8656C>T | 43       | L>L      | 4      | [89]            |
|        |            |          | 9008T>C | 45       | V>V      | 1      | This study      |
|        |            |          | 9262G>A | 47       | E>E      | 1      | 3/306 [28]      |
|        |            |          | 9296A>G | 47       | N>N      | 4      | [89]            |
|        |            |          | 9343A>G | 47       | T>T      | 3      | 5/306 [28]      |
|        |            |          | 9430G>A | 48       | D>D      | 4      | [89]            |
|        |            |          | 9595A>G | 49       | N>N      | 6      | [28]            |
|        |            |          | 10232A>C| 52       | E>E      | >10    | [89]            |
|        |            |          | 11504C>T| 59       | T>T      | >10    | [28]            |
|        |            |          | 11602A>G| 60       | M>M      | >10    | [89]            |
|        |            |          | 11677C>A| 60       | P>P      | 1      | 1/306 [28]      |
|        |            |          | 15091C>T| 70       | R>R      | 2      | 2/306 [28]      |
|        |            |          | 15377T>C| 71       | I>I      | 3      | 2/306 [87]      |
| VLRG1  |            |          |         |          |          |        |                 |
|        |            |          | 365C>T  | 4        | S>S      | >10    | This study      |
|        |            |          | P194H   | 6        | P>P      | 1      | 5/468 [28]      |
|        |            |          | 1033C>A | 7        | Q>Q      | 1      | This study      |
|        |            |          | 2261T>C | 12       | V>V      | 1      | 0/306 [28]      |
|        |            |          | 3289G>A | 17       | G>G      | 1      | 3/478 [28]      |
|        |            |          | 3482C>G | 19       | S>S      | 1      | 0/306 [28]      |
|        |            |          | 4939A>G | 23       | I>I      | >10    | This study      |
|        |            |          | 5780C>T | 28       | T>T      | >10    | [11]            |
|        |            |          | 5851G>A | 28       | V>V      | >10    | [11]            |
|        |            |          | 5953A>G | 28       | N>N      | >10    | [11]            |
|        |            |          | 5960C>T | 28       | P>P      | >10    | [11]            |
|        |            |          | 6012G>T | 28       | L>L      | >10    | [11]            |
|        |            |          | 6695A>G | 30       | Y>Y      | >10    | [11]            |
|        |            |          | 7034A>G | 32       | N>N      | >10    | [11]            |
|        |            |          | 7582C>T | 33       | P>P      | 1      | 1/306 [28]      |
|        |            |          | 7751A>G | 33       | N>N      | 10     | [97]            |
|        |            |          | 8291C>T | 36       | S>S      | 6      | [11]            |
|        |            |          | 8407G>A | 37       | A>A      | 4      | [11]            |
|        |            |          | 9280G>A | 43       | V>V      | >10    | This study      |
|        |            |          | 9743G>A | 45       | G>G      | >10    | [11]            |
|        |            |          | 9650C>T | 45       | A>A      | 2      | [11]            |
|        |            |          | 10411G>A| 49       | E>E      | >10    | [97]            |
|        |            |          | 10429G>T| 50       | D>D      | 1      | This study      |
Table 5 Presumably neutral missense variants (Continued)

| Variation   | Position | ID      | Frequency | Description | Source  |
|-------------|----------|---------|-----------|-------------|---------|
| 10490A>G    | 50       | Q3497R  | 1         | This study  |         |
| 10577T>C    | 51       | M3526T  | 3         | This study  |         |
| 10936T>C    | 52       | S3646P  | 3         | This study  |         |
| 11599G>A    | 56       | E3867K  | >10       | This study  |         |
| 12269C>A    | 59       | T4090N  | 2         | This study  |         |
| 14029T>C    | 69       | F4677L  | 1         | This study  | 2/478   |
| 14905T>C    | 73       | W4969R  | 2         | This study  |         |
| 17626G>A    | 82       | V5876I  | >10       | This study  |         |
| 18475A>G    | 88       | M6159V  | 2         | This study  |         |

WHRN

| Variation   | Position | ID      | Frequency | Description | Source  |
|-------------|----------|---------|-----------|-------------|---------|
| 229A>T      | 1        | T77S    | 1         | 1/468       | [98]    |
| 979G>A      | 4        | L327I   | 1         | This study  |         |
| 1318C>A     | 6        | A440T   | >10       | [99]        |
| 1838T>C     | 9        | M613T   | >10       | This study  |         |
| 2348T>C     | 10       | V783A   | >10       | [99]        |
| 2388C>A     | 10       | N796K   | >10       | [99]        |

Novel mutations are in bold. * Mutations considered pathogenic by prediction Software, but excluded by segregation studies. † Exons 34, 36 and 37 are specific to isoforms CD1, CD2 and CD3, respectively.

The pathogenicity of several exonic variants found in our patients and predicted to be pathogenic in previous studies and/or by prediction software was further investigated. The p.T1209A missense mutation in CDH23 has previously been reported in two affected families and considered as pathogenic [55,58]. However, we found it in five of 486 control alleles from French and Maghreban populations. The p.Y1719C missense mutation in MYO7A seems to represent a frequent sequence variant in the Moroccan population, with an estimated carrier frequency of 0.07 [100], and was observed in three out of 306 control alleles. The p.R302H mutation in MYO7A, which affects a residue within a non-conserved region of the motor domain, was detected in one out of 494 control alleles. Moreover, two of five different MYO7A cDNA clones isolated from three independent libraries were found to encode a histidine residue at codon position 302 [101], which further argues in favor of a non-pathogenic sequence variant. The p.E3088K missense mutation in USH2A, previously described by Dreyer et al., was present in three out of 306 control alleles, which argues in favor of a non-pathogenic sequence variant [26,28]. The missense mutation p.I5126T in USH2A has been reported as likely pathogenic [87]. We found it in two USH1 patients, who in addition carried two pathogenic mutations in MYO7A. We detected it in two individuals from the French control population, suggesting that it is a non-pathogenic sequence variant. The p.L555V mutation in USH2A has been found in homozygous state in one Spanish patient, together with a biallelic splice site variant (c.1841-2A>G) [26]. Numerous, presumably neutral, isocoding and intronic variants were also observed (listed in Additional file 2Table S1).

Figure 2 Segregation of the mutations in MYO7A, USH1G and USH2A in family U3. Arrow indicates the deaf proband.
procedure that includes genes seemingly inconsistent with the clinical classification of USH currently in use.

Notably, our study has revealed one case of likely oligogenic inheritance for USH1, involving MYO7A and USH1G, and possibly USH2A. Three cases of digenic inheritance of USH1 have been reported so far [42], all caused by mutations in CDH23 and PCDH15, in agreement with the contribution of cadherin-23 and protocadherin-15 to the hair bundle transient lateral links and tip-links [31,32,36,67-69]. The pathogenicity of the p.T1209A mutation in CDH23 [18,55] is, however, questionable since we found it in five alleles from the control population. The c.5601delAAC mutation in PCDH15, leading to an in frame-deletion of a threonine residue (p.T1868del) [42] within the intracellular domain of the protocadherin-15 CD1 isoform, also warrants a special mention. Three protocadherin-15 isoforms (CD1-3) that differ in their intracytoplasmic regions have been reported [69]. Already two presumably pathogenic mutations (p.M1853L and p.T1868del) [42,70] have been found in exon 34 that is specific for CD1. The p.T1209A mutation in CDH23 [18,55] is, however, questionable since we found it in five alleles from the control population. The c.5601delAAC mutation in PCDH15, leading to an in frame-deletion of a threonine residue (p.T1868del) [42] within the intracellular domain of the protocadherin-15 CD1 isoform, also warrants a special mention. The pathogenicity of the p.T1209A mutation in CDH23 [18,55] is, however, questionable since we found it in five alleles from the control population. The c.5601delAAC mutation in PCDH15, leading to an in frame-deletion of a threonine residue (p.T1868del) [42] within the intracellular domain of the protocadherin-15 CD1 isoform, also warrants a special mention. Three protocadherin-15 isoforms (CD1-3) that differ in their intracytoplasmic regions have been reported [69]. Already two presumably pathogenic mutations (p.M1853L and p.T1868del) [42,70] have been found in exon 34 that is specific for CD1. Incidentally, the p.T1209A mutation in CDH23 [18,55] is, however, questionable since we found it in five alleles from the control population. The c.5601delAAC mutation in PCDH15, leading to an in frame-deletion of a threonine residue (p.T1868del) [42] within the intracellular domain of the protocadherin-15 CD1 isoform, also warrants a special mention. The pathogenicity of the p.T1209A mutation in CDH23 [18,55] is, however, questionable since we found it in five alleles from the control population. Therefore, even though non-monogenic inheritance of USH appears to be rare, it has to be taken into consideration in the molecular diagnosis strategy. In addition, ten patients had presumably pathogenic mutations in two different USH genes. Seven of them had biallelic mutations in one gene, and carried an additional mutation in a second and, for one of them, a third USH gene. None of these additional mutations were nonsense or frame-shifting mutations, but the conservation of the corresponding amino acid residues in the orthologous genes (ush2a, myo7a, whrn) of Ciona savignyi [72], a cnidarian which is evolutionary distant of about 520 million years from man [73], argues in favor of their pathogenicity (Figure 4). Notably, these mutations were not found in 402 to 666 control alleles from populations of matched geographic origin. A substantial proportion of USH patients thus carry a third, presumably pathogenic mutation which, in some cases, may contribute to worsen the sensory defects resulting from missense mutations present in the “primary” USH gene.

Finally, no mutations were detected in five patients, specifically three USH1 and two USH2 patients. In patient S1823 (USH1), born from consanguineous parents, involvement of any of the nine currently known USH genes could be excluded by segregation analysis of polymorphic markers at the corresponding loci (data not shown). In the four remaining patients, the
mutations in a number of patients, we conclude that exon sequencing (including flanking splice sites) of all currently known USH genes is required for proper molecular diagnosis in every USH patient, both in the context of genetic counseling and in the perspective of retinal and cochlear gene therapy. The activity of the USH gene carrying biallelic mutations may indeed turn out to be only partly restored by gene therapy, and the presence of a third mutation in another USH gene may then critically impact on the benefits of the gene therapy. Moreover, as PDZD7 [76] has recently been reported to modify the phenotype in patients carrying mutations in USH2A or VLGR1 [77], future studies should also take into account modifier genes in the USH exome sequencing strategy.

Additional material

Additional file 1: Figure S1: Sequence alignment of amino acid residues mutated in patients carrying missense mutations in USH1 genes. Representative stretches of amino acid sequences from each of the USH1 proteins from various species were aligned. Identical residues in missense mutations are underlined. Protein ID accession numbers are indicated in parentheses. Orthologs of MYO7A, USH2A and WHRN are present in the cnidarian Ciona savignyi; they encode proteins that have 53.5%, 36.5%, and 24.7% (whirlin short isoform) of sequence identity with the human MYO7A, USH2A and WHRN orthologs, respectively. Notably, the P1220 residue of myosin VIIa, and the G1301 and C3307 residues of usherin, which are involved in the USH patients’ missense mutations, are conserved in C. savignyi. Incidentally, all the new USH2A missense mutations detected in our series of patients affect residues that are also conserved in this species.

Additional file 2: Table S1: Presumably neutral, isocoding and intronic variants in USH1 genes.

List of abbreviations

APEx: Arrayed Primer Extension; CDH23: Cadherin 23; DNA: Deoxyribonucleic Acid; ESE: Exonic Splicing Enhancers; MYO7A: Myosin VIIa; PCR: Polymerase Chain Reaction; PCDH15: Protocadherin 15; SIFT: Sorting Intolerant From Tolerant; USH: Usher syndrome; USH1: USH type I; USH2: USH type II; USH3: USH type III; VLGR1: Very Large G protein-coupled Receptor; WHRN: Whirlin.

Acknowledgements

We thank the patients and their families for participation in the study. We are very grateful to Laurent Abel, Jamila El Baghdadi and Cécile Julien for providing us with control DNA samples from Moroccan and Algerian individuals. We thank Corinne Chauve, Catherine Meunier, France Michel, and Isabelle Sargs for expert technical assistance. We also thank Sophie Bahaban, Anne-Flore Grange, Elizabeth Alden Landis and Patrick Joynt for collaboration. We thank Jean-Louis Mandel for helpful discussions. M.G. was supported by European Commission FP6 Integrated project, EuroHear (LSHG-CT-2004-512063) and C.B. by the French Foundation “Voir et Entendre”. This work was supported by Fondation R & G Strittmatter (under the aegis of Fondation de France), FAUN Stiftung (Scherdt Foundation), EC FP7 TREATUSH (HEALTH-F2-2010-242013), Foundation Fighting Blindness, and the FP7 TREATRUSH (HEALTH-F2-2010-242013), Foundation Fighting Blindness, and Isabelle Sargs for expert technical assistance. We also thank Sophie Bahaban, Anne-Flore Grange, Elizabeth Alden Landis and Patrick Joynt for collaboration. We thank Jean-Louis Mandel for helpful discussions. M.G. was supported by European Commission FP6 Integrated project, EuroHear (LSHG-CT-2004-512063) and C.B. by the French Foundation “Voir et Entendre”. This work was supported by Fondation R & G Strittmatter (under the aegis of Fondation de France), FAUN Stiftung (Scherdt Foundation), EC FP7 TREATUSH (HEALTH-F2-2010-242013), Foundation Fighting Blindness, and the FP7 TREATRUSH (HEALTH-F2-2010-242013), Foundation Fighting Blindness.

undetected mutations might still be located in the unexplored promoter regions or intragenic regulatory sequences of these genes, but may also be located in other, still unknown USH genes, as in patient S1823. Indeed, a new locus, USH1H, at chromosome 15q22-23 [74], and three candidate regions for new USH2 genes (2q32, 4q26 and 15q22-23) have been reported [75].

Conclusion

Direct exon sequencing of a set of specific disease genes is a reliable, easy set-up method, which remains less expensive than full exome sequencing in the patients. Based on the high prevalence of private mutations both in USH1 and USH2 patients, the substantial number of cases displaying genotype/phenotype discrepancy, and the presence of additional, presumably pathogenic
Bolz H, von Brederlow B, Ramirez A, Bryda EC, Kutsche K, Nothwang HG, France. 2Unité de Génétique et Physiologie de l’Hérédité, Hôpital Charles-Nicolle, Rouen, France. 22Service ORL Pédiatrique, Hôpital Charles-Nicolle, Strasbourg, France. 8Service ORL, Hôpital de Hautepierre, Strasbourg, France. 5. Online Mendelian Inheritance in Man (OMIM).

Authors

1. Online Mendelian Inheritance in Man (OMIM). [http://www.ncbi.nlm.nih.gov/omim].

References

1. Online Mendelian Inheritance in Man (OMIM). [http://www.ncbi.nlm.nih.gov/omim].

2. Weil D, Blanchard S, Kaplan J, Guilford P, Gibson F, Walsh J, Mburu P, Ahmed ZM, Riazuddin S, Makishima T, Ghosh M, Bokhari S, Ahmed Z, Smith TN, Riazuddin S, Menon PSN, Deshmukh D, Griffith AJ, Riazuddin S, et al. Nonsyndromic recessive deafness DFNB18 and Usher syndrome type IC are allelic mutations of the novel cadherin-like gene CDH23. Am J Hum Genet 2001, 68(1):36-37.

3. Ahmed ZM, Riazuddin S, Bernstein SL, Ahmed Z, Khan S, Griffith AJ, Morell RJ, Friedman TB, Riazuddin S, Wilcox ER. Mutations of the protocadherin gene PCDH15 cause Usher syndrome type 1F. Am J Hum Genet 2001, 69(1):25-34.
syndromes. Usher Syndrome Consortium. Am J Med Genet 1994, 50(1):32-38.
44. Le Guéraud S, Faugere V, Malcolm S, Claustres M, Roux AF. Large genomic rearrangements within the PCDH15 gene are a significant cause of Usher1F syndrome. Mol Vis 2007; 13:102-107.
45. GenBank. [http://ncbi.nlm.nih.gov/GenBank/].
46. SIFT (Sorting Intolerant from Tolerant). [http://blocks.fhcrc.org/sift/SIFT.html].
47. Polyphen. [http://genetics.bwh.harvard.edu/polyphen/].
48. NetGene2. [http://www.ncbi.nlm.nih.gov/Genbank/seq_tools/splice.html].
49. ESE Finder. [http://rulai.cshl.edu/tools/ESE/].
50. Mutilator. [http://www.humgen.nl/mutilator/1.0.1/].
51. HGVS recommendations. [http://www.hgvs.org/mutnomen].
52. Zheng L, Zhang Y, Whitlon DS, Garcia-Anoveros J, Barlow JS. Targeting of the hair cell proteins cadherin 23, harmonin, myosin VIIa, espin, and prestin in an epithelial cell line. J Neurosci 2010, 30(21):7178-7201.
53. von Bredow B, Bolz H, Jancke A, La OCA, Rudolph G, Lorenz B, Schwenger E, Gal A. Identification and in vitro expression of novel CDH23 mutations of patients with Usher syndrome type 1D. Hum Mutat 2002, 19(3):268-273.
54. Oshima A, Jaito T, Aller E, Millam J, Carney C, Usami S, Moller C, Kimbeling WJ. Mutation profile of the CDH23 gene in 56 probands with Usher syndrome type 1. Hum Mutat 2008, 29(6):537-46.
55. Satoharoy M, Weihofen WA, Gaudet B, Corey DP. Structural determinants of cadherin-23 function in hearing and deafness. Neuron 2010, 66(1):85-100.
56. Ouyang XM, Yan D, Du LL, Heijtmancik JF, Jacobson SG, Nance WE, Li AR, Angeli S, Kaiser M, Newton V, et al. Characterization of Usher syndrome type I gene mutations in an Usher syndrome patient population. Hum Genet 2005, 116(4):292-299.
57. Dreyer B, Tranegraaen B, Lyssett R, Rosenberg T, Moller C, Liu XZ, et al. A common ancestral origin of the frequent and widespread 2299delG USH2A mutation. Am J Hum Genet 2001, 69(1):228-234.
58. Penninger RJ, Te Brinke H, Westen MD, Claassen A, Otten DJ, Ogilher SJ, Razzaq S, Morell RJ, Khan S, et al. CDH23 mutation and phenotype heterogeneity: a profile of 107 diverse families with Usher syndrome and nonsyndromic deafness. Am J Hum Genet 2002, 71(2):262-275.
59. Oshima A, Jaito T, Aller E, Millam J, Carney C, Usami S, Moller C, Kimbeling WJ. Mutation profile of the CDH23 gene in 56 probands with Usher syndrome type 1. Hum Mutat 2008, 29(6):537-46.
60. Satoharoy M, Weihofen WA, Gaudet B, Corey DP. Structural determinants of cadherin-23 function in hearing and deafness. Neuron 2010, 66(1):85-100.
61. Ouyang XM, Yan D, Du LL, Heijtmancik JF, Jacobson SG, Nance WE, Li AR, Angeli S, Kaiser M, Newton V, et al. Characterization of Usher syndrome type I gene mutations in an Usher syndrome patient population. Hum Genet 2005, 116(4):292-299.
waltzer mice during inner ear hair cell development. Dev Biol 2005, 280(2):295-306.
68. Michel V, Goodyear RJ, Weil D, Marotti W, Perfettini L, Wollfum U, Kros C, Richardson GP, Petit C: Cadherin 23 is a component of the transient lateral links in the developing hair bundles of cochlear sensory cells. Dev Biol 2005, 280:281-294.
69. Ahmed ZM, Goodyear R, Riazuddin S, Nazli S, Zafar R, Schneider R, Dreier R, Dreyfus B, Engler JP, Farcas R, Schneider-Ramakrishnan M, Kimberling WJ, Payne AM, Bhattacharya SS. Identification of 11 novel mutations in USH2A among Japanese patients with Usher syndrome type 2. Clin Genet 2007, 72(4):339-344.
70. Ahmed ZM, Goodyear R, Riazuddin S, Nazli S, Zafar R, Schneider R, Dreier R, Dreyfus B, Engler JP, Farcas R, Schneider-Ramakrishnan M, Kimberling WJ, Payne AM, Bhattacharya SS. Identification of 11 novel mutations in USH2A among Japanese patients with Usher syndrome type 2. Clin Genet 2007, 72(4):339-344.
71. Ahmed ZM, Goodyear R, Riazuddin S, Nazli S, Zafar R, Schneider R, Dreier R, Dreyfus B, Engler JP, Farcas R, Schneider-Ramakrishnan M, Kimberling WJ, Payne AM, Bhattacharya SS. Identification of 11 novel mutations in USH2A among Japanese patients with Usher syndrome type 2. Clin Genet 2007, 72(4):339-344.
72. Ahmed ZM, Goodyear R, Riazuddin S, Nazli S, Zafar R, Schneider R, Dreier R, Dreyfus B, Engler JP, Farcas R, Schneider-Ramakrishnan M, Kimberling WJ, Payne AM, Bhattacharya SS. Identification of 11 novel mutations in USH2A among Japanese patients with Usher syndrome type 2. Clin Genet 2007, 72(4):339-344.
73. Ahmed ZM, Goodyear R, Riazuddin S, Nazli S, Zafar R, Schneider R, Dreier R, Dreyfus B, Engler JP, Farcas R, Schneider-Ramakrishnan M, Kimberling WJ, Payne AM, Bhattacharya SS. Identification of 11 novel mutations in USH2A among Japanese patients with Usher syndrome type 2. Clin Genet 2007, 72(4):339-344.
74. Ahmed ZM, Goodyear R, Riazuddin S, Nazli S, Zafar R, Schneider R, Dreier R, Dreyfus B, Engler JP, Farcas R, Schneider-Ramakrishnan M, Kimberling WJ, Payne AM, Bhattacharya SS. Identification of 11 novel mutations in USH2A among Japanese patients with Usher syndrome type 2. Clin Genet 2007, 72(4):339-344.