Microarray-Based Mutation Analysis of 183 Spanish Families with Usher Syndrome

Teresa Jaijo,[1,2] *Elena Aller,*[1,2] *Gema García-García,*[1,2] *María J. Aparisi,*[1] *Sara Bernal,*[2,3] *Almudena Ávila-Fernández,*[2,4] *Isabel Barragán,*[2,5] *Montserrat Baiget,*[2,3] *Carmen Ayuso,*[2,4] *Guillermo Antiñolo,*[2,5] *Manuel Díaz-LLopis,*[6] *Maigi Külm,*[7] *Magdalena Beneyto,*[1,2] *Carmen Nájera,*[8] *and Jose M. Millán*[1,2]

Purpose. The purpose of this study was to test the ability of the genotyping microarray for Usher syndrome (USH) to identify the mutations responsible for the disease in a cohort of 183 patients with USH.

Methods. DNA from 183 patients with Usher syndrome from the Spanish population was analyzed using a genotyping microarray containing 429 previously identified disease-associated variants in eight USH genes. Mutations detected by the array were confirmed by direct sequencing. Haplotype analysis was also performed in families carrying common Spanish mutations.

Results. The genotyping microarray identified 43 different variants, divided into 32 disease causative and 11 probably nonpathologic. Mutations were detected in 62 patients with USH (33.9%). According to the clinical classification of patients, pathologic variants were detected in 31.4% patients with USH1, 39.4% of with USH2, 22.2% with USH3 and 15.8% with unclassified Usher syndrome. Ninety-seven pathologic alleles were detected, corresponding to 26.5% of expected alleles. The *USH2A* mutations p.C3267R and p.T3571M were revealed as common in the Spanish population, and two major haplotypes linked to these mutations were observed.

Conclusions. The genotyping microarray is a robust, low-cost, rapid technique that is effective for the genetic study of patients with USH. However, it also indicates variants of unclear pathologic nature and detection failures have also been observed. Results must be confirmed by direct sequencing to avoid misdiagnosis, and continuous updates of the microarray should be performed to increase the efficiency and rate of detection of mutations. (*Invest Ophthalmol Vis Sci.* 2010;51: 1311–1317) DOI:10.1167/iovs.09-4085

Usher syndrome (USH) is an autosomal recessive disorder characterized by sensorineural hearing loss, retinitis pigmentosa (RP), and, sometimes, vestibular areflexia. Its prevalence ranges between 3.8 and 6.2/100,000, depending on the population,[1–3] and it accounts for more than 50% of individuals who are both deaf and blind.[4,5]

Usher syndrome is clinically and genetically heterogeneous. Three clinical subtypes have been distinguished.[6] Usher syndrome type I (USH1) is the most severe form and is characterized by profound congenital hearing loss, prepubertal onset of RP and vestibular dysfunction. Patients with Usher syndrome type II (USH2) have moderate to severe congenital hearing loss, RP onset around puberty, and normal vestibular responses. Patients with Usher type III (USH3) experience progressive hearing loss with variable vestibular function and onset of RP.

To date, 12 loci have been described, and nine genes have been identified for Usher syndrome. Seven loci are linked to USH1 (*USH1B-USH1H*), and five genes have been reported: *MYO7A*,[7] *USH1C*,[8,9] *CDH23*,[10,11] *PCDH15*,[12,13] and *USH1G*.[14] In USH2, three loci (*USH2A*, *USH2C*, and *USH2D*) have been reported, the three identified genes being: *USH2A*,[15,16] *GPR98*,[17] and *DFNB31*.[18] For USH3, two loci have been described (*USH3A* and *USH3B*), but only the *USH3A* gene has been identified.[19]

Different USH gene prevalence has been observed in the pathogenesis of this disorder. For USH1, *MYO7A* is reported to be the most prevalent gene, causing 29%–55% of cases.[20–24] *CDH23* and *PCDH15* genes are involved in 19%–35% and 11%–19%, respectively, according to different studies,[23,25,26] whereas the prevalence of the *USH1C* gene has been estimated at 6% and 7%.[23,26] *USH1G* was found to be responsible for 7% of USH1 series in a study performed in Britain and the United States[26]; however, it plays a minor role in Spanish and French patients with USH1.[23,27]

Defects in the *USH2A* gene are the most frequent cause of USH2, and a prevalence of 75% has been established.[28,29] Few studies have targeted *GPR98* and *DFNB31* genes, but the genes appear to play a minor role in the pathogenesis of USH2.[17,18]

Finally, *USH3A* does not seem to be responsible for a large proportion of USH cases, with the exception of endogamic populations, like Finnish or Ashkenazy Jews, where it is a common form.[19,30,31]

Genetic analysis of patients with USH is complicated by the large number of genes involved in USH and the many exons comprising the coding regions of these genes. All five identi-

From the [1]Unidad de Genética. Hospital Universitario La Fe, Valencia, Spain; [2]CIBER (El Centro de Investigación Biomédica en Red) de Enfermedades Raras (CIBERER), Valencia, Spain; [3]Servicio de Genética, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; [4]Departamento de Genética, Fundación Jiménez Díaz, Madrid, Spain; [5]Unidad de Gestión Clínica de Genética, Reproducción y Medicina Fetal, Hospitales Universitarios Virgen del Rocío, Sevilla, Spain; [6]Servicio de Oftalmología, Hospital Universitario La Fe, Valencia, Spain; [7]Asper Biotech, Tartu, Estonia; [8]Departamento de Genética, Universitat de Valencia, Valencia, Spain.

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Corresponding author: Jose M. Millán, Unidad de Genética, Hospital Universitario La Fe, Avda. Campanar, 21, 46009 Valencia, Spain; millan_jos@gva.es.

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fied USH1 genes comprise 182 exons and more than 25 kb of coding DNA. For USH2, the three causative genes comprise 179 exons that extend over 37 kb of coding DNA. Consistent clinical differences between the genetic subtypes have not been reported. Therefore, patients with USH are studied genetically by linkage analysis in those informative families, or gene by gene, according to their established prevalence in the analyzed population. This method is costly and a burden on medical resources, as well as being time consuming.

A specific genotyping microarray for USH was developed by Asper Biotech (Tartu, Estonia) to facilitate the genetic study of patients. Cremers et al.[32] evaluated the first version of the microarray, detecting mutations in 46% of patients with USH1, 24% of patients with USH2, 29% of patients with USH3, and 30% of patients with atypical USH. However, this initial version of the genotyping microarray detected only 298 variants in the analyzed sample, which comprised an ethnically heterogeneous mix of patients.

This microarray currently detects 429 previously described mutations in eight of the nine genes linked to USH. In this study, we analyzed a series of Spanish patients with USH with the genotyping microarray to evaluate the ability of this tool to detect the mutations underlying the disease in our cohort.

Methods

Patients

Patients with USH were enrolled as a part of a large study of the genetics of USH. A total of 183 unrelated Spanish patients were recruited from the Federación de Asociaciones de Afectados de Retinosis Pigmentaria del Estado Español (FAARPEE) and also from the Ophthalmology and ENT Services of several Spanish Hospitals.

These 183 patients were divided into 51 cases of USH1, 104 of USH2, and 9 of USH3 according to clinical history and ophthalmic, audiological, neurophysiologic, and vestibular tests. Clinical data could not be obtained for 19 patients, and these remained as nonclassified (USHNC). The *MYO7A* gene had been excluded as being causative of the disease in 36 of the 51 patients with USH1.[33]

This study was approved by the local ethics committees. All the procedures used conformed to the tenets of the Declaration of Helsinki. Informed consent to genetic testing was obtained from all participants after explanation of the nature and possible consequences of the study.

DNA samples from 100 healthy unrelated individuals in the Spanish population who did not show any sign of hearing loss or retinitis pigmentosa were screened as the control.

Molecular Analysis

Genomic DNA was extracted from peripheral blood by standard procedures. One hundred twenty amplicons from eight USH genes (*MYO7A*, *USH1C*, *CDH23*, *PCDH15*, *USH1G*, *USH2A*, *GPR98*, and *USH3A*) were PCR amplified and used in the primer extension reaction (APEX) on the USH genotyping microarray.[32] All amplicons from eight genes were amplified and optimized, as described previously.[34] In the amplification mixture 20% of the dTTP was substituted by ddUTP. The amplification products were concentrated and purified by PCR purification columns (JETquick; Genomed GmbH, Bad Oeynhausen, Germany). The fragmentation of amplification products was achieved by adding thermolabile uracil *N*-glycosylase (Epicenter Technologies, Madison, WI) and heat treatment.[34] Fragmented and denatured PCR products were used for the primer extension reaction on the Usher microarray. Each APEX reaction consisted of a fragmented and denatured PCR product (4 units of ThermoSequenase DNA Polymerase; GE Life Sciences, Freiburg, Germany), 1× reaction buffer and 1.4 μM final concentration of each fluorescently labeled ddNTP: Texas red-ddATP, fluorescein-ddGTP (GE Life Sciences), Cy3-ddCTP, Cy5-ddUTP (NEN). The reaction mixture was applied to a microarray slide for 15 minutes at 58°C. The reaction was stopped by washing the slide at 95°C in deionized water (Milli-Q; Millipore, Billerica, MA).[35] The slides were imaged (Genorama QuattroImager; Asper Biotech, Ltd.) at a resolution of 20 μm. This imager combines a total internal reflection fluorescence-based excitation mechanism with a charge-coupled device camera.[17] The sequence variants were identified by the software.[36,37]

Each mutation/polymorphism is identified on Usher microarray by two unique oligomers designed specifically according to the wild-type sequence of the Usher genes. Oligonucleotides were spotted onto an activated chip surface (VersArray; Bio-Rad Laboratories, Hercules, CA).

The results obtained from the microarray were confirmed. All exons where a mutation was identified were amplified by PCR. Amplicons were directly sequenced with dye termination chemistry (Prism Big Dye Terminator ver.1.1, Applied Biosystems, Inc. [ABI]Foster City, CA) and purified sequencing reactions were resolved in a sequencer (Prism 3130xl; ABI).

To ascertain parental origin, segregation analysis was performed in those cases in which at least two pathologic variants were identified.

Table 1. Pathologic Variants

Gene/Exon	Nucleotide Variant	Codon Variant	Alleles (*n*)
MYO7A			
7	c.640G>A	p.G214R	3
29	c.3719G>A	p.R1240Q	2
40	c.5581C>T	p.R1861X	2
44	c.6025delG	p.A2009PfsX32	1
CDH23			
10	c.1096G>A	p.A366T	2
20	c.2289+1G>A	*Splicing defect*	6
30	c.3625A>G	p.T1209A	2
31	c.4021G>A	p.D1341N	1
46	c.6050-9G>A	*Splicing defect*	5
47	c.6393delC	p.12132SfsX11	4
54	c.7823G>A	p.R2608H	1
PCDH15			
2	c.7C>T	p.R3X	5
USH2A			
2	c.100C>T	p.R34X	1
6	c.1000C>T	p.R334W	1
6	c.921_922insCAGC	p.H308SfsX16	1
8	c.1518T>A	p.Y506X	1
10	c.1663C>G	p.L555V	4
11	c.1841-2A>G	*Splicing defect*	5
13	c.2276G>T	p.C759F	3
13	c.2299delG	p.E767SfsX21	15
13	c.2431_2432delAA	p.K811DfsX11	1
13	c.2522C>A	p.S841Y	1
14	c.2898delG	p.T967LfsX44	2
18	c.3883C>T	p.R1295X	1
28	c.5776+1G>A	*Splicing defect*	2
34	c.6587G>C	p.S2196T	1
41	c.8167C>T	p.R2723X	2
50	c.9799T>C	p.C3267R	9
52	c.10272_10273dupTT	p.C3425FfsX4	1
54	c.10712C>T	p.T3571M	6
61	c.11864G>A	p.W3955X	1
66	c.14453C>T	p.P4818L	2

The number of alleles where each variant has been identified is shown.

Results

The USH microarray identified 43 different variants, but only thirty-two changes were considered as disease causative. Clearly detected pathologic mutations are summarized in Table 1.

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TABLE 2. Summary of Mutant Alleles Identified in the Patients with USH

	USH1				USH2				USH3				USHNC			
	Total	0 Alleles	1 Allele	2 Alleles	Total	0 Alleles	1 Allele	2 Alleles	Total	0 Alleles	1 Allele	2 Alleles	Total	0 Alleles	1 Allele	2 Alleles
n	51	35	8	8	104	63	25	16	9	7	0	2	19	16	2	1
Patients with mutations, *n* (%)	16/51 (31.4)				41/104 (39.4)				2/9 (22.2)				3/19 (15.8)			
Mutated alleles, *n* (%)	24/102 (23.5)				57/208 (27.4)				4/18 (22.2)				4/38 (10.5)			

USHNC, Usher syndrome patients without a clinical classification.

Sixty-two of the 183 patients were found to be carriers of mutations (33.9%). The two mutated alleles responsible for the disease were identified in 35 (56.5%) patients and only one pathologic allele was identified in the remaining 27 (43.5%) patients. The detection rate varied according to the clinical classification of patients. Table 2 shows the number of mutant alleles identified, as well as the number of patients where pathologic variants were detected, according to their clinical subtype. When DNA samples were available from relatives, segregation analysis was performed. Genotypes of patients are indicated in Table 3.

Failures in the Genotyping Microarray Detection

All pathologic variants were verified by direct sequencing. The mutation p.C575S (*USH2A*, exon 10), detected by the microarray, could not be confirmed. That variant was homozygously found in patient RP-1244; however, sequencing revealed that it was not present.

The mutation c.6392_6393insA (*CDH23*, exon 47) was detected in three cases, RP-822, RP-914 and RP-1314. This exon was sequenced in those patients, and we found the deletion c.6393delC, not the insertion.

Two variants showed an incorrect nomenclature according to den Dunnen and Antonarakis.[38] The mutation c.921_922insCAGC (*USH2A*, exon 6) should have been indicated as c.920_923dupGCCA and the mutation c.10273_10274insTT (*USH2A*, exon 52) should have been named c.10272_10273dupTT.

Detected Mutations Not Included in the Microarray

The sequencing of pathologic variants also detected mutations not included in the microarray. These variants are indicated with an asterisk in Table 3. The mutation c.6393delC (*CDH23,* exon 47) was detected in patient RP-914. Sequencing of that exon also revealed there was the deletion c.6346_6347delTT. Similarly, the mutation p.R1240Q (*MYO7A*, exon 40) was identified by the microarray in patient RP-1524. The mutation p.Q1242X (c.3724C>T) was also identified after sequencing. The deletion c.6346_6347delTT has recently been described[39] and the *MYO7A* variant is novel. Therefore, these variants are not included in the array yet.

Common Mutations in the Spanish Population

Two missense variants were revealed as frequent in the Spanish population. The *USH2A* mutations p.C3267R (c.9799T>C) and p.T3571M (c.10712C>T) were detected in nine and six alleles, respectively. It represents an allele frequency of 4.3% and 2.9% for the patients with USH2 enrolled in this study. None of these variants was found in 200 healthy control chromosomes. Segregation analysis could be performed in most families, indicating that these alleles cosegregated with the disease. Haplotype analysis was performed to investigate a possible common origin for those variants. To construct the haplotypes, in addition to the mutations, four intragenic SNPs were used: c.1419C>T_c.3157+35A>G_c.6506T>C_c.9799T>C/c.10712C>T_c.12666A>G. A major haplotype linked to mutations was observed: C-A-C-C-G for the p.C3267R alleles and T-G-T-T-A for the p.T3571M alleles.

Variants with Uncertain Pathogenicity

Eleven variants detected in this study were considered as probably nonpathologic. Table 4 summarizes the assumed noncausative disease variants. The microarray classified two of these variants as polymorphisms. However, nine of them were listed in different ways: *mutation* or *mutation/SNP*. We considered these variants as putative nonpathogenic for a number of reasons, such as the changes did not segregate with the disease in the family, or recent studies identified some of them in control samples. In other cases, those variants were identified in patients with an unexpected clinical classification, some of whom were carriers of mutations in other genes.

DISCUSSION

In this study, 183 patients with USH were analyzed against a genotyping microarray. With this tool, we detected 98 pathologic alleles that correspond to 32 different mutations. Likewise, we detected 148 putative benign alleles, corresponding to 11 different variants considered as nonpathologic.

Mutations were only identified in four of the eight analyzed genes. Most pathologic variants (62.9%) were identified in the *USH2A* gene. This gene shows the highest prevalence in USH2[28,29] and the great majority of patients in our study were classified as USH2. Thus, these results were as expected. Regarding USH1 genes, *MYO7A* is considered the most prevalent gene followed by genes *CDH23* and *PCDH15*, while the remaining USH1 genes play a minor role in the pathogenicity of the disease.[23,24,26,39] In the present study, 23.6% of detected mutated alleles correspond to *CDH23,* followed by genes *MYO7A* and *PCDH15*, with 9% and 5.6% of pathologic alleles, respectively. The low percentage of mutated alleles identified in the *MYO7A* gene might be surprising due to it is the most prevalent USH1 gene. However, it was expected because the *MYO7A* gene had previously been discarded as responsible for the disease in 36 of the 51 patients with USH1.[24,33] In the remaining 15, most pathologic variants (seven of nine) were detected in *MYO7A*, showing the high implication of this gene in the USH1 pathogenicity. No mutation was detected in the

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TABLE 3. Genotypes of USH Patients

USH Type/Patient	Mutation 1	Mutation 2	Gene
USH1			
Homozygous			
RP-822	c.6393delC	c.6393delC	*CDH23*
RP-1267	c.6050-9G>A	c.6050-9G>A	*CDH23*
RP-1430	p.R3X	p.R3X	*PCDH15*
RP-1479	p.R1861X	p.R1861X	*MYO7A*
RP-1738	p.G214R	p.G214R	*MYO7A*
Compound Heterozygous			
RP-212	c.2289+1G>A	c.6050-9G>A	*CDH23*
RP-1361	c.2289+1G>A	c.6050-9G>A	*CDH23*
RP-1537	p.R1240Q	p.C759F	*MYO7A/USH2A*
Heterozygous			
RP-157	c.2289+1G>A	ND	*CDH23*
RP-808	c.6050-9G>A	ND	*CDH23*
RP-914	c.6393delC	(c.6346_6347delTT)*	*CDH23*
RP-1374	p.A366T/p.R3X	ND	*CDH23/PCDH15*
RP-1524	p.R1240Q	(p.Q1242X)*	*MYO7A*
RP-1534	c.2289+1G>A	ND	*CDH23*
RP-1614	p.A2009fs	ND	*MYO7A*
RP-1739	p.D1341N	ND	*CDH23*
USH2			
Homozygous			
RP-597	c.2898delG	c.2898delG	*USH2A*
RP-1246	c.2299delG	c.2299delG	*USH2A*
RP-1592	c.2299delG	c.2299delG	*USH2A*
RP-1608	p.C3267R	p.C3267R	*USH2A*
RP-1639	p.T3571M	p.T3571M	*USH2A*
RP-1735	p.L555V/c.1841-2A>G	p.L555V/c.1841-2A>G	*USH2A*
Compound heterozygous			
RP-933	p.R34X	c.2431_2432delAA	*USH2A*
RP-934	p.C3267R	p.T3571M	*USH2A*
RP-1103	p.A366T	p.T1209A	*CDH23*
RP-1113	p.R334W	c.5776+1G>A	*USH2A*
RP-1114	p.C3267R	p.T3571M	*USH2A*
RP-1242	p.R2723X	p.T3571M	*USH2A*
RP-1314	c.6393delC	p.R2608H	*CDH23*
RP-1351	c.921_922dupGCCA	p.R2723X	*USH2A*
RP-1357	c.2299delG	c.5776+1G>A	*USH2A*
RP-1628	p.C759F	p.C3267R	*USH2A*
Heterozygous			
RP-259	p.C3267R	ND	*USH2A*
RP-527	c.2299delG	ND	*USH2A*
RP-532	p.Y506X	ND	*USH2A*
RP-690M	p.P4818L	ND	*USH2A*
RP-898	p.T3571M	ND	*USH2A*
RP-904	p.C3267R	ND	*USH2A*
RP-987	p.C3267R	ND	*USH2A*
RP-1190	p.P4818L	ND	*USH2A*
RP-1172	c.10272_10273dupTT	ND	*USH2A*
RP-1296	p.S2196T	ND	*USH2A*
RP-1349	c.2299delG	ND	*USH2A*
RP-1360	p.C759F	ND	*USH2A*
RP-1472	c.2299delG	ND	*USH2A*
RP-1518	c.2299delG	ND	*USH2A*
RP-1522	c.2299delG	ND	*USH2A*
RP-1525	c.2299delG	ND	*USH2A*
RP-1527	p.W3955X	ND	*USH2A*
RP-1528	c.2299delG	ND	*USH2A*
RP-1539	p.R1295X	ND	*USH2A*
RP-1540	p.G214R	ND	*MYO2A*
RP-1569	c.2299delG	ND	*USH2A*
RP-1618	p.C3267R	ND	*USH2A*
RP-1631	c.1841-2A>G	ND	*USH2A*
RP-1632	c.2299delG	ND	*USH2A*
RP-1736	c.2299delG	ND	*USH2A*
USH3			
Homozygous			
RP-938	p.R3X	p.R3X	*PCDH15*
RP-1459	p.L555V/c.1841-2A>G	p.L555V/c.1841-2A>G	*USH2A*
USHNC			
Homozygous			
RP-512	c.2289+1G>A	c.2289+1G>A	*CDH23*
Heterozygous			
RP-683	p.T1209A	ND	*CDH23*
RP-1561	p.S841Y	ND	*USH2A*

ND, not determined. Detected variants not included in the microarray are indicated by an asterisk.

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TABLE 4. Variants Assumed to be Nonpathogenic

Gene	Nucleotidic Variant	Proteic Change	Alleles (*n*)	Microarray
MYO7A				
3	c.47T>C	p.L16S	97	SNP
35	c.4697C>T	p.T1566M	1	Mutation
37	c.5156A>G	p.Y1719C	7	Mutation
40	c.5598C>A	p.L1866L	1	Mutation
CDH23				
26	c.3178C>T	p.R1060W	1	Mutation
USH2A				
4	c.688G>A	p.V230M	8	Mutation/ polymorphism?
8	c.1434G>C	p.E478D	7	Mutation
21	c.4560C>T	p.115201	1	Mutation/ polymorphism?
41	c.7685T>C	p.V2562A	8	Mutation/SNP?
43	c.8656C>T	p.L2886F	12	SNP
47	c.9262G>A	p.E3088K	5	Mutation/SNP

The microarray column shows the indication of these variants in the microarray.

USH3A gene, with some patients with USH3 carrying mutations located in other USH genes.

Failures in the Genotyping Microarray Detection

The genotyping microarray detected the presence of variants in 244 alleles. These were divided into 148 non pathogenic and 96 mutant alleles. The sequencing of all mutations failed to confirm two out of the 96 mutated alleles. Therefore, erroneous microarray detection represents 2% of pathologic alleles. Identification of false positives in our series stresses the need to confirm all pathologic variants by direct sequencing.

In some cases incorrect nomenclature was observed for certain detected mutations. Likewise, when the *CDH23* mutation c.6392_6393insA is detected, the array should also indicate the mutation c.6393delC, which is identified with the same probes. This cannot be considered a microarray error because both mutations indicate the location of an adenine at position c.6393 of the *CDH23* gene, a nucleotide investigated by the microarray.

Common Mutations in the Spanish Population

A number of pathologic variants were detected at high frequencies (Table 1), most of them extensively described.[40] However, the *USH2A* variants p.C3267R and p.T3571M were revealed as frequent in the Spanish population. A possible polymorphic nature for these variants was discarded because they were not detected in control samples and cosegregated with the disease in the families. Haplotype analysis was performed, to investigate a possible common origin for these mutations. Two major haplotypes linked to these variants were observed, supporting the hypothesis of the existence of a common origin.

Mutations Identified in Genes Related to Different USH Subtypes

Some patients were found to be carriers of mutations in genes usually involved in other clinical subtype.

Patients with USH2. Five different mutations in USH1 genes were detected in three patients with USH2. Two *CDH23* mutations were identified in patients RP-1103 and RP-1314: p.A366T and p.T1209A in patient RP-1103, and c.6393delC and p.R2608H in patient RP-1314. The clinical history of RP-1103 was recruited. Onset of decreased visual acuity and visual field was at the age of nine, and RP was diagnosed at the age of 11. The patient had moderate hearing loss and normal vestibular function, confirming the USH2 diagnosis. However, detailed clinical data from RP-1314 could not be obtained. A high phenotypic heterogeneity due to *CDH23* variants has been reported,[10,11,41] although only one patient with USH2 with mutations in that gene was described.

The *MYO7A* mutation p.G214R was detected in RP-1540. This patient reported RP, moderate hearing loss and normal vestibular function, signs clearly compatible with USH2. No mutations in *MYO7A* gene have been reported in patients with USH2. Screening of USH2 genes will elucidate whether this mutation is disease-causative or not in this patient.

Patients with USH3. Two USH3 patients were found to be carriers of mutations in the *USH2A* and *PCDH15* genes. Patient RP-1459 was double homozygous for the *USH2A* mutations p.L555V and c.1841–2A>G, which were previously found in linkage disequilibrium in USH2 cases.[42] This patient displayed RP, slowly progressive hearing loss with onset in childhood, and left vestibular hypofunction. These data would confirm the initial diagnosis of USH3 in this patient. Different studies have shown that patients clinically diagnosed as USH3 have mutations or show linkage to the *USH2A* gene. Furthermore, patients with USH2A display different degrees of progression in hearing loss.[43–47] These results confirm that mutations in the *USH2A* gene could display USH3 clinical phenotype. In the present study, mutations c.1841–2A>G and p.L555V were also homozygously found in one typical USH2 patient (RP-1735). The presence of the same mutations in patients with different clinical classification reinforces the idea that there are unknown environmental or genetic factors that could modify the phenotype of patients.

Patient RP-938 was homozygous for the mutation p.R3X in the *PCDH15* gene. The RP was diagnosed at the age of nine. Currently, the patient has tunnel vision, diminished visual acuity, and cataracts. Hearing loss was congenital and progressive, but no information could be obtained about vestibular function. To date, all patients with mutations in the *PCDH15* gene correspond to USH1.[23,36,40,48] However, the *PCDH15* gene may also be responsible for some USH3 cases, similarly to *USH2A*, *MYO7A* and *CDH23*.[41,44–47,49]

Mutations Detected in Two Different USH Genes

Patient RP-1537 was found to be heterozygous for the p.R1240Q and p.C759F variants located in the *MYO7A* and *USH2A* genes, respectively. The USH1 clinical diagnosis of the patient was confirmed. Digenic inheritance has not been postulated for these genes. Thus, the *MYO7A* gene is presumably responsible for the disease. The study of the whole *MYO7A* gene will elucidate the validity of this hypothesis.

Mutations p.A366T and p.R3X, located in *CDH23* and *PCDH15* genes respectively, were detected in heterozygous state in patient RP-1374. Segregation analysis was performed, revealing that both mutations were also carried by the healthy father, discarding the digenic inheritance.

Variants with Uncertain Pathogenicity

Nine variants listed by the microarray as mutation or mutation/SNP were detected in the present study and considered as putative non-pathogenic (Table 2) for several reasons, now described.

The *MYO7A* variant p.T1566M was detected in one patient with USH2 heterozygous for an *USH2A* mutation. This variant was identified in patients with different clinical subtypes and was found in healthy control subjects.[23,32] The missense change p.Y1719C was identified in seven patients classified as USH1 or USH2, and its pathogenic effect was questioned.[23,32,33] The unpublished isocoding variant p.L1866L

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was detected in one patient with USH2 and it does not localize in canonical splicing regions.

The *CDH23* variant p.R1060W was detected in one patient with USH1. However, its pathogenicity was questioned in a previous report.[23]

Regarding to the *USH2A* variants, the changes p.V230M and p.E478D were detected in patients with different clinical subtypes. Furthermore, they were identified in control samples and patients with pathogenic mutations in other gene.[29,32,50] The variant p.I1520I was detected in one USH2 patient, but this change was described as polymorphism[51] and it does not localize in a consensus splicing region. The variants p.V2562A and p.E3088K reported as changes with unclear or nonpathologic implication,[29] were detected in patients with USH1, -2, or -3 from our cohort, some of them with clear mutations in other gene.

These variants were classified as probably non–disease causing, according to different reports and the data obtained in the present study. However, evidences of the existence of third alleles that act as modifiers of the phenotype have been reported in several ciliopathies.[52,53] Because of this, we cannot discard that some of these putative nonpathogenic variants could act as modifier alleles in USH patients.

Advantages and Limitations of the Genotyping Microarray

The genotyping USH-microarray is a robust technique that can analyze a large number of sequence variations simultaneously. It is low-cost and rapid compared to the direct sequencing of USH genes. Analysis is performed independently of the clinical subtype of patients, thus facilitating the detection of mutations located in genes usually associated with other clinical subtypes and of putative digenic inheritances. Furthermore, the detection of one mutation in a patient indicates the gene in which the second mutation is expected to be present.

Despite of these advantages, the USH genotyping microarray displays several limitations. It does not detect insertions or deletions with unknown start and end points. These types of rearrangements must be analyzed by other techniques such as Multiplex Ligation-dependent Probe Amplification (MLPA; http://www.mrc-holland.com). Furthermore, every mutation detected by the microarray must be confirmed by direct sequencing. Besides this, the array incorporates changes of unclear pathologic nature, which could lead to misdiagnosis. In addition to these limitations, the main disadvantage is that the microarray only detects previously identified mutations.

Thirty-two different mutations were detected by the microarray in our cohort of patients. Nineteen (59.3%) of them had previously been identified in Spanish patients. The Spanish population is genetically heterogeneous. This heterogeneity hinders the genetic study of patients with USH, due to the lack of major mutations and founder effects. It is important to stress the need to perform genetic studies of USH genes in specific populations, to incorporate novel mutations to the microarray. Regular updates of the microarray will increase both the efficiency and detection rate of mutations, especially in genetically heterogeneous populations.

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