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MYO7A and *USH2A* gene sequence variants in Italian patients with Usher syndrome

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Purpose: To analyze the spectrum of sequence variants in the *MYO7A* and *USH2A* genes in a group of Italian patients affected by Usher syndrome (USH).

Methods: Thirty-six Italian patients with a diagnosis of USH were recruited. They received a standard ophthalmologic examination, visual field testing, optical coherence tomography (OCT) scan, and electrophysiological tests. Fluorescein angiography and fundus autofluorescence imaging were performed in selected cases. All the patients underwent an audiologic examination for the 0.25–8,000 Hz frequencies. Vestibular function was evaluated with specific tests. DNA samples were analyzed for sequence variants of the *MYO7A* gene (for USH1) and the *USH2A* gene (for USH2) with direct sequencing techniques. A few patients were analyzed for both genes.

Results: In the *MYO7A* gene, ten missense variants were found; three patients were compound heterozygous, and two were homozygous. Thirty-four *USH2A* gene variants were detected, including eight missense variants, nine nonsense variants, six splicing variants, and 11 duplications/deletions; 19 patients were compound heterozygous, and three were homozygous. Four *MYO7A* and 17 *USH2A* variants have already been described in the literature. Among the novel mutations there are four *USH2A* large deletions, detected with multiplex ligation dependent probe amplification (MLPA) technology. Two potentially pathogenic variants were found in 27 patients (75%). Affected patients showed variable clinical pictures without a clear genotype-phenotype correlation.

Conclusions: Ten variants in the *MYO7A* gene and 34 variants in the *USH2A* gene were detected in Italian patients with USH at a high detection rate. A selective analysis of these genes may be valuable for molecular analysis, combining diagnostic efficiency with little time wastage and less resource consumption.

Usher syndrome (USH; OMIM 276900-2276905, 605472) is an autosomal recessive disease in which sensorineural hearing loss is associated with photoreceptor degeneration with the clinical features of retinitis pigmentosa [1-3]. USH is usually distinguished by three subtypes [4]. Individuals with USH1 are generally born completely deaf or lose most of their hearing early. Deafness is then followed by progressive visual impairment caused by retinitis pigmentosa, which usually becomes apparent in childhood. The patients often have difficulty maintaining their balance owing to problems in the vestibular system. USH2 is characterized by variable degrees of hearing loss and progressive visual troubles beginning in adolescence or early adulthood. Vestibular responses are usually normal. Patients with USH3 usually have normal hearing at birth but develop progressive and severe hearing loss over time. Retinitis pigmentosa usually develops in late childhood or adolescence, and balance problems are present in most cases.

The prevalence of the disease has been estimated at about 3.5–8:100,000 in European populations [5] and 4.4:100,000 in the United States [6]. In a recent survey, USH represented 10.8% of inherited retinal dystrophies in southern France [7]. USH is inherited as an autosomal recessive trait, and to date, 15 USH loci have been characterized and 12 causative genes identified (A-genes, last update May 31, 2014). There are six *USH1* identified genes: *MYO7A* (NM_000260.3, OMIM# 276903), coding the protein Myosin VIIa; *USH1C* (NM_153676.3; OMIM# 605242), coding Harmonin; *CDH23* (ID: 64072; OMIM# 605516), coding Cadherin 23; *PCDH15* (ID: 65217; OMIM# 605514), coding Protocadherin 15; *USH1G* (ID: 124590; OMIM# 607696), coding *SANS* and *CIB2* (ID: 10518; OMIM# 605564) coding Ca2+-and integrin-binding protein [8]. Three genes have been associated with USH2: *USH2A* (ID: 7399; OMIM# 608400), coding the protein Usherin; *USH2C* (ID: 84059; OMIM# 602851), coding *GPR98* (formerly known as *VLGR1b* or *MASS1*); *USH2D* (ID: 25861; OMIM# 607928), coding *Whirlin*. Finally

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USH3 phenotype has been associated with the gene *CLRN1* *USH3A* (ID: 7401; OMIM# 606397), coding the protein Clarin-1.4, and more recently with the genes *ABHD12* (ID: 26090; OMIM# 613599) coding the enzyme with the same name [9] and *HARS* (ID: 3035; OMIM#142810) coding histidyl-tRNA synthetase [10].

Although a common phenotype has been described in the literature concerning the different USH types, the identified USH genes encode for proteins from different classes and families with different functions. Growing evidence suggests that these proteins are organized in a protein "interactome" [11-13] in the inner ear and the retina, which is critical for the development, maintenance, and correct function of the sensorineural cells.

Variants in the *MYO7A* gene represent the most common cause of USH1, accounting for approximately 50% of cases [14-16]. The gene is located on chromosome 11q13.5 and has 49 exons. It codes a 2,215-amino-acid protein, myosin VIIA, which is produced in the retina and in the inner ear and acts on the transport processes through the cilia of the retinal photoreceptors. In addition to USH, *MYO7A* variants have been associated with autosomal dominant non-syndromic sensorineural hearing impairment (DFNA11, OMIM 601317) [17] and autosomal recessive deafness (DFNB2, OMIM 60060) [17].

USH2A is the most common mutated gene in USH2 [18,19]. It is located on chromosome 1q41 and contains 72 exons. It codes the protein usherin, which can be found in a short (1,546 amino acids) and a long (5,202 amino acids) isoform, both present in the retina where they are functionally linked with the other proteins of the interactome. *USH2A* gene variants have also been found in autosomal recessive retinitis pigmentosa without hearing loss (MIM 613809) [20].

To date, more than 424 variants have been identified in the coding region of MY07A, and the majority are single-base variants. In contrast, more than 648 *USH2A* variants have been detected, the majority are also single-base variants.

In Italian patients with USH, molecular data were previously obtained using the Asper Biotech microarray that investigated 612 already known variants using APEX technology [21]. In another study, the same group analyzed 12 patients with USH with next-generation sequencing (NGS), highlighting some limitations of this technology for USH [22]. Furthermore, ten Italian families affected by USH1 were screened for *MYO7A* sequence variants within a collaborative investigation that examined patients of different origin [15].

At present, no specific study has reported the complete sequence of *MYO7A* and *USH2A*, the most common USH genes, involving a large series of Italian patients with USH. In the present study, sequence variants of the *MY07A* and *USH2A* genes were determined with direct sequencing in a group of 36 Italian patients affected by USH.

METHODS

Clinical evaluation: Thirty-six Italian patients (from 33 different families) with a clinical diagnosis of USH were recruited through the Hereditary Retinal Degenerations Referring Centre of the Eye Clinic of the University of Florence. Except the visual and hearing impairment they didn't have other health related problems. Ten were males and 26 females. The mean age was 39.7 years (±13.7 years) (range 17-72 years).

Criteria for USH phenotype included hypoacusia of various degrees associated with hemeralopia, diffused retinal dystrophy at fundus examination, visual field loss and abnormal ERG responses. The presence of typical bone spicules could be found in most of the patients but was not considered within the inclusion criteria.

The study adhered to the tenets of the Declaration of Helsinki and to the ARVO statement on human subjects. It is approved by the Local Ethics Committee. Moreover, each patient gave written informed consent.

All subjects included in the study were clinically evaluated with a standard ophthalmologic examination, fundus photography (Zeiss Retinograph, Carl Zeiss, Dublin, CA), optical coherence tomography (OCT) scan (Topcon 3D OCT-1000, Topcon Medical Systems, Oakland, NJ), and ERG (Electrophysiological Diagnostic Unit Retimax, Roland Consult, Brandenburg, Germany) performed according to the existing International Society for Clinical Electrophysiology of Vision (ISCEV) Guidelines [23]. Most patients underwent automated visual field testing (Humphrey Visual Field Analyzer, Carl Zeiss) while selected patients with poor vision were tested with Goldmann perimetry. In most of the cases, electrophysiological and perimetric examinations were performed in our department, but for a few patients, we accepted examinations performed during the previous year in other hospitals and included in the patient's medical documentation. Fluorescein angiography (FA; Zeiss Retinograph with Image Processing Software Visupac, Carl Zeiss) was performed on three patients with sine pigmento fundus appearance to improve the visualization of the retinal dystrophy. Fundus autofluorescence imaging (FAF; Confocal SLO, HRA, Heidelberg Engineering, Heidelberg, Germany) was performed on all affected patients who agreed to collaborate.

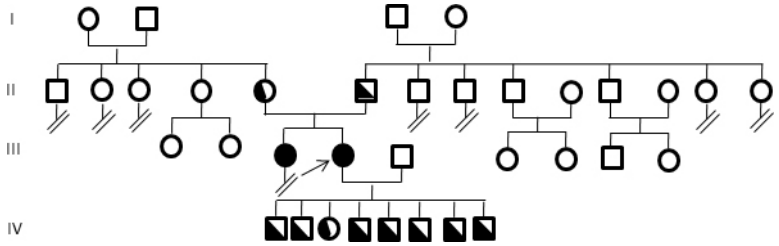

Figure 1. Pedigree of a family with two sisters with USH2. The patients carried in the *USH2A* gene the single sequence variant c.4144T>C (p.Trp1382Arg), which was first supposed to be present in homozygosis. Because this variant was detected in the father but not in the mother of the patients, a deletion was then suspected. The evaluation of the patients with a new MLPA kit for the gene *USH2A* allowed identification in the mother and her two daughters of a heterozygous deletion of exons 17–19.

All patients underwent an audiologic examination for the 0.25–8,000 Hz frequencies. Vestibular function was evaluated with specific tests (caloric test, study of spontaneous and induced nystagmus with Frenzel glasses or video-oculoscopy).

According to the ophthalmologic and audiologic phenotype, the patients were then clinically distinguished in USH1 or USH2. Finally, all patients underwent genetic testing for the complete sequencing of the *MYO7A* gene in USH1 and the *USH2A* gene in USH2. Some patients first classified as USH2 in whom no *USH2A* mutation was detected later received complete sequencing of the *MYO7A* gene.

DNA extraction and PCR amplification: Following informed consent and a complete medical history of each family, 5 ml of peripheral blood was taken from the antecubital vein using EDTA-containing vials. DNA was extracted from 1 ml of peripheral blood using the QiaSymphony DNA Blood Midi kit on the QIAsymphony SP workstation (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Genomic DNA was isolated from peripheral leukocytes, using the QiaSymphony DNA Blood Midi kit on the QIAsymphony SP workstation (Qiagen), according to the manufacturer's protocol. All coding exons, including intron-exon boundaries of the *MYO7A* and *USH2A* genes, were processed with the automated Core System (Beckman Coulter, Fullerton, CA). After purification, amplicons were sequenced on the 3730 DNA Analyzer (ABI, Foster City, CA). The sequences were assembled and analyzed using SeqScape software (ABI).

Variants of unknown pathogenicity were interpreted with Alamut 2.2 (Interactive Biosoftware, Rouen, France), a decision-support software application for medical molecular genetics. The software relies on web-based prediction software, such as Align-GVGD, SIFT, PolyPhen, Mutation Taster (hosted by Interactive Biosoftware). Note that Alamut 2.2 scoring systems provide a predictive evaluation only for missense variants.

Multiplex ligation dependent probe amplification (MLPA) analysis was performed on the patients in whom no variant or only one variant was identified in the *USH2A* gene and on the carriers of a variant in homozygosity in which study of the parents did not confirm the Mendelian inheritance of the variant detected in the proband (Figure 1). The MLPA reaction (P361-A1/P362-A2 SALSA MLPA kit; MRC Holland, Amsterdam, The Netherlands) was performed according to the manufacturer's recommendations. One microliter of each reaction product was separated on a POP-7 polymer with capillary electrophoresis using the 3730 DNA Analyzer (ABI). Freely available software provided by MRC Holland was used to analyze the MLPA data (Coffalyser; MRC Holland; Figure 2).

The deletion was further confirmed with real-time quantitative PCR with a predesigned TaqMan Copy Number Assay specific for *USH2A* genes (Hs00961154_cn, Hs03023043_cn, Hs02691757, Applied Biosystems) using TaqMan® Universal Master Mixes with the following parameters of run : hold 95 °C for 10 min, cycle 95 °C for 15 s and 60 °C for 60 s (40 cycles). Quantitative PCR was performed using an ABI 7900HT instrument (Applied Biosystems) in a 96-well optical plate using six replicates for each sample in a final volume of 20 µl, according to the manufacturer's protocol. Data were analyzed using CopyCaller to estimate the copy number for each sample.

RESULTS

Thirty-six Italian patients with a clinical diagnosis of USH (from 33 families) were clinically examined: ten were male and 26 female. The mean age was 39.7 years (±13.7 years; range 17–72 years). The age of onset of the symptoms (usually hemeralopia and/or perception of visual field constriction) varied, ranging from 4 to 37 years, with an average of 17.5 years (±8.8 years).

Snellen visual acuity ranged from 1.0 to light perception, with an average value of 0.56 (±0.37). In the best eye, 20

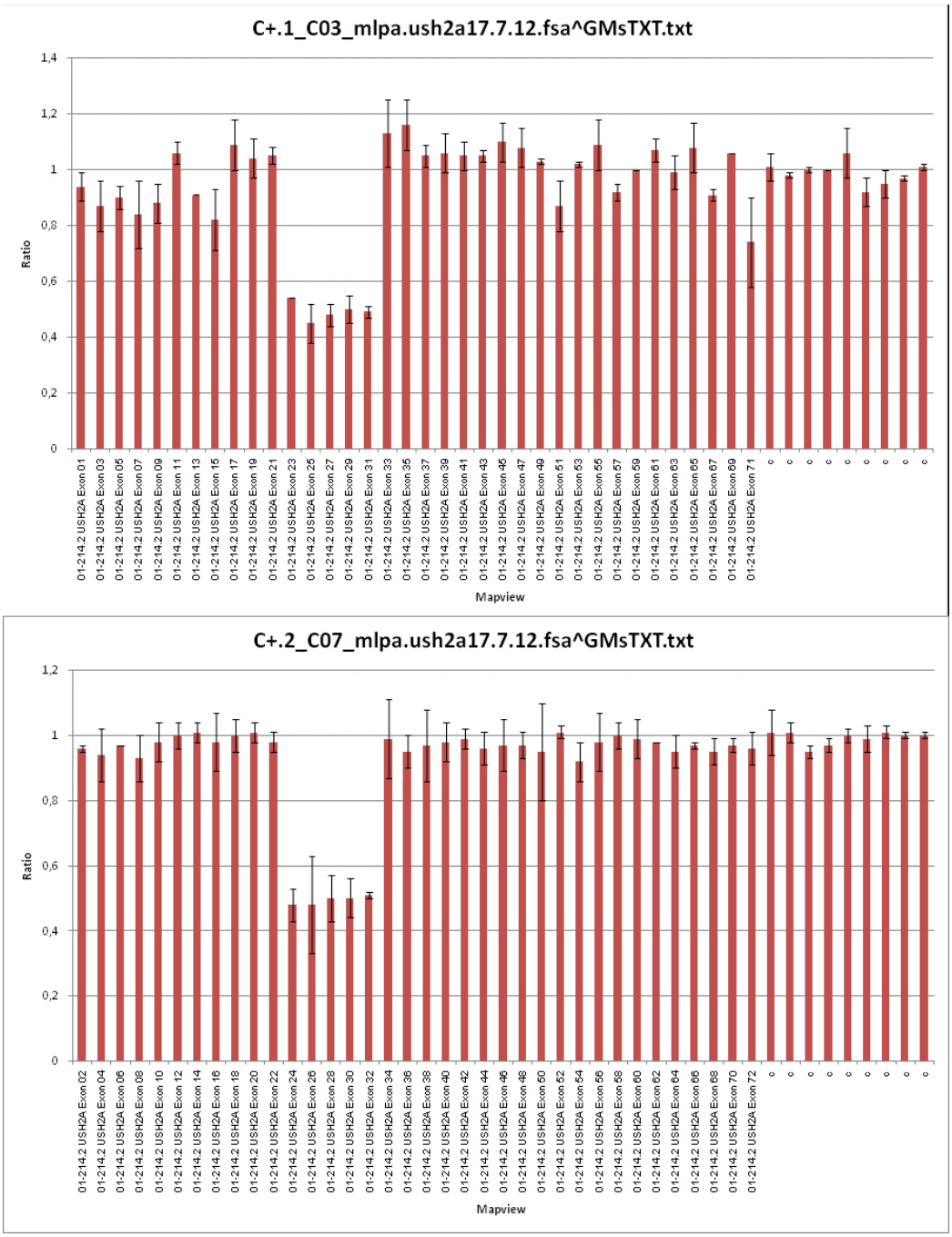

Figure 2. MLPA probe signals and results of the analysis. The Coffalyser Sample Plate Generator was used to automatically create sample plate files for the MLPA analysis. Results showed that probe signals from exons 23 to 32 of the *USH2A* gene (see the x-axis) were significantly decreased (ratio on the y-axis is about 0.5) indicating the presence of a deletion across those exons in patient CF.

(55.6%) patients presented normal visual acuity or mild visual loss (0.9–0.6), nine patients (25%) had moderate visual loss (05–0.2), and the other seven patients (19.4%) showed low vision (<0.2).

At fundus examination, 18 patients (50%) showed typical retinitis pigmentosa with attenuated vessels, diffused retinal dystrophy with evident bone-like spicules, and pale optic disc. The other 18 patients (50%) presented an atypical fundus appearance with rare or absent pigment clumpings (Figure 3).

Posterior lens opacity was found in at least one eye in 15 patients (41.7%) while another nine patients (25%) had

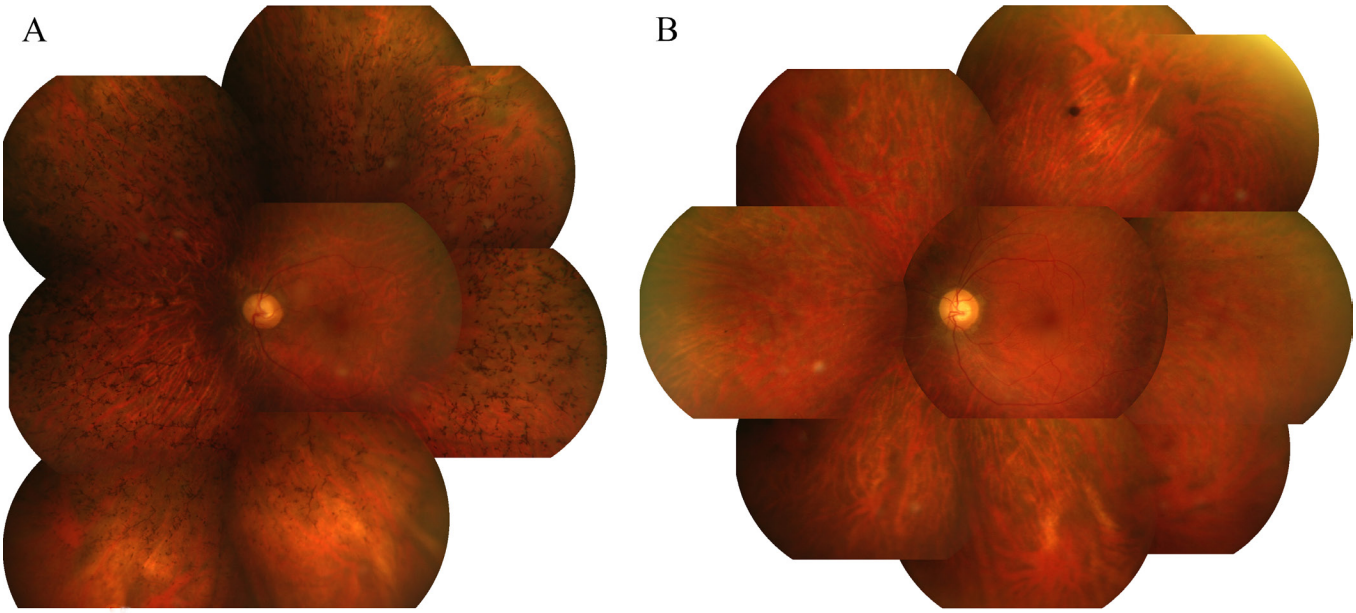

Figure 3. Fundus pictures of two brothers of the same family, who carry the same genotype (c.2299del (p.Glu767Serfs*21) *USH2A* variant on one allele and c.13130C>A (p.Ser4377*) *USH2A* on the other allele). One patient (**A**, patient GF) shows a typical form of retinitis pigmentosa while the other one (**B**, patient GS) presents with an atypical fundus appearance with mild retinal dystrophy and rare pigment deposits. In the second case, the clinical diagnosis is supported by typical visual field and electrophysiological abnormalities. The coexistence of two different clinical pictures in the same pedigree emphasizes the poor genotype-phenotype correlation reported in our series.

already undergone cataract surgery in at least one eye. One (2.8%) patient presented anterior lens opacity.

OCT showed a macular edema in at least one eye in six patients (16.7%) while three cases (8.3%) presented a unilateral macular hole and four patients (11.1%) had macular atrophy.

ERG scotopic response was always bilaterally flat or severely abnormal. Photopic response was abnormal in all patients.

The Goldmann visual field was concentrically reduced in both eyes of 14 patients (38.9%) while 19 (52.7%) showed peripheral loss and pericentral scotomas leaving limited central and peripheral field remnants. In three patients (8.3%) visual field loss was severe, with only a central isle of surviving vision.

Three patients showed early and severe deafness with balance problems and onset of retinal dystrophy in childhood and were clinically classified as USH1. Thirty-three patients were affected with a milder hearing impairment, no vestibular dysfunction, and variable retinal phenotypes and were classified as USH2.

All three patients with USH1 carried two *MY07A* sequence variants. Among the 33 patients clinically classified as USH2, 22 (66.6%) carried two *USH2A* variants, two (6.1%) carried two *MYO7A* variants, one (3%) carried a single *MYO7A* variant, two (6.1%) carried a single *USH2A* variant, one (3%) carried a *MYO7A* variant on one allele and an *USH2A* variant on the other allele while in the remaining five patients no *MYO7A* or *USH2A* variant was detected. The patients in whom two pathogenic *MYO7A* or *USH2A* variants were found were screened for the *USH3A/Clrn1* gene, but no variant was identified.

The *MYO7A* and *USH2A* sequence variants detected in our series are summarized in Table 1. On the whole, out of the 36 patients with USH in our series, 27 (75%) carried two pathogenic variants in the same gene (five in the *MYO7A* gene and 22 in the *USH2A* gene) and therefore could be considered genetically characterized. In the *MYO7A* gene, ten sequence variants were identified in seven patients. Three patients were compound heterozygous, two patients were homozygous, one patient showed the variant only on one allele, and one patient carried the *MYO7A* variant on one allele and an *USH2A* variant on the other allele. All variants were missense. Each family had a specific *MYO7A* variant.

Six *MYO7A* variants have already been reported in the literature [15,22,24] and are therefore considered pathogenic. The other four variants [c.4798G>C (p.Gly1600Arg), c.5810T>C (p.Leu1937Pro), c.977T>C (p.Leu326Pro), c.4039C>A (p.Arg1347Ser)] were novel and could not be

Table 1. The *MYO7A* and *USH2A* sequence variants detected in our series.

Patient	USH phenotype	Gene	Allele 1	Allele 2	Allele 1 reference	Allele 2 reference
LA	USH1	*MYO7A*	c.721C>G (p.Arg241Gly)	c.721C>G (p.Arg241Gly)	[15]	[15]
ZE	USH1	*MYO7A*	**c.4798G>C (p.Gly1600Arg)**	**c.5810T>C (p.Leu1937Pro)**	*	*
TG	USH1	*MYO7A*	**c.977T>C (p.Leu326Pro)**	c.5617C>T (p.Arg1873Trp)	*	[43]
LMa	USH2	*MYO7A*	c.4411T>C (p.Ser1471Pro)	c.4411T>C (p.Ser1471Pro)	[22]	[22]
BN	USH2	*MYO7A*	c.395C>T (p.Pro132Leu)	**c.4039C>A (p.Arg1347Ser)**	[15]	*
IP	USH2	*USH2A*	c.2052A>G (p.=)	Not found	[44]	
		MYO7A	Not found	Not found		
BPi	USH2	*USH2A*	c.2276G>T (p.Cys759Phe)	Not found	[45]	
		MYO7A	Not found	Not found		
NLu	USH2	*MYO7A*	c.5866G>A (p.Val1956Ile)	Not found	[16]	
		USH2A	Not found	Not found		
EM	USH2	*USH2A*	c.7246A>G (p.Asn2416Asp)	Not found	*	
		MYO7A	Not found	c.3979G>A (p.Glu1327Lys)		[46]
BG	USH2	*USH2A*	**c.8906C>G (p.Ser2969*)**	**c.8906C>G (p.Ser2969*)**	*	*
PiF	USH2	*USH2A*	c.2610C>A (p.Cys870*)	**c.1729T>C (p.Cys577Arg)**	[16]	*
ZK	USH2	*USH2A*	c.1663C>G p. Leu555Val, c.1841–2A>G (p.?)	**c.11750_11753del (p. Val3917Glyfs*15)**		*
AF	USH2	*USH2A*	**c.439_445del (p. Ser147Profs*2)**	c.7595–2144A>G (p. Lys2532Thrfs*56)	*	[26]
GF	USH2	*USH2A*	c.2299del (p.Glu767Serfs*21)	c.13130C>A (p.Ser4377*)	[25]	[16]
SF	USH2	*USH2A*	**c.9811del (p. Met3271Cysfs*30)**	**c.9959–1G>C (p.?)**	*	*
RA	USH2	*USH2A*	c.1663C>G (p. Leu555Val), c.1841–2A>G (p.?)	**c.8628G>A (p.Trp2876*)**	[33]	*
JP	USH2	*USH2A*	c.8232G>A (p.Trp2744*)	c.10450C>T (p.Arg3484*)	[16]	[27]
PB	USH2	*USH2A*	c.11864G>A (p.Trp3955*)	c.12067–2A>G (p.?)	[21]	[28]
BV	USH2	*USH2A*	**c.4144T>C (p.Trp1382Arg)**	**c.3317-?_4251+?**	*	*
BM	USH2	*USH2A*	**c.4144T>C (p.Trp1382Arg)**	**c.3317-?_4251+?**	*	*
CP	USH2	*USH2A*	c.1841–2A>G (p.?)	c.1841–2A>G (p.?)	[33]	[33]
BL	USH2	*USH2A*	c.1434G>C (p. Glu478Asp), c.7595–2144A>G (p. Lys2532Thrfs*56)	**c.4628-?_4758+?**	[26,29]	*

Patient	USH phenotype	Gene	Allele 1	Allele 2	Allele 1 reference	Allele 2 reference
FT	USH2	*USH2A*	**c.12859_12863del (p.Pro4287Argfs*4)**	c.13130C>A (p.Ser4377X)	*	[16]
SC	USH2	*USH2A*	**c.9811del (p.Met3271Cysfs*30)**	**c.9959–1G>C (p.?)**	*	*
CF	USH2	*USH2A*	**c.11831C>A p.Ala3944Asp**	**c.4759-?_6325+?**	*	*
FS	USH2	*USH2A*	**c.4046del (p.Ser1349Phefs*17)**	c.5776+1G>A (p.?)	*	[33]
ML	USH2	*USH2A*	**c.1040A>G (p.Asp347Gly)**	**c.9811del (p.Met3271Cysfs*30)**	*	*
LMo	USH2	*USH2A*	c.5776+1G>A (p.?)	**c.14977_14978del (p.Phe4993Profs*7)**	[33]	*
MG	USH2	*USH2A*	c.1663C>G (p.Leu555Val), c.187C>T (p.Arg63*)	**c.11918del (p.Ala3973Valfs*11)**	[33,47]	*
GS	USH2	*USH2A*	c.2299del (p.Glu767Serfs*21)	c.13130C>A (p.Ser4377*)	[25]	[16]
VE	USH2	*USH2A*	**c.11194C>T (p.Gln3732*)**	**c.11194C>T (p.Gln3732*)**	*	*

The sequence variants-identified for the first time in this study are marked in bold on Allele 1 and/or Allele 2 columns and with an asterisk on Allele 1 reference and/or Allele 2 reference columns. Three patients were clinically classified as USH1. Thirty-three patients were classified as USH2. All the 3 USH1 patients carried 2 MY07A sequence variants. Among the 33 patients clinically classified as USH2, 22 carried 2 USH2A variants, 2 carried 2 MYO7A variants, 1 carried a single MYO7A variant, 2 carried a single USH2A variant, 1 carried a MYO7A variant on one allele and an USH2A variant on the other allele. In the remaining 5 patients no MYO7A or USH2A variants could be detected and they were not included in this table.

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found in the 1000 Genomes Database. They were labeled as deleterious by the predictive software Alamut 2.2. The results of the analysis of the novel *MYO7A* variants are summarized in Table 2.

In the *USH2A* gene, 34 sequence variants were identified in 25 patients. The variants were present in compound heterozygosis in 19 patients, in homozygosity in three patients, as a single allele variant in two patients, and in association with a *MYO7A* variant on the other allele in one patient. Eight variants were missense, nine nonsense, six splicing mutations, and 11 deletions. c.1663C>G (p.Leu555Val) and c.9811del (p.Met3271Cysfs*30) were the most common allelic variants of the *USH2A* gene in our Italian patients with USH; these variants were found in 3/25 patients (12%), but almost all patients carried private sequence variants. Fifteen variants have already been reported in the literature [15,16,21,25-30] while the remaining 19 are novel. Among them, 14 [c.8906C>G (p.Ser2969*), c.11750_11753del (p.Val3917Glyfs*15), c.439_445del (p.Ser147Profs*2), c.9811del (p.Met3271Cysfs*30), c.9959–1G>C (p.?), c.8628G>A (p.Trp2876*), c.3317-?_4251+? (deletion of exons 17–18–19 with MLPA), c.4628-?_4758+? (deletion of exon 22 with MLPA), c.12859_12863del (p.Pro4287Argfs*4), c.4759-?_6325+? (deletion of exons 23–32 by MLPA), c.4046del (p.Ser1349Phefs*17), c.14977_14978del (p.Phe4993Profs*7), c.11918del (p.Ala3973Valfs*11), c.11194C>T (p.Gln3732*)] are nonsense, splicing variants, or deletions and can be considered pathogenic. The remaining five missense variants [c.1729T>C(p.Cys577Arg), c.4144T>C (p.Trp1382Arg), c.11831C>A (p.Ala3944Asp), c.1040A>G (p.Asp347Gly), c.7246A>G (p:Asn2416Asp)] could not be found in the 1000 Genomes Database and were labeled as disease-causing by the Alamut 2.2 software. The results of the analysis of the novel *USH2A* variants are summarized in Table 3.

DISCUSSION

A group of 36 Italian patients with a clinical diagnosis of USH received a comprehensive ophthalmological examination and underwent molecular genetic analysis of the genes *MYO7A* and *USH2A*. The age of onset of the symptoms (usually hemeralopia and/or visual field reduction) and the clinical pictures, including best-corrected visual acuity (BCVA), perimetry, and ERG responses varied. On fundoscopy, half of the patients showed typical retinitis pigmentosa while the other half presented an atypical clinical picture with rare or absent pigment clumping. The high prevalence of atypical retinal dystrophy with poor pigment migration is not considered typical of USH, even if it has been reported in other syndromic forms of retinitis pigmentosa such as Bardet-Biedl [31]. It may be related to a peculiar genetic background of USH in Italian patients, although a previous investigation of USH in Italy did not mention this clinical feature [21].

According to the audiologic and ophthalmologic evaluation, the patients were distinguished in USH1 (and screened for *MYO7A* variants, three patients) and USH2 (and screened for *USH2A* variants, 33 patients). Two patients clinically classified as *USH2A* did not carry *USH2A* sequence variants and then underwent an *MYO7A* examination leading to the detection of biallelic potentially pathogenic variants. This finding is in agreement with previous studies [16,32-35] that reported the identification of molecular variants in a gene not corresponding to the clinical USH type and suggests that the procedure of molecular genetics should also include genes inconsistent with the traditional clinical classification of USH.

Ten *MYO7A* sequence variants were identified in seven patients (biallelic in five patients and on a single allele in two patients). In the three patients clinically classified with USH1, all the expected six *MYO7A* variants were identified, in agreement with a previous study [15] that reported a high detection rate in Italian patients with USH1, even if our USH1 series is too small to allow a general statement. They were all missense, but other types of *MYO7A* variants (nonsense, deletions, duplications) have been detected in larger series [15,16,32,34,36,37]. Four variants were novel but were interpreted as pathogenic by the predictive software Alamut 2.2. In our series, all the variants were private, occurring only in isolated families, and were distributed along the gene without a clear indication of a mutational hotspot. The variants c.721C>G (p.Arg241Gly) and c.4411T>C (p.Ser1471Pro) have already been reported in Italian patients with USH ([15] and [22] respectively), and the repeated detection in various small series of Italian patients suggests that these variants may be a recurrent variant in Italian patients with USH, even if c.721C>G (p.Arg241Gly) has been reported in other ethnic groups [16].

Thirty-four *USH2A* sequence variants were identified in 25 patients (biallelic in 22 patients and on a single allele in three patients). Among the 33 patients originally classified as USH2, two potentially disease-causing *USH2A* variants were found in 22 (66.6%) cases while one single variant was detected in another three patients (9.1%) for a total of 47/66 (71.2%) alleles in agreement with the detection rate reported in other studies that investigated other ethnic groups [16,18,19,34,35,38]. The detected variants belonged to different subtypes (missense, nonsense, splicing, deletions). Nineteen were novel, and among them, 14 were nonsense, splicing variants, or deletions while the other four were

Table 2. Pathogenicity clues of the missense variant found in *MYO7A* gene in this study.

DNA Level (cDNA) Protein Level (p.)	Conserved nucleotide (phyloP: −14.1;6.4)	Physico chemical difference Grantham [0–215]	Variation is in protein domain	Align GVGD	SIFT	Mutation Taster
c.4798G>C (p. Gly1600Arg)	Highly (phyloP: 5.86)	Moderate (Grantham dist.: 125)	FERM domain	C65 (GV:0.00 - GD: 125.13)	Deleterious (score: 0, median: 3.70)	disease causing (p value: 1)
c.5810T>C (p. Leu1937Pro)	Highly (phyloP: 4.73)	Moderate (Grantham dist.: 98)	FERM domain (Band 4.1 domain)	C0 (GV:353.86 - GD: 0.00)	Deleterious (score: 0, median: 3.69)	disease causing (p value: 1)
c.4039C>A (p.Arg1347Ser)	Weakly (phyloP: 1.50)	Moderate (Grantham dist.: 110)	FERM, N-terminal	C0 (GV:241.31 - GD: 21.04)	Deleterious (score: 0, median: 3.70)	disease causing (p value: 1)
c.977T>C (p.Leu326Pro)	Highly (phyloP: 5.13)	Moderate (Grantham dist.: 98)	Myosin head, motor domain	GVGD: C0 (GV:234.72 - GD: 50.17)	Deleterious (score: 0, median: 3.71)	disease causing (p value: 1)

All sequence variants highly conserved the amino acid, up to *C.elegans* (considering 15 species). Align-GVGD: a grade of C0-C65 is given where C0 is benign and C65 is most likely pathogenic. SIFT (Sort Intolerant From Tolerant,) Score ranges from 0 to 1. The amino acid substitution is predicted to be damaging if the score is <0.05, and tolerated if the score is 0/>0.05. Mutation Tester: A prediction is given as either ‘disease-causing’ or ‘polymorphism’ along with a p value indicating the security of the prediction (with 1 being most secure). PolyPhen (polymorphism phenotyping): “Probably damaging” (it is believed most likely to affect protein function or structure), “Possibly damaging” (it is believed to affect protein function or structure), “Benign” (most likely lacking any phenotypic effect).

Table 3. Pathogenicity clues of the missense variant found in *USH2A* gene in this study.

DNA Level (cDNA) Protein Level (p.)	Conserved nucleotide (phyloP: −14.1;6.4)	Physico chemical difference Grantham [0–215]	Variation is in protein domain	Align GVGD	SIFT	Mutation Taster
c.1729T>C (p.Cys577Arg)	Moderately (phyloP: 3.92)	Large (Grantham dist.: 180)	EGF-like, laminin	C65 (GV:0.00 – GD: 179.53)	Deleterious (score: 0, median: 3.85)	disease causing (p value: 1)
c.4144T>C (p.Trp1382Arg)	Moderately (phyloP: 3.76)	Moderate (Grantham dist.: 101)	Fibronectin, type III	C0 (GV:268.54 – GD: 82.54)	Deleterious (score: 0, median: 3.83)	disease causing (p value: 1)
c.11831C>A p.Ala3944Asp	Highly (phyloP: 5.61)	Moderate (Grantham dist.: 126)	Fibronectin, type III	C65 (GV:0.00 – GD: 125.75)	Deleterious (score: 0, median: 3.85)	disease causing (p value: 1)
c.1040A>G (p. Asp347Gly)	Moderately (phyloP: 3.35)	Moderate (Grantham dist.: 94)	Laminin, N-terminal	GVGD: C0 (GV:213.16 – GD: 64.73)	Deleterious (score: 0, median: 3.85)	disease causing (p value: 1)
c.7246A>G (p:Asn2416Asp)	Moderately (phyloP: 3.68)	Small (Grantham dist.: 23)	Fibronectin, type III	C0 (GV:213.69 – GD: 22.66)	Deleterious (score: 0, median: 3.85)	disease causing (p value: 1)

All sequence variants highly conserved the amino acid, up to *Chicken* (considering 10 species). Align-GVGD: a grade of C0-C65 is given where C0 is benign and C65 is most likely pathogenic. SIFT (Sort Intolerant From Tolerant,) Score ranges from 0 to 1. The amino acid substitution is predicted to be damaging if the score is <0.05, and tolerated if the score is 0/>0.05. Mutation Tester: A prediction is given as either 'disease-causing' or 'polymorphism' along with a p value indicating the security of the prediction (with 1 being most secure). PolyPhen (polymorphism phenotyping): "Probably damaging" (it is believed most likely to affect protein function or structure), "Possibly damaging" (it is believed to affect protein function or structure), "Benign" (most likely lacking any phenotypic effect). **c.9959–1G>C**, predicted change at acceptor site 1 bps downstream: −100.0% (MaxEnt: −100.0%, NNSPLICE: −100.0%, HSF: −100.0%). The consequence of this change is not predictable, but a skip of exon 51 is very likely.

interpreted as pathogenic by the predictive software Alamut 2.2. The variants spread over the entire gene without a specific distribution, suggesting the absence of mutational hotspots.

Without taking into consideration the three exon deletions detected with MLPA-*USH2A* technology, 13 *USH2A* variants were located in the first 21 exons while the other 18 were found in the remaining 51 exons, supporting the need to sequence the whole gene. The variants c.9811del (p.Met3271Cysfs*30) and c.1663C>G (p.Leu555Val) were the most common allelic variants of the *USH2A* gene in our series and were found in 3/33 patients (9.1%) and then in 3/66 (4.5%) alleles. The first was a novel variant, and all three patients carrying it (two of whom were sisters) came from the area of Bari in southern Italy. The second was a novel variant already reported in Spanish [33] and Italian [21] patients with USH. In our study, the variant was always associated in *cis* with a pathogenic variant (a splicing variant in two patients and a nonsense variant in one patient), and therefore, the variant's pathogenicity is questionable.

The common *USH2A* variant c.2299del (p.Glu767Serfs*21; 25), which was the most prevalent in a group of Spanish patients with USH [19] and accounted for about 30% of all USH2 alleles in Scandinavian [18], American [38,39], and British [16] patients, was detected only in 2/33 patients (6.1%) and then in 2/66 alleles (3%). Similarly, the variants c.4338_4339del (p.Cys1447Glnfs*29) and c.8559–2A>G (p.?), which, respectively, have a high prevalence in French Canadian [40] and Japanese [35] patients with USH was not detected in our series. The high rate of novel variants and the poor prevalence of variants common in other ethnic groups suggest a peculiar mutational spectrum of the *USH2A* gene in Italian patients with USH.

In our series, the majority of the USH variants (24/34, 70%) occurred only in isolated families, which confirmed the predominance of private *USH2A* variants in USH reported in other studies [2,3,18]. The high prevalence of novel variants and the common recurrence of private variants suggest poor utility of microarray chips, which can detect only previously identified mutations for USH molecular diagnosis. This is in agreement with the low detection rate (22.6%) obtained in a previous study in Italian patients with USH with the Asper Biotech microarray [21].

In 13 patients, *MYO7A* and *USH2A* exon sequencing did not identify biallelic mutations on the same gene (six monoallelic *USH2A* variant, one monoallelic *MYO7A* variant, one patient with one *MYO7A* and one *USH2A* on different alleles, five patients without potentially pathogenic variants on both genes). MLPA performed on these patients resulted in the identification of three complete *USH2A* exon deletions in four cases (two patients were brother and sister; 4/13, which is 30.7%), always in *trans* with missense variants on the other allele. This result is in agreement with a recent study [41] that reported large genomic rearrangements (mainly consisting of deletions) in 35% of patients with USH previously identified as carriers of a monoallelic *USH2A* mutation and suggest that in USH MLPA analysis can detect a significant fraction of mutations not identified with exon sequencing. The clinical picture of one patient carrying a heterozygous *USH2A* deletion is illustrated in Figure 4.

One patient was heterozygous for mutations in both USH genes having received the *USH2A* variant from the unaffected father and the *MYO7A* variant from the unaffected mother. This finding supports the hypothesis of a possible digenic/oligogenic inheritance of the syndrome or the possible combined influence of variants of different genes on the clinical phenotype of the patient, as suggested by similar results reported in other studies [16,21,32-34,37].

In three patients, *MYO7A* and *USH2A* sequencing and MLPA analysis detected only a monoallelic variant (one *MYO7A* and two *USH2A* variants) while in five patients no variant of both genes was detected. Unidentified mutations could lie in the promoter, regulatory regions, and deep intronic areas, usually not analyzed during conventional mutation screening. Otherwise, they could be located in other USH genes, already known or yet to be identified. Finally, in a few cases the coexistence of non-syndromic deafness and non-syndromic retinal dystrophy cannot be excluded.

Segregation of the mutant alleles with the disease was reported in all families, particularly considering the close relatives (namely, parents, brothers, and sisters) of the proband. Figure 1 shows the pedigree of a family in whom segregation analysis first suggested the possibility of a large deletion in the *USH2A* gene.

In our series, the USH1 group was too small to evaluate for possible genotype-phenotype associations, and patients with USH2 showed variable clinical pictures (different ages of onset, disease progression, and fundus appearance) so it was not possible to establish reliable genotype-phenotype correlations. This phenotypic heterogeneity has already been reported in other studies [30,38,42] and is probably due to the influence of environmental factors and/or modifier genes.

In conclusion, we identified ten *MYO7A* and 34 *USH2A* sequence variants in a series of 36 patients with USH from 33 independent Italian families; to our knowledge, this is the first large study reporting complete exon sequencing of these genes in the Italian population. The high number of novel and

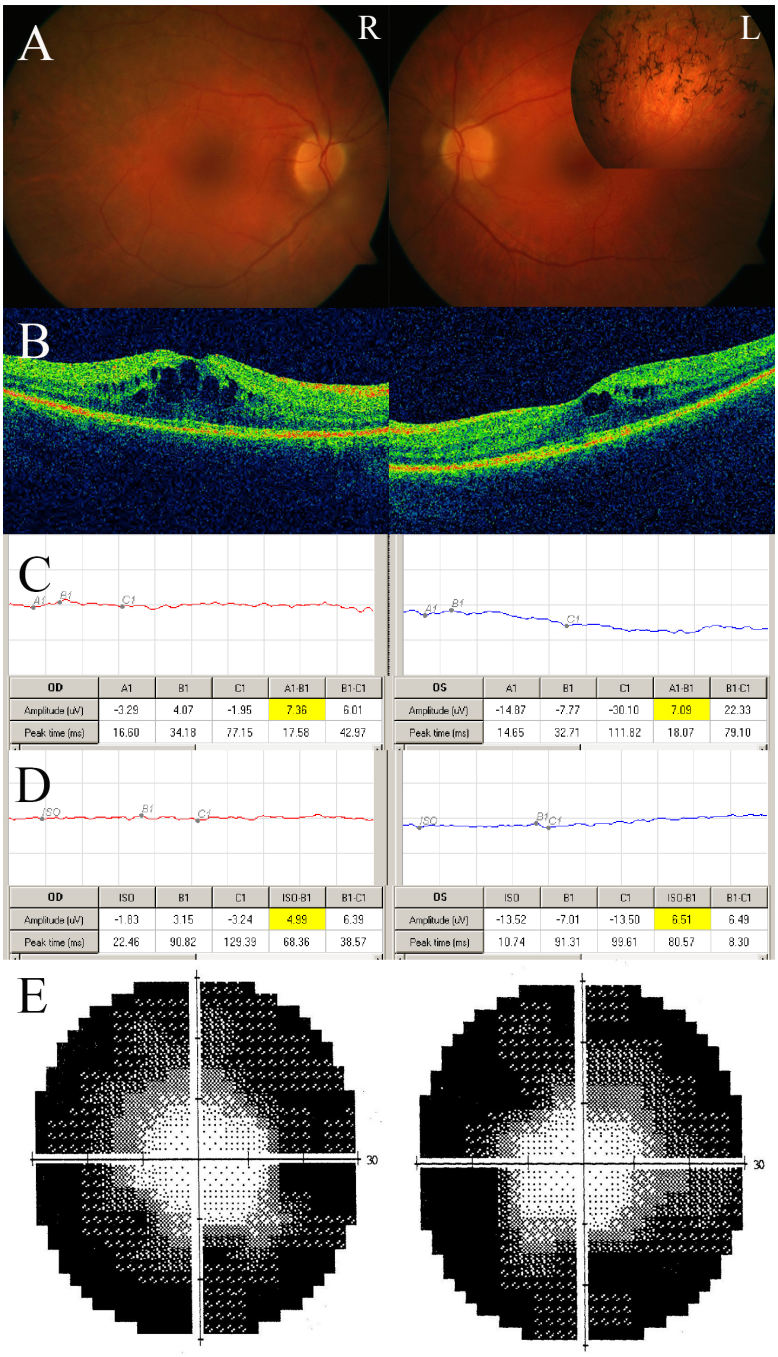

Figure 4. Clinical case of a patient carrying an *USH2A* missense variants (c.1434G>C (p.Glu478Asp), c.7595–2144A>G (p.Lys2532Thrfs*56)) on one allele and an *USH2A* exon deletion (Del EX22) on the other allele (patient BL). **A**: Fundus photographs: typical form of retinitis pigmentosa. **B**: OCT scans: cystoid macular edema. **C**: Photopic electrophysiological responses: severely abnormal. **D**: Scotopic electrophysiological responses: severely abnormal. **E**: Humphrey visual field: concentrically reduced. This patient carrying a monoallelic *USH2A* deletion presented with the clinical picture of typical retinitis pigmentosa, similar to other cases in our series.

private variants and the poor prevalence of variants common in other ethnic groups suggest a difference in the spectrum of *USH2A* sequence variants between Italian patients with USH and other populations. The overall prevalence of patients carrying two pathogenic mutations in different alleles of the same gene was 75%. This high detection rate supports the sequencing of *MYO7A* and *USH2A* genes associated with MLPA-*USH2A* as the first step in the molecular diagnosis of Italian patients with USH. This strategy is significantly more efficient than the microarray investigation and may represent an alternative to the NGS technology that is presently not available in all laboratories. Moreover, amplicon sequencing by NGS investigation cannot presently identify the exon deletions detected with the MLPA-*USH2A* method. The main limit of this diagnostic approach is the impossibility of studying other USH or still unknown genes and to detect modifying variants in additional genes that could influence the phenotype determined by the primary USH genes. Better knowledge of molecular alterations underlying USH in specific populations may lead to more efficient diagnostic strategies and future therapeutic approaches.

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