ONLINE MUTATION REPORT

Identification of 14 novel mutations in the long isoform of USH2A in Spanish patients with Usher syndrome type II

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Mutations in *USH2A* gene have been shown to be responsible for Usher syndrome type II, an autosomal recessive disorder characterised by hearing loss and retinitis pigmentosa. *USH2A* was firstly described as consisting of 21 exons, but 52 novel exons at the 3′ end of the gene were recently identified. In this report, a mutation analysis of the new 52 exons of *USH2A* gene was carried out in 32 unrelated patients in which both disease-causing mutations could not be found after the screening of the first 21 exons of the *USH2A* gene. On analysing the new 52 exons, fourteen novel mutations were identified in 14 out of the 32 cases studied, including 7 missense, 5 frameshift, 1 duplication and a putative splice-site mutation.

Usher syndrome (USH, OMIM276901) is an autosomal recessive disorder, characterised by the association of retinitis pigmentosa, sensorineural hearing loss and, in some cases, vestibular dysfunction. It is thought to be responsible for more than half the cases of deaf-blindness. The prevalence of USH ranges from 3.5 to 6.2 cases per 100 000.[1] In Spain, the estimation is 4.2 per 100 000.[2]

Traditionally, three clinical types of USH have been distinguished—USH1, USH2 and USH3.[3] Usher syndrome type I (USH1) is characterised by profound and congenital hearing loss, absent vestibular responses and retinitis pigmentosa with prepuberal onset. Patients with Usher syndrome type II (USH2) display moderate to severe hearing loss, normal vestibular function and postpuberal onset of retinitis pigmentosa. The traits of Usher syndrome type III (USH3) are variable onset of retinitis pigmentosa, progressive postlingual hearing loss and changes in vestibular responses in 50% of cases.

Three loci for USH2 have been mapped to date, but only the causative genes for *USH2A* (*USH2A*)[4] and *USH2C* (*VLGR1*)[5] have been isolated. USH2 is the most common form of Usher syndrome and the *USH2A* gene is thought to be involved in 74–90% of cases of USH2.[2 6–8] Moreover, it is also responsible for the disease in patients described as having atypical Usher syndrome because they display progressive, rather than stable, hearing loss and/or vestibular arreflexia.[9 10] In addition, this gene has been shown to be responsible for some recessive cases of non-syndromic retinitis pigmentosa.[10–12]

The *USH2A* gene located in 1q41[13] was first described as comprising 21 exons that expanded 259 kb of genomic DNA. The encoded protein was predicted to consist of 1546 amino acids containing laminin epidermal growth factor and fibronectin type III motifs, typical of extracellular matrix proteins.[4 6] However, van Wijk *et al*[14] identified 51 novel exons at the 3′ end of the *USH2A* gene, which indicated alternative splicing. A long open reading frame extends from exon 2 to 72, encoding a putative protein of 5202 amino acids that contains, in addition to the previously known extracellular domains, 2 laminin G and 28 fibronectin type III repeats, as well as a transmembrane region followed by an intracellular domain with a PDZ-binding motif at the C-terminal end. Additionally, Adato *et al*[15] described a new alternatively spliced exon 71, which encodes a 24 amino-acid peptide of the usherin cytoplasmic domain that is highly expressed in the murine inner ear and conserved throughout vertebrate evolution. The functional significance of the USH2A long isoform was shown by the presence of pathological mutations in several of the 51 novel exons in patients with USH2 syndrome.[14] Furthermore, functional studies have shown that the PDZ-binding domain of USH2A long isoform protein binds with harmonin,[15 16] the defective protein in *USH1C* and whirlin,[15 17] which is defective in the non-syndromic hereditary deafness form *DFNB31*.

In previous studies of Spanish patients with USH2, atypical Usher, non-syndromic retinal degeneration and non-syndromic deafness, only one pathological mutation could be found in several cases, after screening exons 2–21 of the *USH2A*

Key points

- Usher's syndrome type II (USH2) is the most common form of Usher syndrome, an autosomal recessive disorder characterised by hearing loss and retinitis pigmentosa. It has been shown that mutations in the *USH2A* gene are responsible not only for USH2 but also for atypical Usher syndrome and non-syndromic retinitis pigmentosa.
- *USH2A* was first described as comprising 21 exons; however, 52 novel exons have recently been identified at the 3′ end of the gene. This paper reports a mutation analysis of the 52 new exons of the *USH2A* gene, carried out in 32 unrelated patients with Usher's syndrome, non-syndromic retinal degeneration or non-syndromic deafness. On screening the first 21 exons of the gene, patients were found not to be carrying both disease-causing mutations.
- On analysing the 52 new exons, 14 novel mutations were identified in 14 of the 32 patients studied, including 7 mis-sense, 5 frameshift, 1 duplication and 1 putative splice-site mutation. The 14 patients were diagnosed with USH2 and 2 of them were found to carry both pathological variants in the 52 new *USH2A* exons.

Abbreviations: PCR, polymerase chain reaction; USH, Usher syndrome

gene.[10 18] Consequently, the aim of the present work was to screen for mutations in the 52 novel exons of the *USH2A* gene in these patients, to detect the second mutation responsible for the disease.

MATERIALS AND METHODS

Subjects

Spanish patients with Usher syndrome, non-syndromic retinal degeneration or non-syndromic deafness were recruited from the Federacion de Asociaciones de Afectados de Retinosis Pigmentaria del Estado Español and from Ophthalmology and ENT Services of several Spanish hospitals as part of a large-scale study on the genetics of Usher syndrome in the Spanish population.

The present study was carried out in 32 unrelated patients, all of whom had previously been screened for mutations in exons 2–21 of *USH2A* gene (unpublished data).[10 18] Twenty nine of them were found to carry only one mutated allele. In another two patients a putative but unconfirmed pathological change was detected. The last patient was found not to carry any pathological variant, but intragenic SNPs showed homozygosity for this region. Consequently, in this patient a putative mutation in homozygotic state could be located in the 52 new exons of the gene.

On the basis of their clinical history and ophthalmological, audiological, neurophysiological and vestibular tests, 25 of these patients were clinically classified as having USH2, three displayed atypical Usher syndrome, another three were diagnosed with non-syndromic retinal degeneration and one as having non-syndromic deafness. Ophthalmological studies included determining visual acuity and visual field, fundus ophthalmoscopy and electroretinography. Audiometric tests included otoscopic exploration, pure-tone audiometry and speech audiometry. Vestibular evaluation included electronystagmography, noting spontaneous, gaze and positional nystagmus, caloric testing and rotatory chair testing.

Fifty unrelated people of the Spanish population were screened as controls to evaluate the frequency of the mutations found in the patient sample.

Mutation screening

Genomic DNA from affected people and family members was extracted from leucocytes of peripheral blood samples using standard phenol-choroform extraction procedures.

Exons 22–72 and their intron–exon boundaries of the *USH2A* gene were amplified using primers described by van Wijk *et al*.[14] Primers 71CD and 71CR were designed to amplify the alternatively spliced exon 71 described by Adato *et al*.[15] These primers and other additional primers designed to amplify some of the exons are available as an online supplementary table (http://jmg.bmjjournals.com/supplemental).

Samples from all affected people were analysed by single-strand conformational polymorphism on polyacrylamide gel electrophoresis after polymerase chain reaction (PCR) amplification. The amplified DNA fragments were heat denatured, separated through 12% polyacrylamide gels at 18°C and silver stained.

Those PCR fragments showing different mobilities were analysed by direct sequencing on an automated sequencer (ABI-PRISM, Applied Biosystems, California, USA, model 310).

Microsatellite-marker analysis

Polymorphic markers from chromosome 1 were amplified using standard PCR conditions and analysed by polyacrylamide gel electrophoresis. Markers D1S1675, D1S199, D1S508 and D1S2734 were located in 1p. Markers D1S2141, D1S1602, D1S229 and D1S490 were located in 1q.

Table 1 Novel mutations found in this study

Mutation	Exon	Nucleotide change	No of families
Mis-sense			
A2249D	35	6746C→A	1
R2354H	37	7061G→A	1
C3251R	50	9751T→C	1
C3267R	50	9799T→C	1
T3571M	54	10712C→T	2
T4337M	63	13010C→T	1
P4818L	66	14453C→T	1
Frameshift			
R2095GfsX2	32	6238delA	1
T2812MfsX17	42	8435_8438delCCTA	1
C3425FfsX4	52	10272_10273dupTT	1
Y3745fsX1	58	11234dupA	1
F4703HfsX6	64	14110_14111insA	1
Duplication			
Y3472dup	53	10414_10416dupTAT	1
Splicing			
		IVS26+1C→G	1

Reference sequence for the *USH2A* gene AY481573.

Prediction of splice scores

A Splice View program was used to assess how likely intron sequence variants were to create or exclude splice sites. This program is accessible at http://bioinfo.itb.cnr.it/oriel/splice-view.html

RESULTS

The mutation screening in our patients showed 14 different mutations, none of which had been reported previously (table 1). Thirteen of these changes were, as expected, found heterozygously and most of them were private.

The missense mutations, the in-frame duplication and the splicing variant were not found in any of the 50 healthy controls. Segregation analysis was carried out in all cases except for families RP504, FRP60 and FRP37 (table 2).

Figure 1 shows a schematic distribution of previously identified (exons 2–21) and novel (exons 22–72) mutations along the USH2A protein domains (appendix).

Only one mutation was found in more than one family. The amino acid change T3571M was detected in two families together with the 2299delG. To find out whether T3571M could have a common origin, intragenic haplotypes were constructed using the SNPs found in *USH2A* gene. Segregation analysis of the intragenic SNPs using healthy relatives showed that although both families shared the same haplotype for 2299delG it was not so for the novel mutation T3571M.

In another family, two additional potentially pathogenic changes were detected. IVS26+1C>G and Y3472dup were found together with the previously identified G713R change. Segregation analysis for the three mutations showed that the mother carried both the G713R and Y3472dup changes, whereas the IVS26+1C→G was carried by the father. An in silico analysis was carried out to predict whether IVS26+1C→G might affect the splicing of the mutant *USH2A* transcript. On analysis, the normal allele gave a score of 72 for the donor splice site, whereas for the mutant allele the splice sequence was not recognised.

One of the 14 novel mutations detected in this study, the amino acid change T4337M, was found homozygously in one patient. In this case, no mutation had been detected on screening the first 21 exons of the *USH2A* gene. This patient did not refer consanguinity, but intragenic haplotype showed homozygosity along the entire gene. Segregation analysis

Table 2 Clinical features of patients in whom both mutated alleles were detected

Family	Mutations	Sensorineural hearing loss	Onset of night blindness	Onset of visual field loss	Visual field	Visual acuity	Eye fundus	ERG	Cataracts
FRP13	2299delG/C3267R	Moderate, progressive	16	16	Marked concentric loss	RE: 0.4; LE: 0.2	1	No response	BE
		Moderate, progressive	16	16	Marked concentric loss	RE: 0.15; LE: 0.1	1	No response	BE
FRP7	2299delG/11234dupA	Mild, progressive	22	25	Marked concentric loss	RE: 0.8; LE: 1	1	No response	BE
		Moderate, progressive	11	16			2	No response	BE
RP504	C759F/6238delA	Slight	26	30	Marked concentric loss	RE: 0.08; LE: 0.2	2	No response	LE
FRP35	2299delG/P4818L	Moderate	14	20	Concentric loss, −10°	RE: 0.8; LE: 1	1	No response	BE
FRP37	239_240insGTAC/ 10272_10273dupTT	Slight, progressive	20	20	Marked concentric loss		1	No response	LE
		Slight, progressive	14	14	Marked concentric loss		1	No response	BE
		Slight, progressive	17	20	Marked concentric loss		1	No response	BE
FRP291	2299delG/ 14110_14111insA	Moderate	10		Marked concentric loss	RE: 0.8; LE: 1	1	No response	BE
FRP292	2299delG/A2249D	Moderate, progressive	20		Concentric loss, −5°		1	No response	BE
FRP293	2299delG/T3571M	Moderate	10		Marked concentric loss	RE: 1/3; LE: 1/2	1		BE
FRP60	2299delG/C3251R	Moderate/severe	20	25	Slight concentric loss	RE: 0.9; LE: 0.9	1	Iregular response	
FRP54	T4337M (homozygotic)	Moderate/severe	27	27	Concentric loss, −10°	<0.1	1	No response	No
FRP186	G713R/Y3472dup/ IVS26+1C→G	Slight	18	17	Marked concentric loss		1	No response	No
FRP220	2299delG/T3571M	Mild	13	13	Marked concentric loss	RE: 0.5; LE: 0.5	1	No response	BE
FRP229	D778Y/R2354H	Moderate/severe	20	22	Marked concentric loss	RE: 0.6; LE: 0.8	1	Altered	No
FRP232	C759F// 8435_8438delCCTA	Moderate	30	33	Marked concentric loss		1	No response	No

BE, both eyes; ERG, electroretinography; LE, left eye; RE, right eye.
Reference sequence for the *USH2A* gene AY481573.
Eye fundus: (1) Bone spicules deposits, attenuation of vessels and waxy pallor of the optic nerve head; (2) 1+ macular affectation.
Onset of night blindness and visual-field loss are expressed in years.

showed that T4337M was not homozygously carried by any of the healthy relatives studied. The patient's mother carried the mutation heterozygously, but there was no DNA sample available from the patient's father. A possible maternal uniparental disomy was discarded using microsatellite markers (see Materials and methods).

Moreover, 26 variants, presumed to be non-pathological, were also found after screening the last 52 exons of *USH2A*. Table 3 summarizes these variants which were considered to be polymorphisms on the basis of their nature, frequency and segregation analysis.

The 14 patients found to carry mutations in the last 52 exons of the *USH2A* gene were clinically diagnosed with USH2. In these patients, the age of onset of night blindness and visual field loss ranged from 10 to 30 years. The sensorineural hearing loss ranged from slight to moderate or severe and, in some cases, was subjectively progressive. Fundus ophthalmoscopy showed typical retinitis pigmentosa

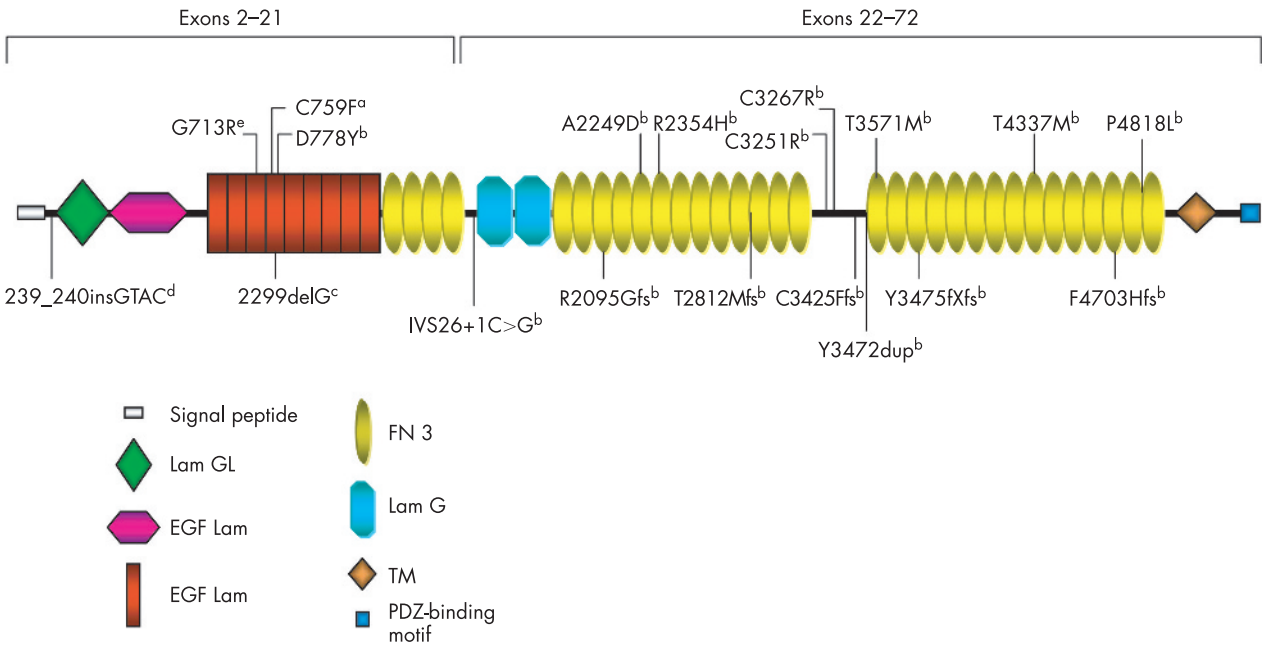

Figure 1 Schematic distribution of previously identified (exons 2–21) and novel (exons 22–72) mutations along USH2A protein domains. [a]Mutation reported by Rivolta *et al*[11]; [b]previously unreported mutations; [c]mutation first reported by Eudy *et al*[4] as 2314delG; [d]mutation previously reported by Najera *et al*[18]; [e]mutation described by Dreyer *et al*[7]. EGF, epidermal growth factor; FN, fibronectin; GL, laminin G-like; TM, transmembrane.

Table 3 Presumed non-pathological variants found in the last 52 exons of *USH2A*

Exon	Nucleotide change	Codon change	Allele frequency
22	4714C→T	L1572F	11/64
25	4994T→C	I1665T	18/64
25	5013C→A	G1671G	7/64
	IVS30+76A→T	—	1/64
32	6317T→C	I2106T	15/64
34	6506T→C	I2169T	ND
35	6713A→C	E2238A	2/64
	IVS36+19A→G	—	4/64
	IVS38–65T→G	—	3/64
40	7506G→A	P2502P	3/64
	IVS40+22C→T	—	3/64
43	8624G→A	R2875Q	2/64
43	8656C→T	L2886F	ND
	IVS44–52_53delTT	—	2/64
47	9296A→G	N3099S	4/64
48	9430G→A	D3144N	2/64
52	10232A→C	E3411A	23/64
	IVS52–26T→C	—	ND
	IVS58+9A→T	—	10/64
	IVS59+98G→A	—	8/64
60	11602A→G	M3868V	8/64
61	11736G→A	E3912E	1/64
61	11907A→T	P3969P	1/64
63	12666A→G	T4222T	14/64
63	13191G→A	E4397E	7/64
	IVS71–194A→T	—	2/64

ND, not determined; –, it can be missed.
Allele frequency referred to patient sample.
Reference sequence for the *USH2A* gene AY481573.

in all cases, but in two of them the macula was also affected, corresponding to the cases in which retinitis pigmentosa was more advanced. Most patients were found to have cataracts. Table 2 summarises the clinical data of these patients (appendix).

DISCUSSION

The first 21 exons of the *USH2A* gene have been screened for mutations in many different populations,[6 7 18–20] with most studies finding that several patients displayed only one mutated allele. In our Spanish patient sample, both pathogenic mutations were detected in 12 patients, whereas 24 patients displayed only one of the two expected mutations.[10 18] This prompted us to screen the 52 novel exons of the *USH2A* gene to find the second mutation responsible for the disease.

A high diversity of mutations was found in our series: seven missense, five frameshift, one duplication and one putative splice-site mutation. Although 2299delG, located at exon 13, is the most prevalent mutation found in the first part of the *USH2A* gene in several populations,[4 6 7 19 21] no predominant mutation was detected for the Spanish population in the last part of the gene, as only one of the mutations was detected in two families and the remaining changes were private. These 14 novel mutations were found to be distributed along the whole new part of the *USH2A* gene, there being no indication of a hot spot for mutations in the new 52 exons. No mutation was found either in the transmembrane or in the intracellular domain of the protein. This fact is hardly surprising, as only the last 161 amino acids from a total of 5202 make up these regions. However, recent studies have shown that this intracytoplasmic domain can interact with harmonin and whirlin, defective proteins in USH1 and non-syndromic sensorineural deafness, respectively.[15–17] Therefore, mutations in the transmembrane and cytoplasmic domain of the USH2A protein could lead to a phenotype that is different from USH2. We also included some patients with non-syndromic retinitis pigmentosa, non-syndromic deafness and atypical Usher in this screening, but no mutations were detected.

Only the amino acid change T3571M was not found privately. This mutation was detected in two unrelated cases and in both the accompanying mutation was 2299delG. Segregation analysis using intragenic SNPs showed that the haplotype linked to 2299delG was identical in both cases, in agreement with the common origin indicated by Dreyer *et al*[22] for this ancestral and widespread mutation. Conversely, a different haplotype linked to T3571M, suggesting a different origin of the mutation for these two families.

Here, we have described one patient with USH2, FRP186, with three potentially pathogenic changes in the *USH2A* gene: G713R (previously identified in exon 13), Y3472dup and the splicing variant IVS26+1C→G. With respect to IVS26+1C→G, no recognition of the splicing site was obtained using the Splice View program for the abnormal sequence, suggesting that this variant may affect *USH2A* transcript splicing. Segregation analysis showed that IVS26+1C→G was carried by the father, and both G713R and Y3472dup were carried by the mother. Although G713R was described as a pathological mutation by Dreyer *et al*,[7] we were able to detect this change in 2 of 200 normal control chromosomes.[18] Seyedahmadi *et al*[23] showed that this variant did not segregate with the disease in some of their families. Furthermore, functional studies performed by Bhattacharya and Cosgrove[24] showed that, as opposed to other missense mutations located in the LE domain of USH2A protein, G713R does not abolish usherin–fibronectin interactions. Consequently, we do not consider that there is convincing evidence for the pathogenic effect of G713R, but believe, rather, that it seems to be a polymorphism without clinical implications.

The amino acid change T4337M was found homozygously in patient FRP54. This change was heterozygously detected in the patient's mother, whereas paternal DNA was not available. No indication of maternal uniparental disomy was obtained. However, it is possible there is a deletion in heterozygotic state involving the *USH2A* region in this patient. Future studies using Southern blot, multiplex ligation probe assay or quantitative PCR might elucidate this question.

All the patients harbouring both mutated alleles displayed features that prompted us to classify them as USH2. The greatest clinical differences between affected people concerned the age of onset of retinitis pigmentosa, ranging from 10 to 30 years. Nevertheless, these data were obtained from personally interviewing the patients and are therefore clearly subjective. Also, differences in the degree of hearing loss must be mentioned. This ranged from slight to moderate or severe, and, in some cases, was slightly progressive. This progression is not as evident as it is in USH3 and, in any event, was subjectively qualified. We do, however; think that it is more important to remark on findings on hearing loss in that, in some cases, hearing loss was only mild or even slight. In these cases, USH2 could be misdiagnosed as non-syndromic retinitis pigmentosa.

Evidently, a high proportion of mutated alleles for the *USH2A* gene are undetected, as the expected complement of two pathological mutations could not be found in a large number of patients. This could be explained by the fact that 52 exons of this gene were not screened in most previously published studies, and it is reasonable to expect the mutation detection rate to improve after screening this part of the gene.[25] In this study, 22–72 *USH2A* exons were screened for mutations in 32 unrelated patients, in which the first 21 *USH2A* exons had previously been screened. Both mutations responsible for the disease were finally detected in 14 of them. All 14 novel mutations belonged to the patients with

USH2. This means that 14 of 25 (56%) cases of USH2 were resolved by typing the additional 52 *USH2A* exons. However, we were still unable to identify the second mutation in 18 of the patients studied. Consequently, additional reasons for this finding must be postulated, such as sensitivity of the detection technique, mutations in non-coding regions or the presence of other isoforms expressed in minimal amounts that have not yet been identified.

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