FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA

TRABALHO FINAL DO 6ºANO MÉDICO COM VISTA À ATRIBUIÇÃO DO GRAU DE MESTRE NO ÂMBITO DO CICLO DE ESTUDOS DE MESTRADO INTEGRADO EM MEDICINA

JÓNI LUÍS SOARES NUNES

PHENOTYPICAL AND MOLECULAR CHARACTERIZATION OF PORTUGUESE USHER SYNDROME PATIENTS

ARTIGO CIENTÍFICO

ÁREA CIENTÍFICA DE OFTALMOLOGIA

TRABALHO REALIZADO SOB A ORIENTAÇÃO DE:

PROFESSOR DOUTOR EDUARDO DUARTE SILVA

MARÇO/2015

Index

List of abbreviations

AONs: Antisense Oligonucleotides

AR: Autosomal Recessive

BBS: Bardet Biedl Syndrome

BCVA: Best Corrected Visual Acuity

CDH23 [605516]: Cadherin 23

CHUC: Centro Hospitalar Universitário de Coimbra

CLRN1 [606397]: *Clarin 1*

DFNB31 [607928]: Whirlin

DNA: Deoxyribonucleic Acid

EDTA: Ethylenediamine Tetraacetic Acid

EGF Lam: Laminin type EGF-like modules

ENT: Ear, Nose and Throat

ERG: Electroretinogram

FAF: Fundus Autofluorescence

F: Female

FN3: Fibronectin domain

GPR98 [602851]: G-protein coupled receptor 98

HL: Hearing Loss

ID: Identification

IOL: Intraocular Lens

ISCEV: International Society for Clinical Electrophysiology of Vision

LamG: Laminin G domain

LCA: Leber Congenital Amaurosis

LSDBS: Locus-specific Databases

M: Male

MF ERG: Multifocal Electroretinogram

MYO7A [276903]: Myosin VIIA

ND: Not Detected

NGS: Next Generation Sequencing

PCDH15 [605514]: Protocadherin 15

PSC: Posterior Sub-capsular Cataract

PZDZ7 [612971]: PDZ domain-containing gene 7

OD: Right Eye

OS: Left Eye

OU: Both Eyes

RP: Retinitis Pigmentosa

RPE: Retinal Pigment Epithelium

TM: Transmembrane Domain

TRIDs: Translational Read-through-inducing Drugs

USH: Usher Syndrome

USH1: Usher Syndrome Type 1

USH2: Usher Syndrome Type 2

USH3: Usher Syndrome Type 3

USH1C [605242]: USH1C gene

USH1G [607696]: USH1G gene

USH2A [608400]: USH2A gene

USMA: Usher Missense Analysis

VA: Visual Acuity

VAr: Vestibular Arreflexia

Wnl: Within Normal Limits

Abstract

Introduction: Usher syndrome (USH) is a recessive inherited disease characterized by sensorineural hearing loss (HL), visual loss due to retinitis pigmentosa (RP) and, in some cases, vestibular dysfunction. This syndrome is the most common cause that affects those two major senses, vision and hearing and encompasses three clinical sub-types (USH1, USH2 and USH3) as well as some additional atypical forms.

Usher type II is the most common clinical form, in all published series; since the *USH2A* gene is the most commonly mutated gene, our aim is to characterize from a molecular and phenotypical standpoint, a cohort of 48 portuguese Usher patients, determine if they carry mutations in *USH2A* gene and establish potential genotype-phenotype correlations.

Methods: Forty eight affected individuals were characterized from a molecular standpoint, and those carrying USH2A underwent complete phenotypical ophthalmological examination including, fundus photography, fundus autofluorescence (FAF), full-field ERG, multifocal electrophysiology (MF ERG), and ENT assessment.

Results: Disease-causing mutations in the *USH2A* gene were identified in 15 patients, of ten independent families. Consanguinity could be documented in 40% of families. Fourteen different *USH2A* mutations were detected (6 missense, 14 nonsense, 1 small rearrangement, 2 large gene rearrangements and 6 splice-site mutations), eight patients were homozygous and 7, either compound heterozygous or heterozygous. Mutations in the *DFNB31* gene (USH2D) were also identified in heterozygosity in two siblings. Five novel mutations were identified: three intragenic deletions encompassing several exons c. 7121_9258del, *USH2A* exon 3-

exon34 del, *USH2A* exon 39 – exon 47 del, a nonsense mutation, c.7932G>A, and a deep intronic homozygous mutation c.4988-19T>G, all of them showing potential to interfere with protein function.

Concerning to ophthalmological phenotype, nyctalopia was consistently the presenting complaint in 100% of patients. Best Corrected Visual Acuity (BCVA) varied from 0.1 and 0.8, at the last evaluation. Fundus appearance was compatible with RP in all probands varying from isolated peripheral bone spicules, patchy retinal pigment epithelium (RPE) areas of atrophy, thin vessels and normal discs (20%), to pale discs, near confluent peripheral bone spicules, extensive atrophic RPE changes and thin vessels (47%) to typical RP with preserved macula (33%). The full field ERG disclosed, in the majority, an unrecordable response (87%) and two probands had a severely attenuated, yet recordable, response (13%). Multifocal ERG analysis showed decreased central peak (100% cohort cases lower than 100microV), and occasional drift of the peak of maximal response to an extrafoveal hexagone. FAF revealed that 77% presented a typical ring of hyperautofluorescence while the remainder disclosed patchy hypoautofluorescence.

Conclusion: We characterized from both clinical and genetic standpoints, the first series of Portuguese patients with type II Usher syndrome. Molecular characterization is essencial to improve early diagnosis of USH and better understand the molecular pathomechanism so that is possible to develop treatment strategies to stop or revert the degenerative process of the retina, in a near future.

Keywords: Usher Syndrome; *USH2A mutations*; rearrangements; NGS; AON.

Resumo

Introdução: A síndroma de Usher é uma condição com transmissão autossómica recessiva caracterizada por surdez neurossensorial, perda de visão por Retinopatia Pigmentar e, em alguns casos, disfunção vestibular. Esta síndroma é a causa mais comum da afeção destes dois sentidos, visão e audição, englobando três sub-tipos clínicos (USH1, USH2 e USH3) bem como formas atípicas adicionais.

USH2 é o sub-tipo clinico mais comum, em todas as series publicadas; sendo *USH2A* o gene mais comummente mutado, o nosso objectivo é caracterizar de um ponto de vista molecular e fenotípico 48 portugueses com Síndrome de Usher, determinar se são portadores de mutações no gene *USH2A* e estabelecer potenciais correlações genótipo-fenótipo.

Métodos: Quarenta e oito indivíduos com a síndroma da Usher foram caracterizados do ponto de vista molecular, submetendo-se os portadores de *USH2A* a um exame oftalmológico fenotípico completo, fundoscopia, auto-fluorescência da retina, eletrorretinograma *full field*, eletrorretinograma multifocal e avaliação otorrinolaringológica.

Resultados: Mutações patológicas no gene *USH2A* foram identificadas em 15 pacientes, de 10 famílias não-relacionadas. Em 40% das famílias foi possível documentar consanguinidade. Detetaram-se 14 mutações diferentes no gene *USH2A* (6 *missense,* 14 *nonsense*, 1 pequeno rearranjo, 2 grandes rearranjos e 6 mutações de *splicing*; em 8 doentes as alterações genéticas eram homozigóticas e em 7, heterozigóticas compostas ou em heterozigotia. Foram igualmente identificadas mutações no gene *DFNB31* em heterozigotia, em 2 irmãos.

Foram identificadas cinco novas mutações: três delecções intragénicas envolvendo vários exões c. 7121_9258*del, USH2A exon* 3- *exon* 34 *del, USH2A exon* 39 – *exon* 47 *del,* uma

mutação nonsense c.7932G>A, e uma mutação homozigótica *deep intronic* c.4988-19T>G, todas mostrando potencial interferência com a função proteica.

No que diz respeito ao fenótipo oftalmológico, a nictalopia foi, de forma consistente, o sinal de apresentação em 100% dos doentes. A melhor acuidade visual corrigida variou entre 0.1 a 0.8, na última avaliação. Os aspetos fundoscópicos eram compatíveis com Retinopatia Pigmentar em todos os doentes, variando de espículas ósseas periféricas isoladas, áreas dispersas de atrofia do epitélio pigmentar da retina, vasos de calibre reduzido e disco óptico normal (20%), disco pálido, espículas ósseas confluentes periféricas, atrofia de áreas extensas do epitélio pigmentar da retina e redução importante do calibre vascular (47%) a um padrão típico de RP com preservação da mácula (33%). No ERG, a maioria dos doentes apresentou uma resposta indetetável (87%), com somente 2 doentes a revelar uma resposta profundamente atenuada mas detetável (13%). O MfERG evidenciou uma redução da amplitude do pico central (100% dos casos inferior a 100microV), e ocasionais picos de resposta maxima detetados fora do hexagono extra-foveal. A auto-fluorescência mostrou em 77% dos casos o típico anel de hiperautofluorescência macular enquanto nos restantes se observou um padrão mosqueado de hipoautofluorescência.

Conclusão: Caracterizámos do ponto de vista clínico e genético, a primeira série de doentes portugueses com Síndroma de Usher tipo 2. A caracterização molecular é essencial para aprimorar o diagnóstico precoce desta síndroma e melhor conhecer os mecanismos patogénicos de forma a permitir o desenvolvimento de estratégias terapêuticas para frenar ou reverter este processo degenerativo da retina, num futuro próximo.

Palavras-chave: Síndrome de Usher; mutações *USH2A*; rearranjos; NGS; AON.

Introduction

Usher syndrome (USH) is the most common form of deaf-blindness (responsible for more than 50%) (Saihan, et al., 2009) as an autosomal recessive disorder with a prevalence range from 3.3 to 6.4 per 100.000 live births (1) (Saihan *et al.* 2009). It is characterized by the association of sensorineural hearing loss (HL) and visual impairment due to retinitis pigmentosa (RP). Some patients will also exhibit vestibular areflexia (VAr).

The earliest descriptions were given by Von Graefe (1858), Liebreich (1861), who observed the syndrome among Jews in Berlin, and Hammerschlag (1907). Named for Charles Usher (1914), a British ophthalmologist who emphasized their hereditary nature (1907). (2-5)

It is clinically and genetically heterogeneous, so is divided into three types: USH1, USH2 and USH3 and nine genes have been identified responsible so far. Five causative genes have been reported for USH1: *MYO7A* (myosin VIIa; USH1B), *USH1C* (harmonin; USH1C), *CDH23* (cadherin 23, USH1D), *PCDH15* (protocadherin 15, USH1F), and *USH1G* (SANS; USH1G), with *MYO7A* being the most prevalent (Le Quesne Stabej, et al., 2012; Roux, et al., 2011). Three genes for USH2: *USH2A* (Usherin; USH2A), *GPR98* (GPR98/VLGR1; USH2C), and *DFNB31* (whirlin; USH2D) with *USH2A* responsible for 70-80% of the USH2 cases (Besnard, et al., 2012; Le Quesne Stabej, et al., 2012); only one gene for USH3: clarin 1 (*CLRN1*) and one additional locus has been mapped (6-10). In addition, one modifier gene (*PDZD7*) has been identified. (11,12). Other responsible genes are expected to be identified, as ~20% of clinical cases remain unsolved at the molecular level.

Three clinical subtypes (USH1, USH2 and USH3) are distinguished by the severity and progression of HL and presence or absence of vestibular dysfunction; this distinction is generally used to guide molecular testing. USH1 is the most severe form with congenital

profound HL and VAr. USH2 appears to be the most common clinical form of the disorder, accounting for over a half of all USH cases and is characterized by congenital moderate-to-severe HL, with normal vestibular function. In USH3, the HL is progressive in association with variable vestibular function. USH3 is rare, except in some populations with recognized founder effect where it may be responsible for over 40% of cases namely in the Finnish and Jewish Ashkenazi USH populations (Saihan, et al., 2009).

Genetics, biochemistry and proteomics have demonstrated that functionally different USH proteins are organized in networks in the eye and inner ear (6,8,13). This knowledge explains why defects in proteins of different families are causative for similar symptoms and contributed to the current insights into the function of USH proteins in inner ear and retina. Since recent studies demonstrate that the USH interactome is molecularly connected to the Bardet Biedl syndrome (BBS) proteins and some proteins involved in Leber congenital amaurosis (LCA), overlapping molecular mechanisms may underlie retinal degeneration in USH, BBS and LCA (8,13).

Since phenotypical heterogeneity is the hallmark of USH, an adequate clinical exam is crucial. A complete ophthalmological examination should include a morphological and functional evaluation of the retina, visual field testing and an electroretinogram (ERG) (14). Audiovestibular function should be assessed as part of a complete auditory and vestibular examination, including pyschoacoustic studies of hearing for puretones and speech, biomechanical measurement of middle ear integrity, including tympanometry and acoustic reflexes and neurophysiological studies of auditory brainstem function as well as vestibular studies such as electronystagmography and rotational chair, when appropriate or feasible.

Molecular genetic diagnosis for Usher syndrome evolved from the restriction studies of USH genes (Adato, et al., 1997) to extensive direct sequencing (Aller, et al., 2006; Baux, et al.,

2007; Besnard, et al.). Because of genetic heterogeneity, prioritization of the genes to be sequenced was facilitated by linkage analysis (Roux, et al., 2006; Roux, et al., 2011). Due to the large size of most Usher genes (totalizing more than 350 exons), Sanger sequencing of genes one-by-one remains expensive and time-consuming. Furthermore, large rearrangements have been described in *MYO7A, CDH23, GPR98, USH2A* and, particularly, in *PCDH15,* and their detection requires array-CGH studies, further complicating the analysis. Taken together, these strategies allow a reliable diagnosis for Usher patients with a mutation detection rate of about 90% for USH1 and USH2 patients (Besnard, et al., 2012; Roux, et al., 2011). However its application in clinical diagnosis is hampered by a very low detection rate, as most USH-causing DNA alterations are private or restricted to one or two families. NGS technology has recently demonstrated its capacity to detect DNA variants in sensorineural disorders known to be genetically heterogeneous (Brownstein, et al., 2011; Neveling, et al., 2012; Redin, et al., 2012), and a targeted NGS has shown potential use as a diagnostic platform applied to hearing loss (HL) (Shearer, et al., 2010).

Due to newborn deafness screening programs, hearing deficiencies are detected early in life. Cochlear implants with recommended bilateral implantation in USH patients is most effective when performed before one year of age. If congenitally deaf children get cochlear implants early in life, the auditory pathway can mature quite normally resulting in close to normal speech perception and vocalization, as well as hearing abilities.

To date, there is no clinical therapy for the retinal degenerative component of USH. However, the postnatal onset of retinal degeneration opens a time window for upcoming therapeutic interventions (9). Currently several non-gene-based therapy options are assessed for the retina (9, 15, 16). Especially, the identification of USH genes opens new doors such as gene-specific therapies, translational read-through-inducing drugs (TRIDs) or Antisense Oligonucleotides (AONs).

In most cases the clinical diagnosis is established in the second decade of life. Early diagnosis of USH supports parents in their choice for cochlear implants instead of learning sign language. Thus, the early diagnosis can improve the life-quality of USH patients and furthermore opens the time-window for upcoming therapeutic interventions to prevent blindness.

As USH2 appears to be the most common clinical form of the disorder, accounting for more than 50% of all USH cases (18,19) and among the three cloned genes, *USH2A* is the most commonly mutated gene. The purpose of this paper is to characterize from a phenotypical and genotypical standpoint, for the first time in this country, 48 Portuguese patients carrying the diagnosis of Usher syndrome, determine if they carry mutations in *USH2A* gene, as it is the most commonly affected gene in all published series, and establish potential genotype-phenotype correlations.

Population and Methods

Patients carrying the tentative diagnosis of Usher syndrome were collected from the Center of Excellence for Hereditary Eye Diseases from the Department of Ophthalmology, Centro Hospitalar Universitário de Coimbra (CHUC), between 2001 and 2014. A total of 48 probands with the diagnosis of retinitis pigmentosa or rod-cone dystrophy, in association with neurosensory deafness of variable severity, were collected during this period.

Detailed phenotypical characterization was performed, including family history, geographic provenance, best-corrected visual acuity, determination of refractive error slit-lamp examination and dilated fundus examination.

Fundus images were acquired in accordance with the internationally accepted guidelines using a TOPCON TRC 50X (Topcon Optical, Tokyo, Japan) and/or a Pan-retinal camera (Optomap R) (Optos PLC, Dunfermline, Scotland, UK).

Electrophysiology (ERG and multifocal ERG)

Ganzfeld ERG was performed in accordance with the ISCEV (International Society for Clinical Electrophysiology of Vision) guidelines. In brief, patients were dark-adapted for a period of 30 minutes followed by scotopic assessment. The full field ERG was then completed with recordings obtained in photopic conditions.

Multifocal ERGs (mfERGs) were recorded using DTL fiber electrodes, after a light adaptation period of 10 minutes and pupil dilation with tropicamide, before fundus photography, with a commercial system (RETIscan System; Roland Consult) (Kutschbach, 1997). Refractive errors were corrected in relation to the viewing distance. The stimulus used in the mfERG

consisted of 61 hexagons covering a visual field of up to 30° and presented on a 20-inch monitor at a viewing distance of 33 cm. Luminance was 120 cd/m2 for white hexagons and approximately 1 cd/m2 for black hexagons, resulting in a Michelson contrast of 99%. The hexagonal areas increased with eccentricity to compensate for local differences in signal amplitude because of differences in cone density across the retina (leading to a fourfold change in hexagon area size). Each hexagon was temporally modulated between light and dark according to a binary m-sequence (frame rate, 60 Hz). Observers were instructed to fixate a small black cross in the center of the stimulus. Fixation was continuously checked by means of online video-monitoring during the approximately 8-minute recording sessions. To improve fixation stability, sessions were broken into 47-second segments; eight trials were recorded in total. Signals were amplified with a gain of 100,000 and were band-pass filtered (5–300 Hz).

Reference and ground electrodes were attached to the ipsilateral outer canthus and forehead, respectively. The surface electrode impedance was less than 10 k_. Analyses were performed with the system software (RETIscan; Roland Consult) and standard statistical packages. First-order kernels were used for mfERG analysis because of their close correlation with the function of the outer retina (Hood, 1997). The obtained local ERGs responses were normalized by the area of stimulus delivery to obtain a density response (nV/deg^2). For each hexagon, the peak amplitude of P1—defined as the difference between N1 and P1 amplitudes—the N1 peak, and the implicit time of P1 component were computed. To easily evaluate spatial differences of the local ERG responses, responses from the 61 elements were divided into averages of five concentric rings around the fovea.

Fundus Autofluorescence

Fundus autofluorescence (FAF) was performed using the HRAII device (Heidelberg Engineering, Dossenheim, Germany) in accordance with the instructions from the manufacturer.

ENT assessment

To evaluate the audiological and vestibular characteristics of the affected individuals, probands underwent complete audiovestibular assessment. This included tonal and vocal audiometric testing, tympanometry to assess the normal middle ear function, transient evoked otoacoustic emissions, and auditory brainstem evoked responses to confirm absence of retrochoclear involvement. ENT evaluation was performed by a single physician at the ENT department of Centro Hospitalar Universitário de Coimbra, Coimbra, Portugal, in accordance to departmental and internationally accepted standards of good practice.

Usher molecular testing

Peripheral blood samples with EDTA anticoagulant were collected from each patient and close relatives (for segregation analysis). Genomic DNA was extracted using an automated DNA extractor (BioRobot EZ1, Qiagen, Hilden, Germany).

Molecular testing was performed in the Laboratoire de Génetique Moleculaire, INSERM, Montpellier, France (senior researcher: Anne F Roux). This is part of a multinational collaborative effort aiming for the identification of new genes and mutations associated with the Usher phenotype. For this purpose, this laboratory has developed a NGS (next generation

sequencing) approach (GS Junior, Roche 454 Sequencing) applied to a laboratory design of the USH-exome (SeqCap EZ Choice Library, Roche NimbleGen). Raw data obtained from this comprehensive molecular strategy are analyzed and deposited in a set of databases LSDBs USHbases freely available at https://grenada.lumc.nl/LOVD2/Usher_montpellier/. This set of databases includes links to tools such as USMA (USH Missense Analysis) that provides *in silico* analyses for any new missense alterations identified.

This study was approved by the local ethics committee and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from the participating individuals prior to the collection of clinical data and genomic samples.

Results

Forty-eight portuguese probands, carrying USH as a tentative diagnosis, were included in this study. As our aim is to characterize the phenotype and establish potential genotype-phenotype correlations in those patients carrying *USH2A* gene mutations, we focused on the fifteen patients (11 males, 4 females) who present *USH2A* mutations/rearrangements in the molecular analysis.

One family had 3 affected individuals in the same generation (no twins), 3 families presented with a pair of affected sibs (both cases non-consanguineous) while the remainder was single affected, comprising a total of 10 unrelated families. Consanguinity was documented in 40% of cases. Table 1 summarizes the molecular results for the 15 affected individuals. Fourteen different *USH2A* mutations were detected: 6 missense, 14 nonsense, 1 small rearrangement, 2 large gene rearrangements and 6 splice-site mutations; eight patients were homozygous and 7, either compound heterozygous or heterozygous. In 3 cases, one simplex and a sibship of 2 brothers, we were only able to identify an heterozygous nonsense *USH2A* mutation. Mutations in the *DFNB31* gene were also identified, in heterozygosity, in the two siblings, in which only one heterozygous *USH2A* mutation could be identified.

Five novel mutations in the *USH2A* gene were identified in this population: three intragenic deletions encompassing several exons c. 7121_9258del, *USH2A* exon 3 - exon34 del, *USH2A* exon 39 – exon 47 del, a nonsense mutation, c.7932G>A, and a deep intronic homozygous mutation c.4988-19T>G. The three intragenic deletions create a novel protein devoid of several highly conserved elements, and is likely to determine an important disruption in the complex protein network of USH genes. The novel nonsense mutation replaces a tryptophan by a stop codon at exon 41, eliminating critical elements of the N-terminus of the wild-type polypeptide, also interfering with protein function. The novel deep intronic mutation appears

to affect normal splicing, thus compromising translation beyond exon 25 of the *USH2A* gene. The basic demographics and clinical findings (ocular and auditory) are summarized in Tables 2 and 3. The average age of diagnosis was 29,8 years, while the average age at the last clinical evaluation was 49,8 years. Regarding the ophthalmological features, nyctalopia was the presenting complaint, observed in 100% of cases, with an average age of presentation of 15,3 years. At the last ophthalmological evaluation, best corrected visual acuity was below normal in all patients, varying from 0.1 and 0.8.

In our cohort, slit-lamp examination revealed significant changes in 80% of cases, mostly cataract - nuclear, cortical and posterior subcapsular (the most prevalent), and 50% were pseudophakic. None of the IOL implanted cases presented significant opacification of the posterior capsule. Dilated fundus examination disclosed a clinical picture compatible with RP in 100% of probands; it varied from isolated peripheral bone spicules, patchy retinal pigment epithelium (RPE) areas of atrophy, thin vessels and normal discs (20%), to pale discs, near confluent peripheral bone spicules, extensive atrophic RPE changes and thin vessels (47%) to typical RP with preserved macula (33%).

Full field ERG demonstrated that the majority presented with an unrecordable response (87%) and just two probands had a severely attenuated, yet recordable, response (13%). On the other hand, multifocal ERG analysis showed decreased central peak amplitude (100% cohort cases below 100 microV), and occasional drift of the peak of maximal response to an extrafoveal hexagone.

FAF revealed that 77% of this cohort presented a macular ring of hyperautofluorescence associated with peripheral hypoautofluorescence while the remainder probands disclosed patchy hypoautofluorescence of the macula.

Concerning to auditory clinical findings, ENT Standard revealed 100% probands with neurosensorial deafness, with no possibility to know a precise presentation age average due to lack of knowledge from patients.

ID	Gene/cDNA/Allele 1/Allele 2	Predicted protein(s)	Type(s)
USH2A1	USH2A/ c.2137G>C / c.2137G>C	p.Gly713Arg	**Missense**
USH2A2	USH2A/ c.923_924insGCCA/ USH2A del exon 3- exon 34	p.His308Glnfs*16/ -	**Nonsense/** **Large Rearrangement**
USH2A3	USH2A/ c.923_924insGCCA/ ND	p.His308Glnfs*16/ ND	**Nonsense/ -**
USH2A4	USH2A/ c.7121_9258del/ USH2A/ c.7121_9258del	p.Val2374_Gln3086delinsGlyfs*1	**Nonsense**
USH2A5	USH2A/ c.7932G>A/ DFNB31:c.19G>A	p.Trp2644X Gly7Ser	**Nonsense/ Missense**
USH2A6	USH2A/ c.7932G>A/ DFNB31:c.19G>A	p.Trp2644X Gly7Ser	**Nonsense/ Missense**
USH2A7	USH2A/ c.2809+1G>A/ USH2A/ c.2809+1G>A	p.Gly937Aspfs*12	**Nonsense**
USH2A8	USH2A/ c.2809+1G>A/ USH2A/ c.2809+1G>A	p.Gly937Aspfs*12	**Nonsense**

USH2A9	USH2A/ c.2299delG/ c.5329C>T	p.Glu767Serfs*21 /p.Arg1777Trp	**Nonsense/ Missense**
USH2A10	USH2A/ c.2299delG/ c.5329C>T	p.Glu767Serfs*21 /p.Arg1777Trp	**Nonsense/ Missense**
USH2A11	USH2A/ c.7932G>A/ USH2A/ c.7932G>A	p.Trp2644X	**Nonsense**
USH2A12	USH2A/ del exon 39-exon 47/ USH2A/ del exon 39-exon 47		**Small Rearrangement**
USH2A13 (a)	USH2A/ c.4988-19T>G/ USH2A/ c.4988-19T>G		**Splice Site Mutation**
USH2A14 (a)	USH2A/ c.4988-19T>G/ USH2A/ c.4988-19T>G		**Splice Site Mutation**
USH2A15 (a)	USH2A/ c.4988-19T>G/ USH2A/ c.4988-19T>G		**Splice Site Mutation**

Table 1. Molecular results

Legends: table 1 summarizes the changes identified in the USH2A gene and respective predicted changes in the protein. (a) Sibship of three

Patient ID	Sex	Age	Age diagnosis	Deafness	Age hearing impairment
USH2A1	M	63	50	NS	Before 10
USH2A2	M	68	40	NS	Moderate hearing loss, 18
USH2A3	F	67	45	NS	Between 10-20
USH2A4	M	48	24	NS	Before 10
USH2A5	M	43	18	NS	Between 10-20
USH2A6	M	44	30	NS	Before 10
USH2A7	M	36	22	NS	Before 10
USH2A8	F	34	20	NS	Between 10-20
USH2A9	M	44	33	NS	Moderate hearing loss, 7.
USH2A10	F	41	30	NS	Moderate hearing loss, 12
USH2A11	F	34	20	NS	Between 10-20
USH2A12	M	42	10	NS	Before 10
USH2A13 (a)	M	60	40	NS	Before 10
USH2A14(a)	M	52	25	NS	Between 10-20
USH2A15 (a)	M	71	25	NS	Between 10-20

Table 2: Basic demographics and auditory findings

.Legends: (a) Sibship of three; M: Male; F: Female; NS: Neurosensory

Patient ID	Age of nyctalopia	VA OD/OS	Slit-lamp Exam	Fundus	ERG	MF ERG	FAF
USH2A1	25	0.5/0.5	Pseudophakia OU	RP with preservation macula	Flat	Central peak 35 microV	Macular ring of hyperautofluorescence
USH2A2	30	0.4/0.5	Pseudophakia OU	Pale disc, peripheral bone spicules, atrophic RPE, thin vessels	Flat	Central peak 26 microV	Patchy hypoautofluorescence
USH2A3	15	0.3/0.2	Pseudophakia OU	Pale disc, peripheral bone spicules, atrophic RPE, thin vessels	Flat	Central peak 18 microV	Patchy hypoautofluorescence
USH2A4	7	0.5/0.4	PSC	Pale disc, peripheral bone spicules, atrophic RPE, thin vessels	Flat	Central peak 40 microV	Macular ring of hyperautofluorescence

USH2A5	8	0.8/0.9	Mild PSC cataract	Peripheral bone spicules, patchy atrophy, thin vessels, normal discs	Flat	Central peak 65 microV	Macular ring of hyperautofluorescence
USH2A6	10	0,6/0,7	Mild PSC cataract	Peripheral bone spicules, patchy atrophy, thin vessels, normal discs	Flat	Central peak 50 microV	Macular ring of hyperautofluorescence
USH2A7	8	0.2/0.1	PSC cataract	Pale disc, peripheral bone spicules, atrophic RPE, thin vessels	Flat	Central peak 15 microV	Patchy hypoautofluorescence
USH2A8	10	0.6/0.6	Mild PSC cataract	Peripheral bone spicules, patchy atrophy, thin vessels, normal discs	Flat	Central peak 45 microV	Macular ring of hyperautofluorescence

USH2A9	12	0.2/0.2	PSC OU	Pale disc, peripheral bone spicules, atrophic RPE, thin vessels	Flat	Central peak 20 microV	Patchy hypoautofluorescence
USH2A10	6	0.8/0.8	Wnl	Pale disc, peripheral bone spicules, atrophic RPE, thin vessels	Severely attenuated	Central peak 42 microV	Macular ring of hyperautofluorescence
USH2A11	10	0.8/0.6	Wnl	RP with preservation macula	Flat	Central peak 55 microV	Macular ring of hyperautofluorescence
USH2A12	3	0.6/0.7	Wnl	Pale disc, peripheral bone spicules, atrophic RPE, thin vessels	Flat	Central peak 17 microV	Macular ring of hyperautofluorescence

USH2A13 (a)	35	OD 0,6/OE 0,6	Pseudophakia OU	RP with preservation macula	Severely attenuated	Central peak 45 microV	Macular ring of hyperautofluorescence
USH2A14(a)	25	0,6/0.5	Pseudophakia OU	RP with preservation macula	Flat	Central peak 51 microV	Macular ring of hyperautofluorescence
USH2A15 (a)	25	0.2/0.3	Pseudophakia OU	RP with preservation macula	Flat	Central peak 24 microV	Patchy hypoautofluorescence

Table 3: Clinical features and ocular findings.

Legends: (a) Sibship of three; ND: Not Detected; VA: Visual Acuity; OD: Right eye; OS: Left Eye; OU: Both Eyes; PSC: Posterior Sub-capsular Cataract; Wnl: Within normal limits; RP: Retinitis Pigmentosa; ERG: Full-field Electroretinogram; MF ERG: Multifocal Electroretinogram; FAF: Fundus Autofluorescence

Discussion

In this study, we present the first mutational study in a large cohort of USH patients ever carried in Portugal. Each individual was extensively characterized from a clinical standpoint in an attempt to correlate the clinical findings with the molecular results.

A comprehensive phenotyical characterization of USH patients must be performed, as it is essential in differentiating patient's clinical characteristics, defining their diagnosis in one of those three clinical different subtypes.

Molecular testing is essential to better understand this condition. Knowing the enormous variability of phenotypes associated with this syndrome, including atypical cases and spurious associations of retinitis pigmentosa with hearing deficits, having a molecular diagnosis can confirm preliminary clinical suspicion, guide treatment options, help predict the prognosis and is very important for couples who wish to have genetic prenatal counselling. Nowadays it is possible, after several years of trying to achieve a technology capable of high detection rates, to perform a reliable molecular analysis using custom optimized NGS technology, putting us on track to reach gene therapy as a solution available for certain patients who have a specific molecular pattern. However, the substantial heterogeneity existent in USH and the high-costs inherent to those cutting-edge technologies can cause severe economic difficulties, therefore hampering the generalized use of such tests.

This analysis led to identification of a total of 11 different pathogenic mutations, 5 unreported in the literature and databases, and 6 previously described: three missense and three nonsense. The analysis of the previously unreported mutations uncovered: three intragenic deletions encompassing several exons c. 7121_9258del, *USH2A* exon 3 - exon34 del, *USH2A* exon 39 – exon 47 del, all inherited in homozygosity; a nonsense mutation, c.7932G>A, homozygous in

one family and heterozygous in a sibship of 2 affected sibs; and a deep intronic homozygous mutation c.4988-19T>G, identified in a sibship of three brothers, with a very late diagnosis of USH2. It is important to underscore that in the case where c.7932G>A is inherited in heterozigosity, a second, also heterozygous missense mutation was identified in the *DFNB31* gene (USH2D). It remains to be demonstrated whether this represents a digenic mechanism, or whether the second mutated allele, either in *USH2A or DFNB31* will be identified, and the heterozygous element represents a genetic modifier.

The three intragenic deletions create a novel protein devoid of several highly conserved elements, including EGF Lam, FN3 and Lam G subunits, of variable extent; this fact is likely to determine novel polypeptides incapable of properly interacting with the remainder elements of the complex protein network of USH genes, thus affecting normal functioning of both photoreceptors and ciliated inner ear cells.

The novel nonsense mutation replaces a tryptophan by a stop codon at exon 41, eliminating critical elements of the C-terminus of the wild-type polypeptide, including several FN3 subunits, the TM element and the PDZ-binding motif. The absence of these critical elements also interferes with protein function and interactions.

The novel deep intronic mutation appears to affect normal splicing, thus compromising translation beyond exon 25 of the *USH2A* gene. This intronic change, c.4988-19T>G, was the most prevalent mutation in this cohort, since it was identified in homozygosity in a sibship of three. This change is extremely interesting as it may be amenable for therapy using a Antisense Oligonucleotide (AON) approach to restore normal splicing. Several in vitro tests, namely a minigene approach will soon be used to fully confirm the pathogenicity of this novel change, as a pre-requisite to design the appropriate treatment.

The two nonsense mutations, c.7932G>A and c. 2809+1G>A, are each one responsible for

13% of allele modifications. If we compare the relative allele frequencies of this Portuguese population with the ones observed in the Spanish population, due to geographical proximity, we didn´t find the c.2299delG mutation, as a widespread mutation, found in 15% of Spanish Population (20) as well as other Southern European populations (21). This mutation is also present in the Portuguese population, although at a much lower frequency (7%). The resulting pathogenic protein functional modification: p.Glu767Serfd*21, derives from that common ancestral mutation, which is the most prevalent in several populations (22).

It should be underscored that the p.Arg1777Trp, also identified in our cohort was described as Probably Pathogenic, and that p. Gly713Arg and p.Gly7Ser have both been described as "Effect Unknown" [https://grenada.lumc.nl/LOVD2/Usher_montpellier, 35].

In an attempt to establish a possible phenotype-genotype correlation between missense mutations and clinical findings, we focused on those five patients carried this class of mutation (one homozygous, four heterozygous) and compared them with all other mutation types. The patient who inherited an homozygous missense USH2A mutation evidenced slightly better clinical features, with later onset nyctalopia and relatively less severe RP. However, no real conclusions can be drawn, as we do not have important longitudinal data that would allow the establishment of a disease progression pattern of the retinal degeneration component. The remainder heterozygous cases did not display better phenotypical features compared to other patients.

Since two distinct patterns were observed for FAF, an attempt was made to correlate the patchy hypoautofluorescence pattern with a specific genotype. We were unable to uncover a trend when comparing the hyperautofluorescence ring group with the other subgroup.

However, the limited number of eyes would not allow the establishment of any statistically significant conclusions. Larger population studies and international collaborative efforts are

needed to allow adequate genotype-phenotype correlations, to further advance knowledge of this condition and promise better treatment and care for Usher patients.

References

1. Moller, C. G., Kimberling, W. J., Davenport, S. L. H., Priluck, I., White, V., Biscone-Halterman, K., Odkvist, L. M., Brookhouser, P. E., Lund, G., Grissom, T. J. Usher syndrome: an otoneurologic study. *Laryngoscope* 99: 73-79, 1989. [PubMed: 2909824] [Full Text: John Wiley & Sons, Inc.]

2. Usher, C. H. On the inheritance of retinitis pigmentosa, with notes of cases. *Roy. Lond. Ophthalm. Hosp. Rep*. 19: 130-236, 1914.

3. Von Graefe, A. Exceptionelles Verhalten des Gesichtsfeldes bei Pigmententartung der Netzhaut. *Graefes Arch. Ophthalm*. 4: 250-253, 1858.

4. Liebreich, R. Abkunft aus Ehen unter Blutsverwandten als Grund von Retinitis pigmentosa. *Dtsch. Klin*. 13: 53 only, 1861.

5. Hammerschlag, V. Zur Kenntnis der hereditaer-degenerativen Taubstummen und ihre differential-diagnostische Bedeutung. *Z. Ohrenheilk*. 54: 18-36, 1907.

6. Kremer, H. *et al.* Usher syndrome: molecular links of pathogenesis, proteins and pathways. *Hum. Mol. Genet.* 15 Spec No 2, R262-R270 (2006).

7. Reiners, J. *et al.* Molecular basis of human Usher syndrome: deciphering the meshes of the Usher protein network provides insights into the pathomechanisms of the Usher disease. *Exp. Eye Res.* 83, 97-119 (2006).

8. Wolfrum, U. Usher Syndrome: Pathogenesis, Diagnosis and Therapy. Satpal A (ed.), pp. 51-73 Nova Science Publishers (2011).

9. Yang, J. Usher Syndrome: Genes, Proteins, Models, Molecular Mechanisms, and Therapies. 293-328. Dr. Sadaf Naz. *Hearing Loss* (2012).

10. Dad, S. *et al.* Identification of a novel locus for a USH3 like syndrome combined with congenital cataract. *Clin. Genet.* 78, 388-397 (2010).

11. Schneider, E. *et al.* Homozygous disruption of PDZD7 by reciprocal translocation in a consanguineous family: a new member of the Usher syndrome protein interactome causing congenital hearing impairment. *Hum. Mol. Genet.* 18, 655-666 (2009).

12. Ebermann, I. *et al.* PDZD7 is a modifier of retinal disease and a contributor to digenic Usher syndrome. *J. Clin. Invest* 120, 1812-1823 (2010).

13. Van Wijk, E. *et al.* Usher syndrome and Leber congenital amaurosis are molecularly linked via a novel isoform of the centrosomal ninein-like protein. *Hum. Mol. Genet.* 18, 51-64 (2009).

14. Marmor MF & Zrenner E (1998-99): Standard for clinical electroretinography (1999 update). International Society for Clinical Electrophysiology of Vision. Doc *Ophthalmol* 97: 143–156.

15. Pennings, RJ. *et al.* Audiologic performance and benefit of cochlear implantation in Usher syndrome type I. *Laryngoscope* 116, 717-722 (2006).

16. Overlack, N. *et al.* Usher Syndrome: Pathogenesis, Diagnosis and Therapy. Ahuja,S. (ed.), pp. 377-395 *Nova Science Publishers*, Inc. USA, (2011).

17. Cohen, M. *et al.* The changing face of Usher syndrome: clinical implications. *Int. J. Audiol.* 46, 82-93 (2007).

18. Rosenberg, T; Haim, M; Hauch, AM; Parving, A: The prevalence of Usher syndrome and other retinal dystrophy-hearing impairment associations. *Clin. Genet* 1997, 51:314-21.

19. Spandau UH, Rohrschneider K: Prevalence and geographical distribution of Usher syndrome in Germany. *Graefes Arch Clin Exp Ophthalmol* 2002, 240:495-498.

20. Garcia-Garcia G; Aparisi M, Jaijo T *et al*. Mutational screening of the *USH2A* gene in Spanish USH patients reveals 23 novel pathogenic mutations. *Orphanet J Rare Dis*. 2011 Oct 17;6:65.

21. Aller E, Larrieu L, Jaijo T, Baux D, Espinos E *et al.* The USH2A c.2299delG mutation: dating its common origin in a Southern European population. *Eur J Hum Genet* 18: 788-793.

22. Liu XZ, Hope C, Liang CY, Zou JM, Xu LR, Cole T, Mueller RF, Bundey S, Nance W, Brown SD: A mutation (2314delG) in the Usher syndrome type IIA gene: high prevalence and phenotypic variation. *Am J Hum Genet* 1999, 64:1221-1225.