LETTER TO JMG

Survey of the frequency of USH1 gene mutations in a cohort of Usher patients shows the importance of cadherin 23 and protocadherin 15 genes and establishes a detection rate of above 90%

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Background: Usher syndrome, a devastating recessive disorder which combines hearing loss with retinitis pigmentosa, is clinically and genetically heterogeneous. Usher syndrome type 1 (USH1) is the most severe form, characterised by profound congenital hearing loss and vestibular dysfunction.
Objective: To describe an efficient protocol which has identified the mutated gene in more than 90% of a cohort of patients currently living in France.
Results: The five genes currently known to cause USH1 (*MYO7A*, *USH1C*, *CDH23*, *PCDH15*, and *USH1G*) were tested for. Disease causing mutations were identified in 31 of the 34 families referred: 17 in *MYO7A*, 6 in *CDH23*, 6 in *PCDH15*, and 2 in *USH1C*. As mutations in genes other than myosin VIIA form nearly 50% of the total, this shows that a comprehensive approach to sequencing is required. Twenty nine of the 46 identified mutations were novel. In view of the complexity of the genes involved, and to minimise sequencing, a protocol for efficient testing of samples was developed. This includes a preliminary linkage and haplotype analysis to indicate which genes to target. It proved very useful and demonstrated consanguinity in several unsuspected cases. In contrast to *CDH23* and *PCDH15*, where most of the changes are truncating mutations, myosin VIIA has both nonsense and missense mutations. Methods for deciding whether a missense mutation is pathogenic are discussed.
Conclusions: Diagnostic testing for USH1 is feasible with a high rate of detection and can be made more efficient by selecting a candidate gene by preliminary linkage and haplotype analysis.

Usher syndrome type 1 (USH1) is the most severe form of Usher syndrome and is characterised by profound congenital hearing loss, vestibular dysfunction, and prepubertal onset of retinitis pigmentosa. To date six loci, USH1B–G, have been mapped and five causative Usher genes have been cloned: myosin VIIA (*MYO7A*), harmonin (*USH1C*), cadherin 23 (*CDH23*), protocadherin 15 (*PCDH15*), and *SANS (USH1G)* known to be involved in USH1B, USH1C, USH1D, USH1F, and USH1G, respectively.[1 2]

It is not possible to distinguish clinically between the forms of USH1. To date mutations in *MYO7A* and *CDH23* have been reported most often. Patients with USH1 typically have mutations in *CDH23* and *PCDH15*, resulting in truncated proteins (nonsense, frameshift, or splice mutations).[2–6] This is, however, not true for the *MYO7A* gene, where many missense mutations have been reported.[7–11] Demonstrating that these are truly pathogenic changes and not non-functional polymorphic variants complicates the interpretation.

MYO7A has 49 exons, *CDH23* has 69 exons, and *PCDH15* has 33 exons, posing a considerable problem for direct sequencing. Once the extent of genetic heterogeneity became apparent, haplotype analysis was introduced before mutation screening to assist in identification of candidate genes. This enabled us to search for regions of homozygosity in families with known or suspected consanguinity and to check for co-segregation in the six families with more than one affected child (exclusion mapping). It would also have been valuable if there had been a founder gene mutation in the population, such as the *PCDH15* Arg245X mutation in Ashkenazi Jews[12] or the *CDH23* IVS4+1G→A and/or Arg1502X mutations in Swedes.[5]

Using this strategy we identified 46 different pathogenic mutations in four of these genes (of which 29 are novel) and unambiguously genotyped 31 of 34 patients. *MYO7A* accounted for 55% of cases and the cadherin genes, *CDH23* and *PCDH15*, accounted for 19% each. The *USH1C* gene is rarely involved and no mutation was identified in the *USH1G* gene.

METHODS

Patients

Patients were referred from medical genetic clinics distributed all over France. The parents were available in almost all cases. All patients had audiograms and fundus examination or electroretinograms (ERGs), or both. USH1 was diagnosed on the basis of congenital profound sensorineural deafness, vestibular dysfunction, and retinal degeneration. The degree of retinitis pigmentosa was variable among the patients, whose ages varied from 3 to 40 years (see table in the supplemental material, which can be viewed on the journal website, http://www.jmedgenet.com/supplemental).

This study was approved by the local ethics committee and consent to genetic testing was obtained from adult probands or parents of minors. Ethnic origins included Turkish, North African, Senegalese, Guinean, French West Indian, and white (Europid). A questionnaire was completed in the clinic to evaluate the possibility of consanguinity.

Haplotype analyses

In all, 32 microsatellite markers were used to build haplotypes at each known USH1 locus. The majority of markers correspond to Genethon markers, their relative order was confirmed by the ensembl genome server www.ensembl.org, and the detailed list is available in the supplemental material.

PCR amplification of the USH1 genes and sequencing

Genomic DNA was extracted from peripheral blood using standard procedures.

The coding exons and flanking intronic sequences of *MYO7A*, *PCDH15*, *CDH23*, *USH1C*, and *USH1G* were analysed by direct sequencing of 189 amplicons. Most of the primer sequences were obtained from published reports but a few had to be modified to ensure specific amplification or avoid any risk of allele dropout owing to the presence of a single nucleotide polymorphism (SNP) (identified on the Ensembl Genome server). The list of primers used and the conditions used for polymerase chain reaction (PCR) are available in the supplemental material. We customised the SCAIP (single condition amplification/internal primer) method, initially described by Flanigan (2003)[13] which allows a single amplification condition for all exons of a gene, followed by sequencing on a single 96-well plate.

The same PCR primers were used for sequencing unless previous sequence analyses had shown poor quality results, in which case internal primers were designed. Sequences were run on an ABI 3100 DNA analyser and assembled using the ABI Prism Seqscape 2.1 using reference sequences of the *USH1* genes extracted for the NCBI databases. Setting of the basecaller was according to the manufacturer, and records any base with a second peak of >5% as mixed.

URLs and GenBank accession number

http://www.ensembl.org/Homo_sapiens/;

MYO7A: NM_000260; *CDH23*: AY010111; *PCDH15*: NM_033056; *USH1C*: NM_153676; *USH1G*: NM_173477

Control DNAs

Guthrie cards were obtained from the neonatal screening centre GREPAM in Montpellier. All samples were anonymised and neither phenotypic nor ethnic origin data were available. DNA was extracted using standard procedures. The Ensembl server was also scanned for SNPs.

In silico studies

The SIFT (Sorting Intolerant From Tolerant) program developed by Ng[14] was used to predict the consequences of the amino acid substitutions on the protein function (http://blocks.fhcrc.org/sift/SIFT.html).

Analysis in parents and siblings

Whenever substitutions were identified in a patient, segregation analysis was carried out on all available family members to ascertain parental origin, by sequencing, denaturing high performance liquid chromatography, or restriction fragment length polymorphism.

RESULTS

Haplotype analyses

Initially, molecular analyses were focused on *MYO7A* and *CDH23* genes by direct sequencing. However, as some patients had no mutation in either of these genes, it became necessary to analyse further USH1 genes as they were identified. It appeared useful to undertake indirect studies with markers surrounding the USH1 genes (table 1). Even when there was no known consanguinity it helped to prioritise the order in which genes were screened. Examples are U402 and U332, where mutations were found in *PCDH15* and *CDH23*, respectively. Consanguinity or a founder effect, as shown by homozygosity for the haplotype and the mutation, was found in 10 families, although only in five of the cases had it been suggested by the family history. It was useful in a further six of the 23 families screened where there was more than one sibling. In addition, in patient U153, apparent non-inheritance was noticed for one intragenic marker of PCDH15 (D10S2536 localised in intron 3). Analysis using exonic and intronic SNPs confirmed a deletion spanning at least exons 3 to 5 of the *PCDH15* gene (tables 1 and 2). Further deletions of *PCDH15* were identified using quantitative PCR. D10S2522, adjacent to *PCDH15*, failed to amplify in patient U382 and a homozygous deletion of exon 1 was confirmed.

Mutational analysis

Eight families had mutations in *MYO7A* or *CDH23* (U94, U95, U98, U155, U178, U179, U310, and U20). For the remaining families, sequencing was carried out on the genes selected or at least not excluded by haplotyping, in the following order: *MYO7A, CDH23, PCDH15, USH1G*, and *USH1C*.

The 46 mutations identified which could confidently be called disease causing are presented in table 2. Family data are reported in table 1. Twenty nine mutations are novel and include three deletions, eight nonsense and eight missense mutations, seven frameshift alterations, and three splicing aberrations. Mutations found in *CDH23*, *PCDH15*, and *USH1C* were overwhelmingly truncation mutations. A G→A mutation in the last nucleotide of exon 45 of *CDH23* is most likely to act by altering splicing rather than by making the predicted Gly2017Ser change.

Genes were deemed to be disease causing if both alleles were predicted to code for prematurely truncated products by the presence of either a nonsense mutation, a splice defect, or a deletion leading to a frame shift; or if one allele with a missense mutation was detected *in trans* with an allele containing a nonsense mutation or the equivalent (in this study or in published work, as referenced in table 2). Missense changes that were found on both alleles were assessed on an individual basis.

In addition none of the missense changes was identified in a minimum of 168 chromosomes.

Because only one parent was available for U297 and U402, quantitative PCR was carried out for the exons carrying the deleterious mutations. U402 was shown to have the Arg991X mutation on both alleles, but U297 was in fact hemizygous for Arg290X and carried a deletion, *in trans*, spanning exon 8. The apparent homozygosity of the linked markers was caused by a combination of hemizygosity and non-informativeness.

Likely non-pathogenic variations

Various exonic sequence variants, listed in table 3, predicted to be non-pathogenic were identified during the study. They were either also found in controls or additionally in a patient who already had two disease causing mutations identified. Table 3 includes three variants previously described as disease causing but for which our data suggest they are likely to be polymorphisms (see Discussion).

Several intronic variants have also been identified and can be communicated on request.

DISCUSSION

This study confirms that providing a diagnostic service for USH1 is a challenging but soluble problem, both because three genes are involved at a significant level and because a wide range of mutations is found in each of these genes, with few if any hotspots. However, as we were able to detect putative disease causing mutations in more than 90% of families referred it implies that there is no additional major gene for USH1. Using the rapid prescreening test based on linkage and haplotype analysis, we show that a diagnostic test based on the three genes *MYO7A*, *CDH23*, and *PCDH15* is feasible, even with their complex pattern of exons. Homozygosity for markers around a single gene proved useful in 10 families, indicating that the disorder arose by consanguinity or resulted from a founder effect. This was subsequently confirmed by the demonstration of a homozygous change in the relevant gene. In only five cases had the close relation been reported in advance. In the other cases there was either no family history available or no known consanguinity.

Table 1 Haplotype analyses and disease causing genotypes in the USH1 patients

Family	MYO7A	CDH23	PCDH15	USH1C	SANS
U94	[p.His133Asp] + [p.Gly519Asp]				
U95	[p.Glu1170Lys] + [p.Phe1962del]				
U98	[p.Gln1433fsX115] + [p.Gln1798X]				
U155	[p.Asp75ThrfsX30] + [p.Thr165Met]				
U178	[p.Asp75ThrfsX30] + [p.Arg1240Gln]				
U179	[p.Glu1170Lys;] + [p.Leu1858Pro;]				
U310	[p.Leu1878X] + [p.Pro2108fsX4]				
U210	H				
	[p.Arg1240Gln] +[p.Arg1240Gln]				
U102	NE	NE	NE	NE	NE
	[p.Arg1240Gln] + [p.Arg1873Trp]				
U142	H	E	NE	E	E
	[p.Lys164Arg] + [p.Lys164Arg]				
U154	NE	E	E		
	[p.Gly163Arg] + [p.Ala198Thr]				
U226	NE	E	E	NE	E
	[p.Thr204Ala] +[c.2283-1G→T]				
U314	H	NE	NE	NE	NE
	[p.Gly163Arg] + [p.Gly163Arg]				
U320	H	NE	NE		
	[p.Ala335AlafsX37]+[p.Ala335AlafsX37]				
U386	NE	NE	NE	NE	NE
	[p.Ala2009ProfsX31] + [c.1555-8C→G]				
U408	H	NE	NE	NE	NE
	[p.Glu1170Lys] + [p.Glu1170Lys]				
U411	NE	NE	NE	NE	E
	[c.1555-8C→G]+ [c.5886_5889delCTTT]				
U20	†	[c.6050-9G→A] + [p.Leu2207GlnfsX26]			
U97	E	NE	E		
		[p.Arg1437X] + [p.Gly2017Ser]			
U132	NE	NE	NE	E	E
	†	[p.Glu2103X] + [c.8064 + 2T→C]			
U332	NE	H			
		[p.Trp22X] + [p.Trp22X]			
U335	NE	NE	NE	NE	NE
	†	[p.Glu247Lys] + [p.Glu2554X]	†	†	†
U366	NE	H	NE	NE	NE
		[p.Arg1161X] +[p.Arg1161X]			
U153	†	†	LOH	NE	NE
			[p.Ser144LeufsX15] + [E3-E5 del]		†
U177	†	NE	NE	NE	NE
		†	[p.Arg643X] + [Arg1041X]		
U297	†	†	H	H	NE
			[p.Arg290X] + [E8 del]		
U308	NE	NE	NE	NE	NE
	†	†	[c.866_876+6del] + [p.Glu346X]		

Table 1 Continued

Family	MYO7A	CDH23	PCDH15	USH1C	SANS
U382	E	E	H No amplification for D10S2522 [E1del] + [E1del]	NE †	H †
U402	NE	NE	H [p.Arg991X] + [p.Arg991X]	NE	NE
U369	NE	E	E	H [c.238_239insC] + [c.238_239insC]	E
U227	E	NE †	NE †	NE [p.Arg103His] + [c.522-2A→T]	NE †

†Gene fully sequenced but no pathogenic genotype identified.
E, locus is excluded; H, homozygosity is found for all the markers at the locus; LOH, loss of heterozygosity; NE, locus is not excluded.

The interpretation of nucleotide changes in *MYO7A* is particularly difficult because of the large number of missense mutations. The Ala198Thr variation caused by a G to A substitution at position c.592 of *MYO7A* is predicted to alter the normal splicing of exon 6 (the Shapiro and Senepathy score[15] is reduced from 83.6 to 71.2), as it lies in the relatively highly conserved last position of the exon donor site. First, we have interpreted the missense changes as disease causing if the missense alteration was identified *in trans* of a deleterious mutation in either this study or previous ones. None of the changes was found in at least 352 control chromosomes. This applied to Gly163Arg, Thr165Met, Thr204Ala, Glu1170Lys, and Arg1240Gln.[11 16] Second, we have interpreted them as disease causing if they are *in trans* to one of the above. This applies to Leu1858Pro, Arg1873Trp, and the in-frame deletion Phe1962del. Three patients remain where the pathogenic nature of the changes is less firmly based. U94 is a compound heterozygote for two missense mutations, His133Asp and Gly519Asp. Neither mutation was found in 300 control chromosomes. Their deleterious effect remains to be proven, but it is unlikely that these anomalies, localised *in trans*, are not related to the disease. Patient U314, whose parents were known to be consanguineous, was homozygous for Gly163Arg. Similarly U142 was homozygous for a Lys164Arg mutation. These fall in a cluster of mutations involving conserved residues GESGAGKTE (position 158 to 166) which form part of the ATP binding site.[17] In particular residues GKT (163–165) are not only conserved among more than 10 species (including pig, drosophila, and yeast) but also among numerous myosins (not shown, ClustalX[18]). We consider both anomalies likely to be deleterious mutations.

Tyr1719Cys and Thr1566Met (*MYO7A*) have both previously been reported as mutations, but we have found them in controls (2/982 and 8/1104, respectively) and consider they should in future be considered non-pathogenic variants. Arg1060Trp (*CDH23*) was identified as a heterozygous mutation in a patient presenting with non-syndromic hearing loss.[5] We found this variant in patient U177 who carries two truncating mutations in *PCDH15*. Neither of the parents was recorded as having hearing loss, although audiograms were not done.

Two missense changes were identified in *CDH23*. The c.6049G→A change (Gly2017Ser) corresponds to the last nucleotide of exon 45 and reduces the Shapiro and Senepathy score from 84 to 71.5. We consider it is most likely to affect splicing. There remains the Glu247Lys anomaly identified *in trans* of a truncating mutation in patient U335. No other pathogenic effect was found in the four other USH1 genes which were completely sequenced, but we cannot exclude the possibility of a missed mutation, allelic to Glu247Lys. Neither can we exclude the possibility of a deleterious genotype in an unknown USH1 gene. This patient had a particularly severe retinitis pigmentosa phenotype and we also excluded the two most common mutations, Cys759Phe and c.2299delG, found in patients with non-syndromic retinitis pigmentosa linked to *USH2A*. It is possible that the missense change causes an usually severe phenotype by a dominant negative mechanism.

Two patients were found with mutations in *USH1C*. One was homozygous for 238_239insC frameshift which has been reported previously.[19 20] The other patient was a compound heterozygote for a missense Arg103His *in trans* of a c.522-2A→T anomaly. This was the only potentially pathogenic anomaly identified after sequencing *CDH23*, *PCDH15*, *USH1G*, and *USH1C*. *MYO7A* was ruled out by linkage exclusion analysis in the patient and two normal siblings.

Relative involvement of the USH1 genes

Among the 34 families genotyped in this study, *MYO7A* remains the most frequently involved gene, but only accounts for 55% of the cases. *CDH23* and *PCDH15* are also common

Table 2 Pathogenic mutations identified in USH1 genes

Gene	Exon/intron	Nucleotide exchange	Translation effect	In silico analysis*	Number of patients, origin	Alleles in control chromosomes (n)	Original reference
MYO7A	4	c.223delG	p.Asp75ThrfsX30		2, France		22
	5	c.397C→G	p.His133Asp	Affects protein function (0.00)	1, France†	0/352	23
	6	c.487G→C	p.Gly163Arg	Affects protein function (0.00)	1, Algeria	0/664	This study
	6	c.487G→A	p.Gly163Arg	Affects protein function (0.00)	1, Turkey	0/664	§
	6	c.491A→G	p.Lys164Arg	Affects protein function (0.00)	1, North Africa	0/664	This study
	6	c.494C→T	p.Thr165Met	Affects protein function (0.00)	1, France	0/664	21
	6-7	c.592G→A	p.Ala198Thr	Affects protein function (0.00) and splicing	1, Algeria	0/664	This study
	7	c.610A→G	p.Thr204Ala	Affects protein function (0.00)	1, Algeria	0/352	This study
	10	c.1005_1012delins40	p.Ala335AlafsX37		1, Senegal		This study
	Intron 13	c.1555-8C→G		Affects splicing	2, France		7
	14	c.1556G→A	p.Gly519Asp	Affects protein function (0.01)	1, France	0/336	11
	Intron 19	c.2283-1T→G		Affects splicing	1, Algeria		This study
	28	c.3508G→A	p.Glu1170Lys	Affects protein function (0.00)	2, France 1, North Africa	0/352	16
	29	c.3719G→A	p.Arg1240Gln	Affects protein function (0.00)	3, France	0/1414	10
	32	c.4297delC	p.Gln1433SerfsX115		1, France		This study
	39	c.5392C→T	p.Gln1798X		1, France		10
	40	c.5573T→C	p.Leu1858Pro	Affects protein function (0.00)	1, France	0/1176	11
	40	c.5617C→T	p.Arg1873Trp	Affects protein function (0.00)	1, France	0/1176	This study
	40	c.5632delC	p.Leu1878X		1,France/Antilles		This study
	43	c.5886_5888delCTT	p.Phe1962del		1, France		§
	43	c.5886_5889delCTTT	p.Phe1962LeufsX6		1, France		24
	44	c.6025delG	p.Ala2009ProfsX31		1, France		11
	46	c.6324_6339del	p.Pro2108fsX4		1, France		This study
CDH23	1	c.65G→A	p.Trp22X		1,France/Britany		This study
	7	c.739G→A	p.Glu247Lys	Tolerated (0.28)	1, France	0/168	This study
	29	c.3481C→T	p.Arg1161X		1, France		This study
	34	c.4309C→T	p.Arg1437X		1, France		This study
	45	c.6049G→A	p.Gly2017Ser[§]	Tolerated (0.68) , affects splicing	1, France		This study
	Intron 45	c.6050-9G→A		Affects splicing	1, France		5, 25
	47	c.6620delT	p.Leu2207GlnfsX26		1, France		This study
	47	c.6307G→T	p.Glu2103X		1, France		5
	53–54	c.7660G→T	p.Glu2554X		1, France		This study
	Intron 55	c.8064+2T→C		Affects splicing	1, France		This study
PCDH15	1		E1 del		1, France		This study
	3–5		E3-E4-E5 del		1, France		This study
	5	c.423_430dup	p.Ser144LeufsX15		1, France		This study
	8	c.866_876+6del	unknown		1, France		This study
	8	c.868A→T	p.Arg290X		1, France		This study
	8		E8 del		1, France		This study
	10	c.1036G→T	p.Glu346X		1, France		This study
	16	c.1927C→T	p.Arg643X		1, France		6
	22	c.2971C→T	p.Arg991X		1, France		This study
	23	c.3121A→T	p.Arg1041X		1, France		This study
USH1C	3	c.238_239insC	p.Arg80ProfsX68		1, Guinea		19, 20
	4	c.308G→A	p.Arg103His	Affects protein function (0.03)	1, France	0/352	This study
	Intron 7	c.522-2A→T		Affects splicing	1, France		This study

Because truncating mutations are most likely to be pathogenic, no control DNAs were tested for these variations. In silico studies were performed using SIFT program on missense mutations. Statistical value is $p<0.05$ in favour of the pathogenic nature of the mutation. Splicing effects were calculated using http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html
*SIFT prediction (score) on missense mutations and/or splicing effect.
†Patient already reported in Maubaret *et al.*, 2005.
§Mutations mentioned in GeneReviews (Kimberling, www.genetests.org).

with 19% each. In our cohort, *USH1C* was only involved in 6% of cases and no mutation was identified in *USH1G*. Our results suggest a higher level of mutations in cadherin genes, particularly *PCDH15*, than in previous reports.[2 21]

No mutational hotspot was identified apart from a clustering of mutations in the ATP binding site, nor any recurrent mutation (no allele frequency was above 6%). Twenty nine of 46 mutations are new, reinforcing the fact that most mutations are "private" or restricted to populations not studied so far.

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The supplementary material is available on the journal website (http://www.jmedgenet.com/supplemental).

Table 3 Likely non-pathogenic exonic variations identified in USH1 genes

Gene	Exon/intron	Nucleotide exchange	Translation effect	In silico study (SIFT)	No of alleles in patients	No of alleles in controls	Original reference
MYO7A	6	c.510G→A	p.Leu170Leu		1	2/664	This study
	17	c.2035G→A	p.Val679Ile	Tolerated (0.16)	2		This study
	35	c.4697C→T	p.Thr1566Met	Affects protein function (0.03)	1	2/982	16
	37	c.5156A→G	p.Tyr1719Cys	Tolerated (0.08)	1	8/1104	10
CDH23	13	c.1307G→A	p.Ser436Asn	Tolerated (0.06)	1	0/176	This study
	26	c.3178C→T	p.Arg1060Trp	Affects protein function (0.02)	1	0/352	5
	41	c.5418C→G	p.Asp1806Glu	Tolerated (0.56)	1	3/348	This study
PCDH15	2	c.55T→G	p.Ser19Ala	Tolerated (0.13)	4	34/146	This study
	15	c.1910A→G	p.Asn637Ser	Tolerated (0.29)	1	18/348	This study
	21	c.2786G→A	p.Arg929Gln	Tolerated (0.60)	2	3	This study
	23	c.3018G→T	p.Val1006Val		1	0/352	This study

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