Usher syndrome: An effective sequencing approach to establish a genetic and clinical diagnosis

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ABSTRACT

Usher syndrome is an autosomal recessive disorder characterized by retinitis pigmentosa, sensorineural hearing loss and, in some cases, vestibular dysfunction. The disorder is clinically and genetically heterogeneous and, to date, mutations in 11 genes have been described. This finding makes difficult to get a precise molecular diagnosis and offer patients accurate genetic counselling. To overcome this problem and to increase our knowledge of the molecular basis of Usher syndrome, we designed a targeted resequencing custom panel. In a first validation step a series of 16 Italian patients with known molecular diagnosis were analysed and 31 out of 32 alleles were detected (97% of accuracy). After this step, 31 patients without a molecular diagnosis were enrolled in the study. Three out of them with an uncertain Usher diagnosis were excluded. One causative allele was detected in 24 out 28 patients (86%) while the presence of both causative alleles characterized 19 patients out 28 (68%).

Sixteen novel and 27 known alleles were found in the following genes: *USH2A* (50%), *MYO7A* (7%), *CDH23* (11%), *PCDH15* (7%) and *USH1G* (2%). Overall, on the 44 patients the protocol was able to characterize 74 alleles out of 88 (84%).

These results suggest that our panel is an effective approach for the genetic diagnosis of Usher syndrome leading to: 1) an accurate molecular diagnosis, 2) better genetic counselling, 3) more precise molecular epidemiology data fundamental for future interventional plans.

1. Introduction

Usher syndrome (USH) is an autosomal recessive disorder characterized by the association of visual loss due to retinitis pigmentosa (RP), sensorineural hearing loss (SNHL) and in some cases, vestibular dysfunction. The disease has a prevalence of approximately 3.2–6.2/100,000 or even more (Kimberling et al., 2010), accounting for more than 50% of deaf-blind cases, about 18% of RP cases, and 5% of all cases of congenital deafness (Yan et al., 2010).

The disorder is clinically and genetically heterogeneous and USH was historically divided into three subtypes, according to disease severity and progression. Type I (USH1) is the most severe form characterized by profound congenital hearing loss, prepuberal onset of RP, and vestibular dysfunction. Type II (USH2) is characterized by moderate to profound (sloping pattern) congenital hearing loss, later onset of RP and normal vestibular function (Besnard et al., 2014). Finally, type III (USH3), the less common form with a prevalence of 2–4% among Usher patients (Yan et al., 2010) displays variable a) onset of progressive hearing loss, b) onset of RP and c) vestibular function (normal to absent). This classification of USH remains in clinical use, but atypical cases have been described

Abbreviations: USH, Usher syndrome; RP, retinitis pigmentosa; SNHL, sensorineural hearing loss; USH1, Usher syndrome Type I; USH2, Usher syndrome Type II; USH3, Usher syndrome Type III; TRS, targeted resequencing; ASHA, American Speech-Language-Hearing Association; PGM, Personal Genome Machine; VCF, Variant Call Format; IGV, Integrative Genomics Viewer; HSF, Human Splicing Finder; MAF, minor allele frequency

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(Yan et al., 2010). To date, at least 12 genetic loci have been mapped for the three types of USH and 11 genes have been identified so far (Bonnet et al., 2012). In particular, seven loci have been described for type 1 (*USH1B–USH1J*), three for type 2 (*USH2A*, *USH2C* and *USH2D*) and only one for type 3.

Due to the high degree of genetic and clinical heterogeneity, genetic screening with traditional methods such as direct sequencing or genotyping microarrays (i.e. able to detect only a given number of mutations) is challenging. Recently, targeted or whole exome sequencing was shown to be a powerful tool for discovering novel disease-related genes or genetic mutations in large genomic regions (Bowne et al., 2011). In this light, to increase our knowledge on the molecular basis of USH and to provide an accurate and reliable molecular diagnosis for USH patients, we developed a targeted resequencing (TRS) protocol for the simultaneous analysis of USH genes, demonstrating its usefulness in discovering novel alleles as well as in routine molecular diagnosis which in turn opens up for new perspectives in the clinical assessment of these patients.

2. Methods

2.1. Patients

In our study we recruited 47 individuals affected by a combination of hearing loss and visual impairment from different Italian clinical genetics centres. Specifically, 16 patients that had enrolled at the U.O. Genetica Medica Policlinico S. Orsola – Malpighi of Bologna and that had previously been characterized clinically and genetically as Usher patients, were used to validate our USH custom panel. The remaining 31 patients came from Bologna (see above), IRCSS Burlo Garofolo (Trieste) and Fondazione IRCSS Ca' Granda Ospedale Maggiore (Milano). Moreover, in order to confirm mutation segregation, first degree relatives were genotyped wherever possible. Hearing loss was evaluated in line with the American Speech-Language-Hearing Association (ASHA) (Clark, 1981). All participants underwent a vestibular test, with evaluation of spontaneous and evoked eye movements as well as a caloric test. Thorough ophthalmic exams were performed, including measurement of central visual acuity, a fundus exam and electroretinography. Information on ethnic background was not provided, but all subjects were of Italian origin. Blood samples were drawn for DNA extraction. All patients provided written informed consent for both genetic counselling and molecular genetic testing prior to enrolment. In the case of minors/children, a written informed consent was obtained from the next of kin. A total of 13 females (42% – mean age 51 years old) and 18 males (58% – mean age 37 years old) were enrolled in our study. The mean age of USH1 was less than 15 years old, whereas, in USH2, it was less than 28 years old (Table 1).

The study was performed in accordance with the tenets of the Declaration of Helsinki and the ethical guidelines of our institution.

2.2. DNA extraction

Genomic DNAs were extracted from peripheral whole blood using the QIAsymphony DSP DNA midi kit v1 and QIAsymphony robotic device (Qiagen, Milan, Italy) following manufacturer's instructions. DNA sample were stored at −20 °C until use. DNA integrity was evaluated with 1% agarose gel electrophoresis. DNA concentration was measured with a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, DE, USA). These concentrations were confirmed with Qubit fluorometer (Life Technologies, CA, USA).

Table 1
Clinical features of Usher patients. USHER ID: ID of each patient; Degree: severity of hearing loss; Visual field: type of visual impairment, RP: Retinitis Pigmentosa; Balance: vestibular function. N/A: Not available; *: Uncertain Usher diagnosis.

Hearing loss					Visual impairment		
Usher ID	Sex	Subtype	Degree	Onset	Visual field	Onset	Balance
USH 17	F	Usher 1	Profound	Congenital	RP	2nd decade	Areflexia
USH 18	M	Usher 2	Moderate-Severe	Infant	RP	2nd decade	Normal
USH 19	M	Usher 1	Profound	Congenital	RP	4th decade	Areflexia
USH 20	M	Usher 1	Profound	Infant	RP	3rd decade	Areflexia
USH 21	F	Usher 1	Profound	N/A	RP	N/A	Areflexia
USH 22	M	Usher 2	Moderate	2nd decade	RP	2nd decade	Normal
USH 23	M	Usher 1	Profound	Congenital	RP	2nd decade	Areflexia
USH 24	M	Usher 2	Moderate-Severe	N/A	RP	N/A	Normal
USH 25	F	Usher 2	Moderate-Severe	N/A	RP	N/A	Normal
USH 29	M	Usher 1	Profound	Congenital	RP	Infant	Areflexia
USH 32	F	Usher 1	Profound	Congenital	RP	Infant	Areflexia
USH 36*	F	Usher 2	Mild	6th decade	RP	4th decade	Normal
USH 45	F	Usher 1	Profound	Congenital	RP	Infant	Areflexia
USH 46	M	Usher 2	Moderate	Infant	RP	2nd decade	Normal
USH 48*	F	Usher 2	Moderate	6th decade	RP	3rd decade	Normal
USH 49	M	Usher 2	Moderate	3rd decade	RP	4th decade	Normal
USH 52	F	Usher 2	Moderate-Severe	2nd decade	RP	2nd decade	Normal
USH 53	M	Usher 1	Profound	N/A	RP	2nd decade	Areflexia
USH 54	M	Usher 2	Severe	2nd decade	RP	2nd decade	Normal
USH 60	F	Usher 2	Moderate-Severe	Congenital	RP	2nd decade	Normal
USH 62*	M	Usher 2	Moderate-Severe	4th decade	RP	3rd decade	Normal
USH 63	M	Usher 2	Moderate-Severe	2nd decade	RP	3rd decade	Normal
USH 66	F	Usher 1	Severe	Congenital	RP	2nd decade	Areflexia
USH 67	F	Usher 2	Moderate-Severe	Infant	RP	6th decade	Normal
USH 68	M	Usher 2	Moderate	N/A	RP	4th decade	Normal
USH 69	F	Usher 1	Profound	Congenital	RP	N/A	Areflexia
USH 83	F	Usher 2	Moderate	2nd decade	RP	2nd decade	Normal
USH 84	M	Usher 2	Moderate	Congenital	RP	3rd decade	Normal
USH 85	M	Usher 2	Moderate	Congenital	RP	4th decade	Normal
USH 86	M	Usher 2	Moderate	Infant	RP	2nd decade	Normal
USH 87	M	Usher 2	Moderate	Infant	RP	2nd decade	Normal

2.3. Targeted resequencing (i.e. Ion Torrent)

2.3.1. Usher genes panel

We defined a TRS panel including 10 genes involved in USH: *MYO7A*, *PCDH15*, *CDH23*, *USH2A*, *USH1C*, *PDZD7*, *GPR98*, *USH1G*, *WHRN* and *CLRN1*. These genes were selected according to data obtained from comprehensive public mutation databases such as LOVD, UMD-USHbases and HGMD (grenada.lumc.nl/LOVD2/Usher_montpellier/, www.umd.be/usher.html, www.hgmd.org/) and the most updated scientific literature. The TRS panel ensures an overall coverage of the targeted regions equal to 95,8% using 872 pairs of multiplex-PCR primers. The gene panel was defined using Ion Ampliseq Designer v1.2 (Life Techologies, CA, USA). Targeted regions include coding regions (CCDS), 3′UTRs, 5′UTRs and 50 bp exons/introns boundaries of each of the 10 selected genes, spanning over 95 Kbp.

DNA libraries were realized employing Ion AmpliSeq Library Kit 2.0 and were indexed with an Ion Xpress Barcode Adapters Kit (Life Technologies, CA, USA) according to the manufacturer's protocols. Using the Ion Personal Genome Machine (PGM) Template OT2 200 kit, the Template Ion Sphere Particles were created. A single-end 200 bp read sequencing run was obtained using the Ion PGM sequencing 200 kit v2 (Life Technologies, CA, USA) on Ion PGM System (Life Technologies, CA, USA). Each Ion 316™ Chip allowed for the simultaneous sequencing of eight indexed patients' libraries. Sequencing data were analysed in accordance with the Ion Torrent Suite TM v3.6; Small Insertions and Deletions (INDELs) and Single Nucleotides Variations (SNVs) were pooled into a Variant Call Format (VCF) version 4.1 (Danecek et al., 2011).

Human genome build 19 (hg19) was used as a reference. Integrative Genomics Viewer (IGV) was used to visualize the read alignment and confirm the variant calls.

ANNOVAR (Wang et al., 2010) was used to annotate INDELS and SNVs. The most probable disease-causing INDELs/SNVs were subsequently confirmed by direct bidirectional Sanger sequencing on an ABI PRISM 3500Dx Genetic Analyzer sequencer (Life Technologies, CA, USA), using ABI PRISM 3.1 Big Dye terminator chemistry (Life Technologies, CA, USA) according to the manufacturer's instructions. Sanger sequencing was also used to evaluate the segregation within the families.

2.3.2. Pathogenic effect of the identified mutations

In order to exclude genetic variants previously reported as polymorphisms, we compared the identified alleles with data reported in NCBI dbSNP build137 (www.ncbi.nlm.nih.gov/projects/SNP/) as well as in 1000 Genomes Project (www.1000genomes.org/), NHLBI Exome Sequencing Project (ESP) (esp.gs.washington.edu) and Exome Variant Server (evs.gs.washington.edu/EVS/). The impact of the missense mutations on protein structure was evaluated using sevelal *in silico* prediction tools: Mutation Taster (Schwarz et al., 2010), Polyphen-2 (Adzhubei et al., 2010) and SIFT (Ng et al., 2003). Furthermore, PhyloP algorithm (Pollard et al., 2010) allowed for evaluation of the conservation of residues across the species. In order to exclude variants not segregating within the analysed family, SNVs/INDELs were filtered by vcf-tools (vcftools.sourceforge.net/). Human Splicing Finder (HSF) version 2.4.1 (www.umd.be/HSF/) and Splice Site Prediction by Neural Network (NNSPLICE) version 9 (www.fruitfly.org) were used to predict the effects of the mutations on splicing.

3. Results

We designed a TRS custom panel for Usher Syndrome to analyse the coding regions, intron/exon boundaries and UTRs of 10 USH-related genes, with an overall coverage of the targeted regions equal to 95,8% (see Table S1). This custom panel was developed with the intent of creating an accurate molecular diagnostics tool for USH. A mean of 48 mega bases of raw sequence data was produced for each patient. The coverage was greater than or equal to 20-fold for each base, and the mean total coverage was 350-fold. An average of 120 genetic variants (SNVs/INDELs) were called for each patient; we excluded SNVs/INDELs with quality score (QUAL) < 20 as well as synonymous nucleotide substitutions. In addition, all variants previously reported as polymorphisms in the dbSNP v137 with a minor allele frequency (MAF) > 0.03 were excluded. After quality control filtering, we had an average of 4 residual SNVs/INDELs for each subject.

Initially, we validated our gene panel using sixteen USH control patients whose molecular genotype was already known. Our protocol was able to identify 31 out of 32 (97%) expected alleles (Table S2); only the mutation c.986dupG (p.N330Qfs*5) in the *MYO7A* gene was not detected. These findings clearly demonstrate that the protocol is sufficiently robust to be applied to the analysis of new USH cases. In this light, 31 participants were enrolled in the study and clinically classified as USH1 (11 subjects) and USH2 (20 cases). No USH3 cases were detected. The classification was uncertain for three USH2 cases due to their atypical presentation. They had particularly late onset of visual impairment followed by markedly late onset of hearing loss. Further clinical details are given in Table 1. At the molecular level, we detected 43 out of 56 alleles (77%) (Fig. 1), identifying 16 novel and 27 known alleles distributed as follows: 50% (28/56 alleles) in *USH2A*, 7% (4/56 alleles) in *MYO7A*, 11% (6/56 alleles) in *CDH23*, 7% (4/56 alleles) in *PCDH15* and 2% (1/56 alleles) in *USH1G* (see Table 2).

3.1. Molecular diagnosis for the USH 1 subjects

Within the group of clinically diagnosed USH1 patients, we were able to identify mutations in 8 cases out of 11 (73%) (Table 2). Briefly, two subjects (18%) had mutations in *MYO7A*, three (27%) in *CDH23*, two (18%) in *PCDH15* and one (9%) in *USH1G*. In seven cases both mutated alleles were detected while in one case (USH 45) only one mutated allele was identified. The second allele is most likely lacking being located in gene regions not yet analysed or due to the presence of large deletions, duplications, multiple exon insertions or sequence alterations, which adversely affects primer binding.

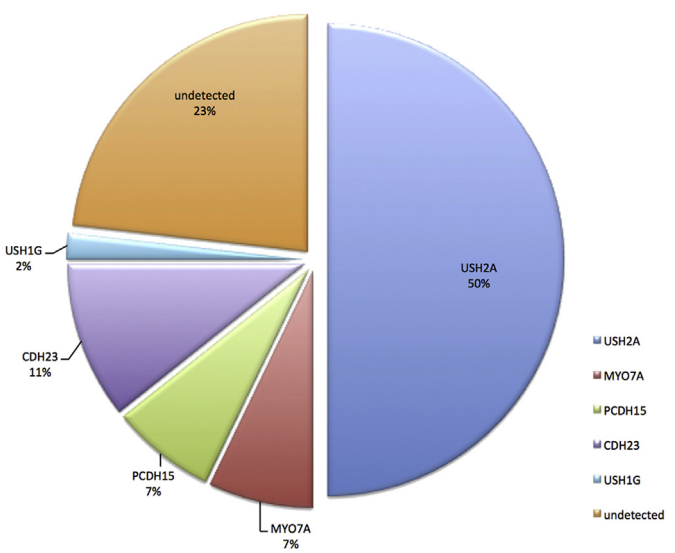

Fig. 1. The percentage of alleles detected in Usher genes. The 50% of the detected alleles were in *USH2A*, the 11% in *CDH23*, the 7% in *PCDH15* and *MYO7A*, the 2% in *USH1G* (overall 77%), 23% was undetected.

Table 2
Genotype Usher subjects. Novel mutations are in boldface and indicated by "†". Segregation: inherited alleles from the parents; N/A: parents not available; Classification: UV3 (likely pathogenic) - Pathogenic. Prediction tools: a: Mutation Taster; b: SIFT; c: Polyphen-2; d: NNSPLICE; e: HFS; f: previously described variant.

USH ID	Gene	Mutation	Segregation	Class	Prediction tools
Usher type 1					
USH 17	*CDH23*	c.9167delT p. V3056Afs*31	inherited from the mother	Pathogenic	f
		c.9167delT p. V3056Afs*31	inherited from the father	Pathogenic	f
USH 19	*PCDH15*	**c.1063delA p.S355Vfs*29**†	N/A	UV3	a
		c.1063delA p.S355Vfs*29†	N/A	UV3	a
USH 20		Unknown			
		Unknown			
USH 21	*CDH23*	c.9167delT p. V3056Afs*31	inherited from the mother	Pathogenic	f
		c.9167delT p. V3056Afs*31	inherited from the father	Pathogenic	f
USH 23		Unknown			
		Unknown			
USH 29	*CDH23*	**c.G484A p.G162R**†	inherited from the mother	UV3	a,b,c
		IVS62-4G > A†	inherited from the father	UV3	a,d,e
USH 32	*PCDH15*	**IVS30 + 1G > T**†	inherited from the mother	UV3	a,d,e
		c.3229_3230insA p.F1077Ifs*8†	inherited from the father	UV3	a
USH 45	*USH1G*	**c.C1339T p.R447W**†	inherited from the father	UV3	a,b,c
		Unknown			
USH 53	*MYO7A*	c.C1900T p.R634X	inherited from the father	Pathogenic	f
		c.C5581T p.R1861X	inherited from the mother	Pathogenic	f
USH 66	*MYO7A*	c.G73A p.G25R	inherited from the mother	Pathogenic	f
		c.C1708T p.R570X	inherited from the father	Pathogenic	f
USH 69		Unknown			
		Unknown			
Usher type 2					
USH 18	*USH2A*	c.IVS28 + 1G > A (c.5776 + 1G > A)	inherited from the mother	Pathogenic	f
		c.9811delA p.M3271Cfs*30†	inherited from the father	UV3	a
USH 22	*USH2A*	c.C9815T p. P3272L	inherited from the mother	Pathogenic	f
		c.2299delG:p.E767fs*21	inherited from the father	Pathogenic	f
USH 24	*USH2A*	c.5189_5199del p.Y1730Wfs*6	inherited from the mother	Pathogenic	f
		c.5189_5199del p.Y1730Wfs*6	inherited from the father	Pathogenic	f
USH 25		Unknown			
		Unknown			
USH 46	*USH2A*	**c.5416_5422delAAAAAGG p.K1807Afs*8**†	inherited from the mother	UV3	a
		c.5416_5422delAAAAAGG p.K1807Afs*8†	inherited from the father	UV3	a
USH 49	*USH2A*	c.1841-2G > A	N/A	Pathogenic	f
		Unknown			
USH 52	*USH2A*	**c.A5153C p.Q1718P**†	inherited from the mother	UV3	a,b,c
		Unknown			
USH 54	*USH2A*	c.2299delG p.E767fs*21	inherited from the father	Pathogenic	f
		c.C949A p.R317R	inherited from the mother	Pathogenic	f
USH 60	*USH2A*	IVS10-2A > G c.1841-2A > G	inherited from the father	Pathogenic	f
		c.G5399A p.W1800X	inherited from the mother	Pathogenic	f
USH 63	*USH2A*	**c.A5153C p.Q1718P**†	inherited from the mother	UV3	a,b,c
		Unknown			
USH 67	*USH2A*	c.C949A p.R317R	N/A	Pathogenic	f
		Unknown			
USH 68	*USH2A*	c.C12006A p.Y4002X	inherited from the mother	Pathogenic	f
		c.G1655C p.C552S†	inherited from the father	UV3	a,b,c
USH 83	*USH2A*	**c.A12700C p.T4234P**†	inherited from the mother	UV3	a,b,c
		c.A12700C p.T4234P†	inherited from the father	UV3	a,b,c
USH 84	*USH2A*	c.9424G > T p.G3142X	N/A	Pathogenic	f
		c.6029delT p.V2010Afs*10†	N/A	UV3	a
USH 85	*USH2A*	c.C10712T p.T3571M	N/A	Pathogenic	f
		c.C10712T p.T3571M	N/A	Pathogenic	f
USH 86	*USH2A*	c.G11864A p.W3955X	N/A	Pathogenic	f
		c.C1055T p.T352I	N/A	Pathogenic	f
USH 87	*USH2A*	c.G11864A p.W3955X	inherited from the mother	Pathogenic	f
		c.G11864A p.W3955X	inherited from the father	Pathogenic	f

In particular, one case (USH29) was compound heterozygote for two novel mutations in *CDH23* (NM_52836), c.G484A p.G162R and IVS62-4G > A. The missense mutation c.G484A leads to a substitution in amino acid from glycine (G), a non-polar amino acid, to arginine (R) a polar amino acid with a side-chain. The splice site mutation IVS62-4G > A gives a substitution from guanine to adenosine in intron 62, which is classified as disease-causing according to the *in silico* prediction tools (Table 2). The analysis of the effects of this variant by Human Splicing Finder (HSF) and NNSPLICE indicated that the score for acceptor site recognition would be increased slightly from 89.22 to 89.29 and from 0.84 to 0.93, respectively.

Three novel mutations were identified in *PCDH15* (NM_0011442765) in two USH1 subjects (USH 19 and USH 32). Patient USH 19 is homozygote for the novel frameshift deletion c.1063delA p.S355fs*29 in exon 10 leading to a stop codon 29 amino acids downstream (Table 2). The other subject (USH32) is compound heterozygote for two different novel mutations in *PCDH15*. The first allele is a frameshift insertion (c.3229_3230insA p.F1077Ifs*8) in exon 26 leading to a new stop codon 8 amino acids downstream, most likely giving an abnormally short, non-functional protein. The intronic mutation IVS30 + 1G > T is a splice site mutation in intron 30 (Table 2). Finally, subject USH 45

had a novel missense mutation c.C1339T p.R447W in *USH1G* (NM_173477) on one allele, while the second allele remained undetermined. This novel missense mutation in exon 2 leads to a substitution in amino acid from arginine (R), a polar amino acid with positive charge, to tryptophan (W) an essential amino acid with hydrophobic side chains.

3.2. Molecular diagnosis for the USH 2 subjects

Twenty USH2 cases were analysed; three of them were characterized by uncertain clinical diagnosis (i.e. markedly late onset of hearing loss) thus were excluded and 17 cases with a definite USH2 diagnosis were analysed (Table 2). Among these, 12 (71%) patients were successfully characterized at the molecular level with both causative alleles identified (Table 2). In 4 cases (24%), only one mutation was found, while in one case no causative alleles were detected (5%). Interestingly, all identified mutations were located in the *USH2A* gene and 9 novel alleles were identified (Table 2). Patient USH 18, along with the known splicing mutation (c.5776 + 1G > A; IVS28 + 1G > A) of maternal origin (Jaijo et al., 2010), had one novel mutant allele, c.9811delA p.M3271Cfs*30 inherited from the father. This frameshift deletion in exon 50 lies in a Cystein-rich domain, and causes a stop codon 30 amino acids downstream, presumably resulting in a truncated, non-functional protein (Table 2). Cases USH 63 and USH 52 had a novel missense mutation in heterozygosis (c.A5153C p.Q1718P), where an adenosine is substituted by cytosine in exon 25, leading to a change in amino acid from glutamine (Q), a polar amino acid to proline (P), a non-polar amino acid with cyclic side chain. Both patients have moderately severe SNHL with onset during adolescence, and visual impairment due to RP with onset during adolescence for USH 52 and in the third decade for USH 63: no balance difficulties were present (Table 1).

USH 68, carrying a nonsense mutation (c.C12006A p.Y4002X) (McGee et al., 2010) inherited from the mother and a novel missense mutation c.G1655C p.C552S in exon 10 leading to an amino acid change from cysteine (C) to serine (S), in a Laminin-type epidermal growth factor-like domain. Both residues are polar and uncharged. Patient USH 46 was homozygote for a novel deletion c.5416_5422delAAAAAGG p.K1807Afs*8 in exon 27 and he has had moderate SNHL since he was an infant, along with visual impairment since adolescence and normal vestibular reflexes (Table 1).

Case USH 83 carries a novel homozygous missense mutation (c.A12700C p.T4234P) due to a substitution of an adenosine with a cytosine in exon 63, leading to a change in amino acid from threonine (T), a polar, uncharged amino acid containing aliphatic hydroxyl group, to proline (P), a non-polar residue. This patient has moderate SNHL and visual impairment due to RP since adolescence, and normal vestibular reflexes (Table 1).

Finally, subject USH 84 carries a known nonsense mutation c.9424G > T p.G3142X (Baux et al., 2007) on one allele, and on the other a novel frameshift deletion (c.6029delT p.V2010Afs*10) which lies in a fibronectin type III6 domain, and causes a stop codon 10 amino acid downstream, most likely resulting in a truncated, non-functional protein. This patient shows moderate SNHL from birth, visual impairment with onset in his thirties and normal vestibular reflexes (Table 1).

Recently a novel gene, *CIB2* (NM_006383), was found to be implicated in the disorder (Riazuddin et al., 2012). Not being included in our TRS panel and in order to have conclusive data, we screened our negative patients for mutations in *CIB2* using Sanger sequencing. No causative alleles in this gene were detected in the whole series of USH cases under investigation.

Finally, despite a clinical outcome not typical for USH syndrome prompted us to exclude three cases from the analysis, they were later analysed with TRS panel and, as expected, they were negative for the presence of causative alleles.

4. Discussion

The high degree of genetic heterogeneity causes a substantial portion of USH patients to remain without an identified genetic cause, underlining the importance of exploring all possible causative genes to find the underlying genetic cause. Unfortunately, no therapy is currently available to stop the evolution of the disease or restore hearing or vision. However, numerous intervention strategies are available, and aim at slowing down the degenerative processes, treat complications and help patients cope with the social impact of the sensory deficits (Hamel, 2006). Although the therapeutic options are limited, it is nevertheless important for affected patients and their families to receive an early diagnosis of USH, and it would therefore be useful to establish an efficient method to identify the underlying molecular cause. As regards the molecular epidemiology, USH 1 is the most severe form accounting for around 30–40% of all subjects (Yan et al., 2010). USH 2 accounts for at least half of all USH cases (Rosenberg et al., 1997) while USH 3 is extremely rare being mostly found in given ethnic groups such as the Ashkenazi Jews (Ness et al., 2003) and the Finnish population (Pakarinen et al., 1996). In line with this distribution, our cohort consists of 18 individuals clinical defined as USH 1 (41%) and 26 as USH 2 (59%).

To date, different approaches to identify mutations within USH genes have been used. Before NGS era, standard methods were based on the analysis of a series of selected known mutations/short sequences (Rong et al., 2014; www.asperbio.com/) or they were restricted to specific gene with expensive and time consuming methods such as Sanger sequencing. Moreover, the large genetic heterogeneity underlying Usher syndrome coupled with the large size of some Usher genes (i.e. *USH2A*) further made these methods were not offered routinely thus lacking the ability to provide an appropriate molecular diagnosis. Recently, whole exome sequencing was applied to the analysis of genes involved in retinal degeneration diseases, such as genes related to the Usher syndrome (Corton et al., 2013; Huang et al., 2013). The overall exome variants were initially filtered taking into consideration only those belonging to a subset of candidate genes, already involved in Usher syndrome. Over the last year, a targeted sequencing approach was applied to the USH genes (Besnard et al., 2014; Yoshimura et al., 2014; Qu et al., 2014; Chen et al., 2014) being an ideal method for sequencing portions of the genome with high sensitivity and very affordable cost. Here, we further demonstrate the reliability of our custom Usher genes TRS panel allowing the successful identification of the vast majority of clinically characterized USH cases. In particular, after the exclusion of the three cases with an uncertain Usher diagnosis, one of the highest detection rate so far reported (84% overall) was obtained, a proportion not detectable with previous methodologies in a time and cost-effective way (i.e. without spending months of work and a significant amount of money).

For both USH1 and USH2 patients, no particular genotype–-phenotype relationship was observed. In fact, among the group of USH1 patients, the phenotype was homogeneous and no differences were found depending on the type of the involved gene and type of mutation. All subjects carrying novel mutations were affected by profound sensorineural hearing loss with congenital onset as well as balance difficulties, and three of them displayed an onset of RP during infancy. As regards USH2 subjects, they displayed clinical manifestations consistent with data reported in the literature, with a larger variation in the phenotype characterized by normal vestibular function, moderate to severe hearing impairment with a variable onset, and RP with onset no earlier than the second decade.

Pooling together the first and the second series of cases, our protocol was able to detect at least one causative allele in 40 out of 44 cases under investigation (91%), while it was conclusive (i.e. both causative alleles detected) in 34 out of 44 cases (77%) leading to an overall alleles characterization of 84% (74 out of 88). Interestingly, 16 novel alleles were identified thanks to this approach. For the cases in which only one causative allele was detected, several possible explanations may be taken into account: gene region not properly covered by sequencing data, the presence of large deletions, duplications, multiple exon insertions or any sequence alterations that could adversely affecting primer binding, and that went unnoticed using TRS. Moreover, for the patients that were completely negative for the presence of mutations among the known USH genes, we cannot rule out the possible presence of another not yet identified USH gene.

The absence of a clear genotype/phenotype relationship which could theoretically help the clinician, as well as the negative molecular data on the three cases with a highly peculiar and uncertain USH clinical presentation clearly underline the relevance of an accurate clinical examination/classification before starting the process of searching for a molecular diagnosis.

In conclusion, the development of a cost-effective, reliable and high throughput TRS approach, largely improves the availability of molecular tools for the diagnosis of USH. This method may lead to an earlier diagnosis along with better genetic counselling, with significant benefits for affected patients and their families. Finally, a more precise molecular epidemiologic overview is fundamental for planning future therapeutic interventions.

Disclosure

The authors declare no conflict of interest.

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