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(30)

Next-generation sequencing reveals three novel variants in Polish patients with Usher syndrome

Nowe mutacje wykryte u pacjentów z zespołem Ushera przy użyciu techniki sekwencjonowania następnej generacji

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Abstract: Aim: Usher syndrome is an autosomal recessive disorder which manifests as sensorineural hearing impairment with retinitis pigmentosa and, in some cases, also vestibular dysfunction.
Material and methods: We studied the molecular basis of the disease in seven unrelated Polish patients with Usher syndrome. Patients underwent audiologic and ophthalmic examination. Next-generation sequencing on the diagnostic panel for Usher syndrome was performed in all patients.
Results: Next-generation sequencing enabled identification of mutations on both alleles in five patients (71.4%). We describe three novel potentially pathogenic variants: c.14219G>T (p.(Gly4740Val)) in the *GPR98* gene, c.5206_5207insC (p.(Lys1737Glnfs*28)) in the *MYO7A* gene and c.11780A>G (p.(Asp3927Gly)) in the *USH2A* gene. In one patient, we identified two variants in two different genes: *GPR98* and *USH2A*.
Conclusions: Our results expand the mutational spectrum associated with Usher syndrome by a description of three novel likely pathogenic alterations and support the use of targeted next-generation sequencing in genetic diagnosis of patients with this syndrome.

Key words: Usher syndrome, next generation sequencing, *USH2A*, *GPR98*, *MYO7A* genes.

Abstrakt: Cel: przeprowadzenie analizy molekularnej w celu potwierdzenia klinicznego rozpoznania zespołu Ushera u siedmiu polskich pacjentów.
Materiał i metody: prezentujemy siedmioro pacjentów z niespokrewnionych ze sobą rodzin, którzy chorują na zespół Ushera. Przeprowadzono u nich badania audiologiczne i okulistyczne. Sekwencjonowanie następnej generacji na panelu dedykowanym dla zespołu Ushera wykonano u wszystkich pacjentów.
Wyniki: badanie molekularne za pomocą sekwencjonowania następnej generacji pozwoliło na wykrycie mutacji na obu allelach u pięciorga z siedmiorga pacjentów (71,4%). Opisaliśmy trzy nowe, potencjalnie patogenne, warianty: c.14219G>T (p.(Gly4740Val)) w genie *GPR98*, c.5206_5207insC (p.(Lys1737Glnfs*28)) w genie *MYO7A* i c.11780A>G (p.(Asp3927Gly)) w genie *USH2A*. U jednego pacjenta zidentyfikowaliśmy dwa warianty w dwóch różnych genach: *GPR98* i *USH2A*.
Wnioski: wyniki naszych badań poszerzają spektrum mutacji znanych dla zespołu Ushera, jednocześnie wskazują na zasadność stosowania metody sekwencjonowania następnej generacji w diagnostyce pacjentów z tym zespołem.

Słowa kluczowe: zespół Ushera, sekwencjonowanie następnej generacji, geny: *USH2A*, *GPR98*, *MYO7A*.
The authors declare no conflict of interest/ Autorzy zgłaszają brak konfliktu interesów w związku z publikowaną pracą

Introduction

Usher syndrome (USH) is an autosomal, recessively inherited condition, which manifests as deafness, retinitis pigmentosa (RP) and, in some cases, vestibular dysfunction. The prevalence has been estimated from 1/ 6000 to 1/ 25000 (1). There are three clinical subtypes classified by the severity and onset of hearing loss and retinitis pigmentosa. USH1 is the most severe form, characterized by congenital severe to profound deafness and prepubertal onset of RP. Furthermore, children also have vestibular areflexia and absent vestibular function. Seven genes associated with this form have been identified: *MYO7A, CDH23, PCDH15, USH1C, USH1G*, *CIB2* and *ESPN* (2–5). USH2 is a more frequent type, which manifests as congenital, mild to profound hearing loss, RP in the first or second decade of life and normal vestibular function. Three genes associated with this form have been described: *USH2A*, *GPR98* and *DFNB31* (4, 6–8). USH3 is the less common form of Usher syndrome, which manifests as progressive hearing loss, usually with the post-lingual onset as well as variable age of onset of visual loss and variable vestibular dysfunction (9). Two causal genes have been identified: *CLRN1* and *HARS* (10–12).

We utilized targeted next-generation sequencing (NGS) to find disease-causing sequence variants in seven patients affected with Usher syndrome, demonstrating a high variant detection rate with this method.

Methods

Clinical studies

Seven patients from unrelated Polish families (one patient from each family) manifesting clinical features of Usher syndrome participated in this study. Their parents did not show any symptoms of USH syndrome or other ocular symptoms. The patients received genetic counseling with detailed medical data analysis. Ocular assessment including central visual acuity measurement, fundus examination, electroretinography (ERG) and optical coherence tomography (OCT) was performed. Pure tone audiometry (PTA) was performed in all patients. One patient (p5) underwent neuro-imaging with high-resolution MRI to exclude brain and inner ear malformations. All patients agreed to participate in the study and to use their anonymized data in the publication. This study conforms to the Helsinki declaration and was approved by the Poznan University of Medical Sciences Institutional Review Board. Informed consent was obtained from them prior to molecular testing.

Molecular genetic analysis

Blood samples were collected and genomic DNA was extracted from peripheral blood leukocytes using the salting-out method. NGS with Illumina NextSeq on the diagnostic panel for Usher syndrome (Asper Biotech, Tartu, Estonia) was performed in all patients. A panel of 20 genes including *ABHD12, CDH23, CIB2, CLRN1, COL4A6, DFNB31, DSPP* (excluding exon 5), *GIPC3, GPR98, HARS, KARS, LHFPL5, LOXHD1, MYO7A, PCDH15, PDZD7, TNC, USH2A, USH1C* and *USH1G* was analyzed. The read depth of the tested genes included in the panel was between 70-120x. The sequences were verified by comparing them to the human reference sequence of the *GPR98* gene (NM_032119.4), *MYO7A* (NM_001127180.1) and *USH2A* (NM_206933.2) genes. The identified sequences variants were referred to the Human Gene Mutation Database (HGMD), the Exome Variant Server (NHLBI Exome Sequencing Project ESP), the 1000 Genomes Project database (1000 Genomes Project Consortium 2012) and ExAC Browser Beta (Exome Aggregation Consortium 2015). The *in silico* analyses using SIFT, PolyPhen-2 and MutationTaster 2 software were performed to predict the possible effect of the novel missense variants. The evolutionary conservation of nucleotide positions was analyzed by PhyloP and GERP ++ scores. To predict the impact of substitution between amino acids, the Grantham score was analyzed. Novel variants identified in this study were classified according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG) guidelines (13). A control cohort of 100 healthy, ethnically matched individuals from the Genetics Department of Poznan University of Medical Sciences database, was used to verify novel variants identified in this study. Segregation analysis for the presence of altered alleles was performed in the families (families 1, 2, 3) with the novel potentially causative variants in *GPR98, MYO7A* and *USH2A* genes by Sanger sequencing of the appropriate exons (Fig. 1).

Results

The diagnosis was based on clinical, audiometric and ophthalmic data. Patients were identified as having USH1 and USH2 type according to the clinical features. The audiometric test demonstrated a congenital, bilateral hearing loss ranging from moderate to severe in the USH2 patients. In a USH1 patient (p2), deafness was defined as bilateral and profound, and was reported to be present in infancy. Parents of a p2 patient reported that the gait and balance problems were present from early childhood. All patients presented with typical signs and symptoms of RP, including night blindness, visual field narrowing, bone spicule-like pigmentation, retinal vessel attenuation and optic disc pallor. Furthermore, in three patients (p1, p5 and p6) cystoid macular edema (CME) was diagnosed. The clinical data has been shown in Table I. An example of typical ERG, OCT and audiometric findings (sourced from the patient p3) are presented in Figure 2.

Molecular results

In our cohort of seven patients diagnosed with USH, mutations in a compound heterozygous state were identified in five patients (71.4%) while in two patients (p5 and p7) only one mutated allele was identified. Three novel variants were detected in three different genes (Tab. I).

In patient p1 the NGS revealed a novel missense variant c.14219G>T (p.(Gly4740Val)) and a known nonsense mutation c.7669C>T (p.Gln2557*) in the *GPR98* gene (Fig. 1a., b.). The novel variant was predicted to be probably damaging by *in silico* analyses (Tab. I). Patient p2 is a compound heterozygous for a previously reported nonsense mutation c.5392C>T (p.Gln1798*) and a novel frameshift insertion c.5206_5207insC (p.(Lys1737Glnfs*28)) in the *MYO7A* gene (Fig. 1c., d.).

In patient p3 a novel variant c.11780A>G (p.(Asp3927Gly)) and one known mutation c.775_776delAG (p.Ser259Phefs*63) in the *USH2A* gene (in a heterozygous status) were identified (Fig. 1e., f.). *In silico* analysis of the missense substitution p.(Asp3927Gly) by PolyPhen2 and Mutation Taster 2 software showed that this variant is predicted to have a deleterious effect on usherin protein, while analysis by SIFT software indicated that this substitution is tolerated. PhyloP and GERP ++ score showed that nucleotide positions of the novel missense substitutions identified in patients p1 and p3 are evolutionarily conserved (Tab. I). None of the novel variants identified in this study was listed in the used databases. Furthermore, these variants were not detected in 100 control individuals from our in-house database.

Two known variants c.2299delG (p.Glu767Serfs*21) in *USH2A* and c.12704A>G (p.Tyr4235Cys) in the *GPR98* gene were identified in patient p4. In two patients (p5 and p7), a known heterozygous variant in the *USH2A* gene was detected: c.11864G>A (p.Trp3955*), while the second allele remained undetermined. Finally, patient p6 had two different known mutations, c.9676C>T (p.Arg3226*) and c.9424G>T (p.Gly3142*), in the *USH2A* gene. All detected variants in USH patients are summarized in table I. Segregation analysis of novel variants performed in their healthy parents revealed that they segregate with the phenotype according to the autosomal recessive mode of inheritance (Fig. 1).

Discussion

Here we report the results of targeted NGS of seven Polish patients with USH1 and USH2. Genetic background of the disease was identified in 71.4% patients with USH, which is consistent with the findings of other studies using the NGS method (14, 15). Patient examinations demonstrated audiometric and ophthalmic findings typical for Usher syndrome. Molecular studies confirmed

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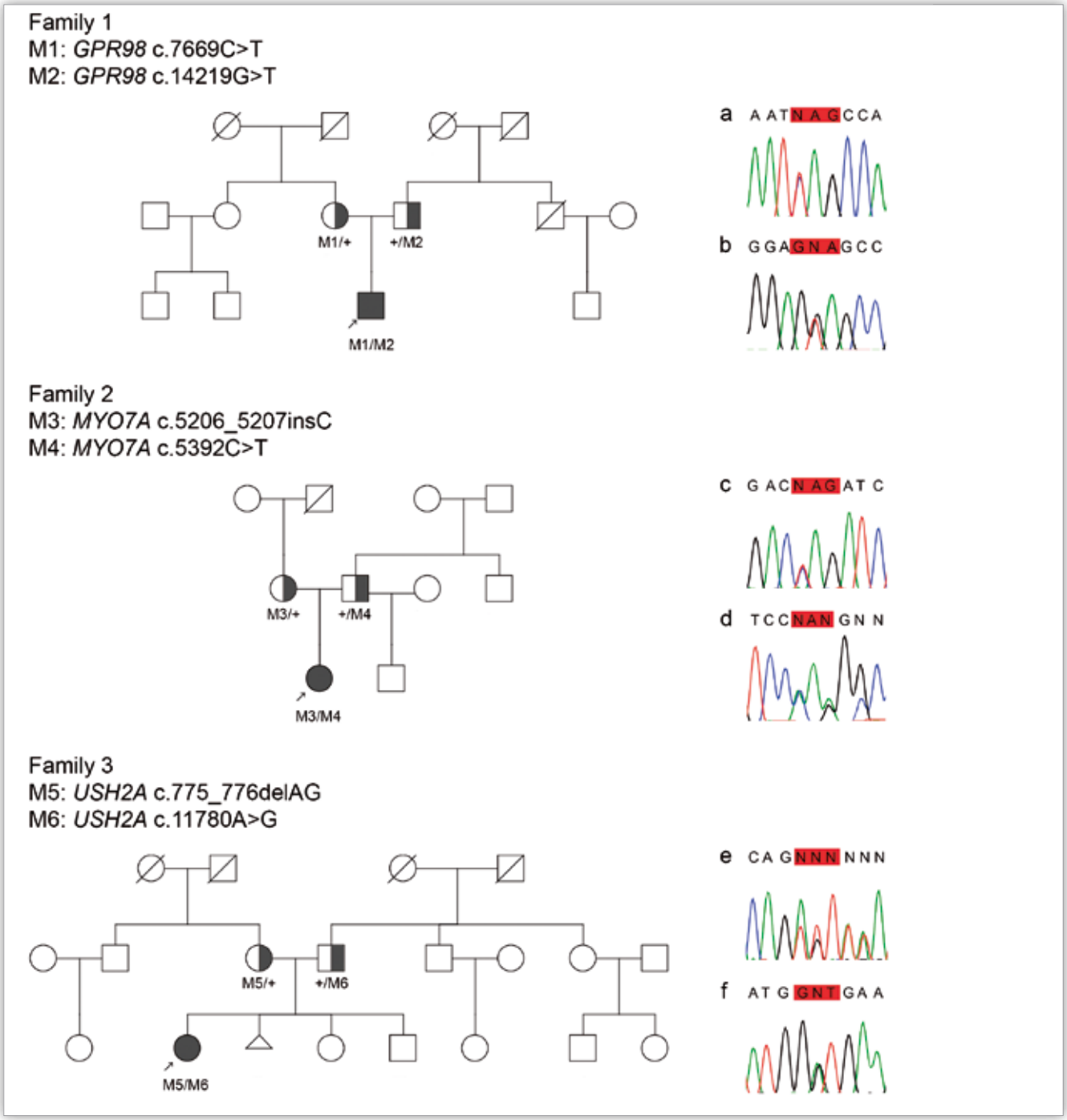

Fig. 1. Pedigrees and sequencing results with chromatograms for USH patients with novel variants. Pedigrees – the genotypes are provided for all subjects available for molecular genetic analysis. The black square and circles represent affected males and females respectively. White squares and circles represent unaffected family members. Triangle represents a miscarriage. Arrows point to probands. M1, M2, indicate variants detected in *GPR98* gene (family 1), M3, M4, indicate variants detected in *MYO7A* gene (family 2), M5, M6 indicate variants detected in *USH2A* gene (family 3). Patient parents are asymptomatic carriers of the identified mutations. Chromatograms – a. b. Sequence variants detected in patient p1 in the *GPR98* gene. a. c.7669C>T (p.Gln2557*). b. Novel variant c.14219G>T, p.(Gly4740Val). c. d. Sequence variants detected in patient p2 in the *MYO7A* gene. c. c.5392C>T (p.Gln1798*). d. Novel variant c.5206_5207insC, p.(Lys1737Glnfs*28). e. f. Sequence variants detected in patient p3 in the *USH2A* gene. e. c.775_776delAG (p.Ser259Phefs*63). f. Novel variant c.11780A>G, p.(Asp3927Gly).

Ryc. 1. Rodowody oraz chromatogramy pacjentów z nowymi zidentyfikowanymi wariantami sekwencji. Rodowody – genotypy przedstawione u pacjentów dostępnych do badań. Czarne kwadraty oraz koła przedstawiają odpowiednio chorych mężczyzn i kobiety. Białe symbole oznaczają zdrowych członków rodziny. Trójkąt oznacza poronienie. Strzałki wskazują probandów. M1, M2 oznaczają warianty sekwencji zidentyikowane w genie *GPR98* w rodzinie 1. M3, M4 oznaczają warianty sekwencji zidentyfikowane w genie *MYO7A* w rodzinie 2. M5, M6 oznaczają warianty sekwencji zidentyikowane w genie *USH2A* w rodzinie 3. Rodzice pacjentów są bezobjawowymi nosicielami mutacji zidentyfikowanych u dzieci. Chromatogramy – a. b. warianty sekwencji zidentyfikowane u pacjenta p1 w genie *GPR98*, a. c.7669C>T (p.Gln2557*). b. nowy wariant c.14219G>T, p. (Gly4740Val). c. d. warianty sekwencji zidentyfikowane u pacjenta p2 w genie *MYO7A* c. c.5392C>T (p.Gln1798*). d. nowy wariant c.5206_5207insC, p.(Lys1737Glnfs*28). e. f. warianty sekwencji zidentyfikowane u pacjenta p3 w genie *USH2A*. e. c.775_776delAG (p.Ser259Phefs*63). f. Nowy wariant c.11780A>G, p.(Asp3927Gly).

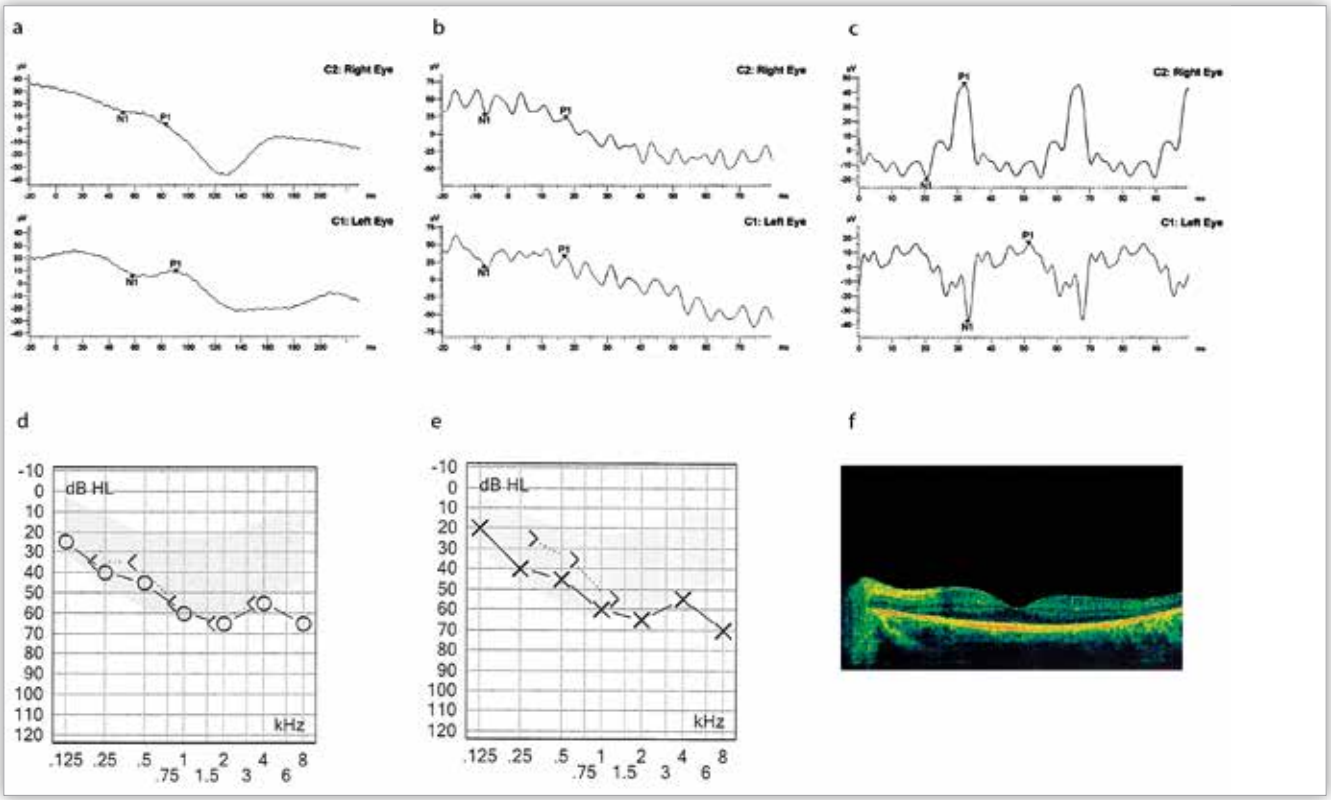

Fig. 2. Clinical findings of the patient p3 with mutations in *USH2A* gene, a, b, c severely abnormal scotopic white flash, scotopic and photopic white 30 Hz flicker electroretinograms (ERG) respectively, d, e audiograms: d – right ear, e – left ear, cross or circle labels indicate air-conduction hearing, right angle labels indicate bone-conduction hearing, f – optical coherence tomogram (OCT) showing a mild foveal hypoplasia.

Ryc. 2. Wyniki badań klinicznych u pacjenta p3 z mutacjami w genie *USH2A*, a, b, c znacznie obniżone zapisy ERG błyskowego, skotopowego oraz flicker 30 Hz, d, e audiogramy: d – prawe ucho, e – lewe ucho, krzyżyki lub kółka oznaczają przewodnictwo powietrzne, symbol kąta oznacza przewodnictwo kostne, f – optyczna koherentna tomografia dna oka (OCT) wskazuje na łagodną hipoplazję dołka.

the clinical classification of the patients, USH2 with variants detected in the *USH2A* and *GPR98* genes and USH1 with a variant in the *MYO7A* gene. Despite a small sample size, we observed that the most frequent sequence variants in USH2 patients were identified in the *USH2A* gene (7 out of 9 variants in USH2). The results are consistent with the previous studies indicating that mutations in this gene are the most common cause of USH2 (16, 17).

Two mutated alleles were identified in five out of seven patients. We detected three novel variants c.14219G>T in the *GPR98* gene, c.11780A>G in the *USH2A* gene and c.5206_5207insC in the *MYO7A* gene. A novel missense variant c.14219G>T (p.(Gly4740Val)) in exon 70 of the *GPR98* gene (p1) changes glycine to valine, both nonpolar amino acids, at amino acid residue 4740 that is highly conserved among species. Frameshift variant detected in one patient (p2), c.5206_5207insC (p.(Lys1737Glnfs*28)), located in exon 38, leading to a stop codon 28 amino acid downstream, causes premature termination of translation. A novel missense variant c.11780A>G identified in exon 61 of the *USH2A* gene (p3) (p.(Asp3927Gly)) is located in the fibronectin type 3 domain, which is involved in cell adhesion, its morphology, migration, thrombosis and embryonic differentiation. In one patient (p4), we identified heterozygous known variants in two different genes *GPR98* and *USH2A.* A pathogenic frameshift variant c.2299delG in *USH2A* has been previously reported in USH patients. A missense substitution c.12704A>G in the *GPR98* gene is a very rare variant (allele frequency based on ExAC is 0.0001968), which was predicted to be probably damaging by *in silico* analyses. The GERP score of 4.45 indicated that nucleotide position 12704 of the identified change p.Tyr4235Cys was likely damaging. The Grantham score of 194 means that Tyrosine and Cysteine are evolutionarily very distant from each other. Therefore, their substitution is predicted to be more damaging. The two detected trans-heterozygous variants are present in two genes, on two different chromosomes. Both genes are known to cause USH2. In view of all these results, the two detected variants may be considered pathogenic in this patient. Digenic inheritance in ciliopathies like Usher syndrome and Nephronophthisis has been reported previously (4, 18, 19). Digenic inheritance of *CDH23* and *PCDH15* has been shown to cause Usher syndrome type I in humans (20). As the presence of two additional variants in yet undiscovered USH genes, intragenic deletion or duplication cannot be excluded in the patient in question, further studies, especially in animal models, are required in order to confirm our assumptions of digenic inheritance in our patient. Many studies suggest that copy number variants can be significant *USH2A* mutations type that are missed by sequencing assays (14, 21, 22). In the molecular analysis performed by Austin-Tse et al. in a group of 700 patients with hearing loss, copy number variants were identified in a significant proportion of the USH patients with a previously detected heterozygous variant only (23).

In our cohort, we were unable to detect the second putative mutation in two patients, who were positive only for heterozygous mutations. This can most likely be explained by technical limitations of targeted sequencing. The panel sequencing is designed to target the coding sequence and exon-intron borders. For deep-

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Patient/ Usher type	Mutation, gene/ Mutacja, gen	Effect of mutation/ ACMG guidelines/ efekt mutacji	Allelic state/ Układ alleli	In silico prediction score (PolyPhen2, SIFT, Mutation Taster)/ Programy *in silico*	PhyloP/ GERP++ score	Sensourineural hearing loss/Deafness/ Niedosłuch odbiorczy/ Głuchota	Visual field/ Pole widzenia	Visual acuity/ Ostrość wzroku	Fundus appearance/ Dno oka	ERG	Balance problems/ Problemy z równowagą
p1/ type 2	c.7669C>T GPR98 c.14219G>T GPR98	p.Gln2557* p.(Gly4740Val) /likely pathogenic	compound heterozygous/ heterozygota złożona	– probably damaging/ prawdopodobnie uszkadzające	– 4.88/5.7	congenital, profound, stable/ wrodzony głęboki stabilny	concentric narrowing/ zawężenie pola widzenia 25-350	0,8/0,1-0,2	typical RP + CME	photopic 50%, scotopic nonrecordable/ zapisy fotopowe obniżone do 50%, skotopowe niewykrywalne	–
p2/ type 1	c.5392C>T MYO7A c.5206_5207insC MYO7A	p.Gln1798* p.(Lys-1737Glnfs*28)/ pathogenic	compound heterozygous/ heterozygota złożona	– –	– –	congenital, profound/ wrodzona, głęboka	ND	0,4-0,5/0,4-0,5	typical RP	photopic diminished, scotopic residua/ zapisy fotopowe obniżone, skotopowe resztkowe	+
p3/ type 2	c.775_776delAG USH2A c.11780A>G USH2A	p.Ser259Phefs*63 p.(Asp3927Gly)/ likely pathogenic	compound heterozygous/ heterozygota złożona	– probably damaging/ prawdopodobnie uszkadzające tolerated (SIFT)/ tolerowane	– 4.5/5.52	congenital, moderate, stable/ wrodzony, umiarkowany, stabilny	concentric narrowing/ zawężenie pola widzenia <100	1,0/1,0	typical RP	photopic and scotopic residua/ zapisy fotopowe i skotopowe resztkowe	–
p4/ type 2	c.12704A>G GPR98 c.2299delG USH2A	p.Tyr4235Cys p.Glu767Serfs*21	digenic inheritance (probable)/ dziedziczenie dwugenowe (prawdopodobnie)	probably damaging/ prawdopodobnie uszkadzające –	4.89/4.45 –	congenital, moderate, stable/ wrodzony, umiarkowany, stabilny	concentric narrowing/ zawężenie pola widzenia 50	0,8/0,6	typical RP	ND	–
p5/ type 2	c.11864G>A USH2A	p.Trp3955*	heterozygous/ heterozygota	–	–	congenital, moderate, stable/ wrodzony, umiarkowany, stabilny	concentric narrowing/ zawężenie pola widzenia 100	1,0/1,0	typical RP + CME	photopic and scotopic nonrecordable/ zapisy fotopowe i skotopowe niewykrywalne	–
p6/ type 2	c.9676C>T USH2A c.9424G>T USH2A	p.Arg3226* p.Gly3142*	compound heterozygous/ heterozygota złożona	– –	– –	congenital, moderate, slightly progressive/ wrodzony, umiarkowany, powoli postępujący	concentric narrowing/ zawężenie pola widzenia 50	1,0/0,6	typical RP + CME	photopic and scotopic nonrecordable/ zapisy fotopowe i skotopowe niewykrywalne	–
p7/ type 2	c.11864G>A USH2A	p.Trp3955*	heterozygous/ heterozygota	–	–	congenital, moderate, stable/ wrodzony, umiarkowany, stabilny	concentric narrowing/ zawężenie pola widzenia	1,0/1,0	typical RP	ND	–

ND – no data, CME – cystoid macular edema. RP – retinitis pigmentosa. Balance problems - normal balance, + abnormal balance. Novel mutations identified in this study are indicated in bold. They are given in parentheses because the consequences of these mutations are only predicted, not proven by functional experiments. Classification of novel variants according to American College of Medical Genetics and Genomics (ACMG) guidelines./ND – brak danych, CME – torbielowaty obrzęk plamki. RP – zwyrodnienie barwnikowe siatkówki. - prawidłowa równowaga, + problemy z równowagą,. Nowe mutacje zidentyfikowane w pracy zostały pogrubione. Nowe mutacje umieszczono w nawiasach, ponieważ konsekwencje mutacji przewidziano jedynie w programach predykcyjnych, nie zostały one natomiast eksperymentalnie udowodnione. Klasyfikację nowych wariantów wykonano na podstawie wytycznych American College of Medical Genetics and Genomics (ACMG).

Tab. I. Identified sequence variants and clinical information of USH patients.

Tab. I. Informacje kliniczne oraz warianty sekwencji zidentyfikowane u pacjentów chorujących na zespół Ushera.

-intronic and intragenic deletions or duplications, only such methods as whole genome sequencing (WGS) or array CGH could be effective. Additional whole exome sequencing (WES) could be a good alternative in order to identify mutations in known or novel genes which are not included in the USH panel.

The effective method which enables a reliable molecular diagnosis of USH can improve genetic counseling contributing to earlier diagnosis in children, as well as pre-symptomatic USH2 and USH3 patients, which can be crucial for preventive or treatment modalities, which are being developed. Novel variants identified in this study expand the spectrum of mutations in USH related genes, thereby enhancing the current knowledge of USH heterogeneity, and further support the use of targeted NGS in genetic diagnosis of Usher syndrome.

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