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Molecular studies of a novel mutation in MYO7A gene in Usher Syndrome type I

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Index

Figure Index

Abbreviations

A – Adenine

Arg – Arginine

bp – Base pair

C – Cytosine

cDNA – Complementary Deoxyribonucleic acid

CHUC – Centro Hospitalar e Universitário de Coimbra

DNA – Deoxyribonucleic acid

DNAse – Desoxirribonuclease

dNTP – Deoxynucleotide triphosphate

EDTA – Ethylenediamine tetraacetic acid

FERM – Four-point-one, ezrin, radixin, moesin domain

Fw – Forward

G – Guanine

Gly – Glycine

min – Minute(s)

Myo7a – Myosin VIIa protein

MyTH4 – Myosin Tail Homology 4 domain

NCBI – National Center for Biotechnology Information

PCR – Polymerase Chain Reaction

PROVEAN – Protein Variation Effect Analyzer

RCF – Relative centrifugal force, it is expressed in units of gravity

Rev – Reverse

RFLP – Restriction fragment length polymorphism

RIN – RNA integrity number

RNA – Ribonucleic acid

RNAse – Ribonuclease

RP – Retinitis Pigmentosa

RPE – Retinal pigment epithelium

RT-PCR – Reverse transcriptase-polymerase chain reaction

sec – Second(s)

SH3 – SRC Homology 3 domain

SIFT – Sorting Intolerant from Tolerant

SNHL – sensorineural hearing loss

T – Timine

TBE – Tris Borate EDTA

TR – Targeted-Resequencing

USH – Usher syndrome

USH1 – Usher syndrome type I

USH2 – Usher syndrome type II

USH3 – Usher syndrome type III

UV – Ultraviolet

Resumo

Introdução: O Síndrome de Usher (USH) é uma doença autossómica recessiva caracterizada por um quadro de défice auditivo neurosensorial e retinite pigmentar, associado ou não a disfunção vestibular. USH é dividido em três tipos, sendo o USH tipo I o mais grave, caraterizado por surdez grave a profunda bilateral congénita, disfunção vestibular e retinite pigmentar diagnosticada durante a infância. Nas famílias com USH tipo I, *MYO7A* é o gene mais frequentemente mutado (50%). Este gene codifica para a proteína Miosina VIIa, previamente descrita como uma proteína motor de transporte e participando na formação da adesão célula a célula.

Num estudo anterior foi encontrada uma nova alteração (c.4489G>C) no gene *MYO7A* num doente português com USH tipo I. Este trabalho propôs-se a avaliar a possibilidade de esta alteração ser responsável pelo fenótipo.

Métodos: Uma avaliação clínica completa foi feita de forma a confirmar o fenótipo do doente. As análises por Sequenciação do exão 34 do gene *MYO7A* da amostra do doente e por PCR-RFLP das amostras do doente e de 250 indivíduos normais sem USH foram efetuadas para avaliar a presença da alteração c.4489G>C. Adicionalmente, foram realizados estudos *in silico,* usando software disponíveis online e a conservação evolutiva num grupo de primatas e não primatas. Finalmente, para determinar a expressão da alteração c.4489G>C nos transcritos do gene *MYO7A*, foram estudadas amostras de epitélio nasal do doente e de dois indivíduos normais.

Resultados/Discussão: Foi confirmada a presença da variante c.4489G>C no doente com USH do tipo I e a sua ausência em 250 indivíduos normais. Os softwares online usados demostraram que a variante era lesiva, provavelmente deletéria ou causadora da doença. O estudo da conservação evolutiva demonstrou uma região altamente conservada no genoma e na proteína, em todas as espécies estudadas. Foi ainda possível identificar a expressão da

variante c.4489G>C nos transcritos da amostra do doente e a sua ausência nas amostras dos indivíduos normais.

Conclusão: Assim, foi possível concluir que a nova alteração c.4489G>C do gene *MYO7A* é uma mutação *missense* homozigótica, provavelmente responsável pelo USH do tipo I no doente português e que a sua expressão foi encontrada nos transcritos do epitélio nasal do doente.

This work was prepared for submission in the format of an Original Paper and according to the Guidelines for Authors of the journal *Ophthalmic Research* (www.karger.com/ore_guidelines).

Molecular studies of a novel mutation in *MYO7A* gene in Usher Syndrome type I

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Abstract

Usher syndrome (USH) is an autosomal recessive disorder characterized by the association of a sensorineural hearing impairment and retinitis pigmentosa, with or without vestibular dysfunction. USH is divided in three types, being USH type I the most severe form, characterized by severe to profound congenital and bilateral sensorineural hearing loss, congenital vestibular dysfunction and retinitis pigmentosa diagnostic during childhood. In USH type I families, *MYO7A* is the most commonly mutated gene (50%). This gene codes for Myosin VIIa protein, previously described as a motor transport protein and participating in the establishment of cell-cell adhesions.

In a previous study, it was found a novel homozygous variant (c.4489G>C) in *MYO7A* gene in an USH type I Portuguese patient. This work purposed to appraise the possibility of this variant be responsible for the phenotype.

A complete evaluation was performed to ascertain the clinical phenotype of the patient. Patient's sample Sequencing analysis of *MYO7A* gene exon 34 and PCR-RFLP analysis of patient and 250 DNA samples from normal individuals without USH was accomplished to assess the c.4489G>C variant presence. Additionally, *in silico* studies using available internet software and evolutionary conservation in a group of primates and non-primates was performed. Finally, in order to determine if the c.4489G>C variant allele was expressed in *MYO7A* gene transcripts, nasal epithelium samples from the patient and two normal individuals were studied.

The presence of c.4489G>C variant in an USH type I patient and its absence in 250 normal individuals was confirmed. Internet software used determined that this variant was probably damaging, deleterious or disease causing. Evolutionary conservation study showed a highly conserved region both in nucleotide and amino acid sequences from *Homo sapiens* to

Caenorhabditis elegans. Expression of c.4489G>C variant in patient's cDNA sample was identified as well as its absence in the two normal individuals cDNA samples.

In conclusion, this study revealed that the novel c.4489G>C *MYO7A* gene variant is a homozygous missense mutation, probably responsible for USH type 1 in a Portuguese patient and its expression found in patient's nasal epithelium transcripts.

Key words

Usher Syndrome, *MYO7A* gene, Missense mutation, PCR-RFLP, Sensorineural Hearing Loss, Retinitis Pigmentosa

Introduction

Usher syndrome (USH) is an autosomal recessive disorder characterized by the association of sensorineural hearing loss (SNHL) and progressive retinal degeneration called retinitis pigmentosa (RP), with or without vestibular dysfunction [1].

USH is responsible for 5% of congenital hearing loss [2] and approximately 18% of all RP cases [3,4]. Considering this, USH is the most frequent (more than 50%) cause of simultaneous deaf and blindness in humans [5,6]. It was first reported by Albrecht von Graefe in 1858 as an association of congenital deafness and progressive pigmentary retina dystrophy and its heritability was established in 1914 by Charles Usher, a British ophthalmologist [7,8,9]. According to previous reports, its overall prevalence ranges from 3,2 to 6,2/100.000 [10,11]. In Portugal, a recent study showed a prevalence of 9,7/100.000 [12].

Clinically, USH is divided into three types: USH type 1 (USH1), USH type 2 (USH2) and USH type 3 (USH3), based on the disease severity and progression of the clinical course, mainly the sensorineural hearing loss, the vestibular dysfunction and the age of onset of the RP. USH1 (33-44%), the most severe phenotype, is characterized by severe to profound congenital and bilateral SNHL, congenital vestibular dysfunction and RP diagnosed during childhood. USH2 (56-67%) is characterized by moderate to severe hearing impairment that remains stable, no vestibular dysfunction and a later onset of RP that occurs during or shortly after puberty. USH3 (very small percentage) is characterized by progressive post-lingual hearing loss that can be identified between the first and third decade of life, variable vestibular dysfunction and variable onset of RP, which usually occurs by the second decade of life [13,14,15].

Genetically, USH is highly heterogeneous and it has been associated with thirteen different loci, from which ten genes have been identified with disease-causing mutations. For USH1, mutations were identified in six genes – *MYO7A* (*USH1B*) [16], *USH1C* (*USH1C*)

[17], *CDH23* (*USH1D*) [18], *PCDH15* (*USH1F*) [19], *USH1G* (*USH1G*) [20] and *CIB2* (*USH1J*) [21]. Three genes with mutations were identified in USH2 – *USH2A* (*USH2A*) [22], *GPR98* (*USH2C*) [23], *WHRN* (*USH2D*) [24]. And in USH3, only one gene was found with mutations – *CLRN1* (*USH3A*) [25].

In USH1 families, *MYO7A* is the most commonly mutated gene, close to 50 % of the cases, followed by *CDH23*, *PCDH15*, *USH1C*, *USH1G* and *CIB2* [14,21,26].

MYO7A gene is located on the long arm of chromosome 11 (11q13.5) and it consists in 49 exons encoding for a 2,215 amino acid protein named Myosin VIIa (Myo7a). Myo7a is an unconventional myosin motor protein, expressed in several different tissues like retina, inner ear, testis, kidney, lung and olfactory epithelium. Myo7a is composed by three myosin typical domains: the N-terminal head or motor domain, the neck or regulatory domain consisting of five IQ motifs and the tail which begins with a short coiled coil domain followed by two large repeats, each containing a MyTH4 (Myosin Tail Homology 4) and a FERM (Four-point-one, ezrin, radixin, moesin) domain, separated by a SH3 (SRC Homology 3) domain [27,28].

In the hair cells, Myo7a has been described as a receptor transporter [29], stereocilia organizer [30] and a generator of a large resting tension in the hair bundles [31].

In retina, Myo7a works as opsin transporter from the inner to the outer segment through connecting cilium, in the photoreceptor cells [32] and as melanosomes and phagosomes transporter in the retinal epithelium [33].

Prior to this study, a Targeted-Resequencing (TR) analysis of all the coding exons from 9 of the known genes responsible for USH (*MYO7A*, *USH1C*, *CDH23*, *PCDH15*, *USH1G*, *USH2A*, *GPR98*, *WHRN* and *CLRN1*) was performed in a group of Usher Patients. After this procedure, a novel homozygous variant (c.4489G>C) [34] in *MYO7A* gene was found in a sample of a patient with clinical diagnosis of USH1, which was posteriorly confirmed by Sanger sequencing.

This work purposed to appraise the possibility of c.4489G>C variant be responsible for the phenotype in the USH1 patient. Accordingly, Molecular Genetics, Bioinformatics and Molecular Biology approaches were used to evaluate this hypothesis.

Methods

Human subjects and samples

The patient (male, 61 years, deaf and speechless) was subjected to a complete ophthalmological, audiological and vestibular evaluation, at the Otorhinolaryngology and Ophthalmology Departments of the Centro Hospitalar Universitário de Coimbra (CHUC) by Prof. Dr. João Carlos Ribeiro and Prof. Dr. Eduardo Silva. It was possible to identify a profound and congenital sensorineural deafness, vestibular dysfunction (vertigo), early onset of RP with abnormal retinal pigmentation, peripheral bone spicules, macula and peripheral areas fairly preserved, nyctalopia and tubular visual field. These features allowed making the clinical diagnosis of USH1. The patient also had a background of right eye cataracts surgery and left ocular prosthesis due to steel traumatism.

Samples from two hundred and fifty individuals without any hearing or visual disorder, 140 males and 110 females with an average age of 49.2 ± 16.34 years, ranging from 12 to 84 years old, were used as normal controls.

Additionally, nasal epithelium samples were collected from the patient and two normal individuals without USH during surgery at the Otorhinolaryngology Department of the CHUC by Prof. Dr. João Carlos Ribeiro.

This study had the approval of the Faculty of Medicine, University of Coimbra Ethics Committee, following the Tenets of the Declaration of Helsinki, and a written consent for genetic testing was obtained from the participants.

Genetic studies

DNA extraction: Peripheral blood was collected in EDTA Tubes (SARSTED) from the patient and the 250 controls, followed by DNA extraction using a standard phenol-chloroform extraction procedure [35] in Prof. Drª. Manuela Grazina Biochemical Genetics Laboratory,

Center for Neurosciences and Cellular Biology of Coimbra. The extracted DNA was quantified by spectrophotometry at optical density of 260 nm and 280 nm using the NanoDrop™ 1000 (Thermo Fisher Scientific, Inc.) and the GeneQuant™ II (Pharmacia Biotech).

Polymerase Chain Reaction (PCR) amplification: Specific primers were designed with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) to amplify *MYO7A* gene exon 34, using *MYO7A* gene reference sequence obtained from NCBI database (http://www.ncbi.nlm.nih.gov/gene/). Primer sequences are available upon request to corresponding author. The PCRs were run on a Veriti® Thermal Cycler (Applied Biosystems) and the PCR reactions were performed using 100ng of genomic DNA mixed with 1X Taq Buffer 10X (with $(NH_4)_2SO_4$) (Fermentas), 0.2µM of Forward and Reverse Primers (Sigma-Aldrich), 1.5mM $MgCl_2$ (Fermentas), 0.2mM dNTPs (5PRIME), 1U Taq Polymerase (Fermentas) and RNAse/DNAse free Water (AccuGENE) to a final volume of 10µl. The reaction mixtures were subjected to a specific PCR program with an initial denaturation step of 5 minutes (min) at 95ºC followed by 35 cycles, each with denaturation at 95°C for 30 seconds (sec), annealing at 59°C for 30 sec, and extension for 30 sec at 72°C, with a final elongation step of 10 min at 72°C. PCR products underwent an electrophoresis on a 1% agarose gel. The products with a Loading Buffer (Bio-Rad) migrated under an electric current of 200 volts for 15 minutes (min). A NZY DNA Ladder VI (NZYTech) was used to estimate the size of the fragments and respective quantification by comparison with the Molecular Weight Marker. The fragments were visualized through UV light in the ChemiDoc™ XRS System and respective Image Lab™ Software (Bio-Rad).

Purification of PCR products for Sequencing reaction: The PCR amplified patient's sample, later used for Sequencing reaction, was purified using 1µl of ExoSAP-IT® (USB)/4µl

of amplified PCR product, incubated at 37ºC for 1h followed by 15 min at 75ºC on a Veriti® Thermal Cycler (Applied Biosystems).

Sequencing reaction: Sequencing reaction was performed with 10ng of PCR product, 1X BigDye® Terminator 5X Sequencing Buffer (Applied Biosystems), 0.16µM of Forward or Reverse Primer (Sigma-Aldrich), BigDye® Terminator v3.1 according to manufacturer recommendations (Applied Biosystems) and RNAse/DNAse free Water (AccuGENE) for a final volume of 10µl. The Primers used were the same as for PCR products amplification. Sequencing reaction was run on a Veriti® Thermal Cycler (Applied Biosystems) using the following conditions: an initial denaturation step of 2 min at 96ºC followed by 25 cycles each of a denaturation at 96ºC for 5 sec, annealing at 61ºC for 10 sec, followed by one last step of elongation at 60ºC for 4 min. After that, sequencing reaction products were purified by an ethanol precipitation method according to standard protocols [35]. Purified Sequencing reaction products were re-suspended with 10µl of HiDi™ (Applied Biosystems) and denatured at 96ºC for 5 min on a Veriti® Thermal Cycler (Applied Biosystems), previously to be analysed by Capillary electrophoresis.

Capillary electrophoresis: Automated capillary DNA Sequencing was performed on Genetic Analyzer 3130 (Applied Biosystems) equipment. DNA sequencing data obtained was analysed using Sequencing Analysis Software v.5.4® (Applied Biosystems) that allowed the identification of *MYO7A* gene variants by comparison with gene reference sequence available at the National Center of Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/sites/entrez).

Restriction fragment length polymorphism (RFLP) analysis: Following the PCR amplification of patient's and the 250 normal individuals without USH DNA samples, an RFLP analysis with endonuclease BstU I (New England BioLabs Inc., Beverly, MA) incubating at 60ºC overnight was performed, according to the manufacturer's instructions.

The RFLP reaction was performed in a final volume of 15µl containing 5µl of PCR product, 1.5µl of NEBuffer 4, 1U of BstU I enzyme and RNAse/DNAse free Water (AccuGENE) up to the final volume. Digested PCR products mixed with Loading Buffer (Bio-Rad) were submitted to an electrophoresis on a 2% agarose gel under an electric current of 200 volts for 20 min. A NZY DNA Ladder VI (NZYTech) was used as a Molecular Weight Marker. The fragments were visualized through UV light in the ChemiDoc™ XRS System and respective System with Image Lab™ Software (Bio-Rad).

***In silico* studies**

In silico analysis of sequence variants: The SIFT (Sorting Intolerant from Tolerant) [36], Polyphen-2 [37], PROVEAN (Protein Variation Effect Analyzer) [38] and MutationTaster [39] software programs were used to predict the influence of the amino acid substitution in the protein structure and function.

Evolutionary conservation studies, with both nucleotides and amino acid sequences, were performed in a group of primate species, such as Chimpanzee (*Pan troglodytes*), Gorilla (*Gorilla gorilla*), Macaque (*Macaca mulatta*) and Orangutan (*Pongo abelli*), and in a group of non-primate species, such as Mouse (*Mus musculus*), Rat (*Rattus norvegicus*), Zebrafish (*Danio rerio*), Fruitfly (*Drosophila melanogaster*) and Nematode (*Caenorhabditis elegans*), using the reference sequences available at the Ensembl database (http://www.ensembl.org).

Transcript studies

RNA extraction: Total RNA from nasal epithelium samples of the patient and two normal individuals was extracted with RNeasy minikit® (QIAGEN®) including an additional step with RNAse-Free DNAse Set (QIAGEN®), after homogenization of nasal epithelium samples with the TissueLyser II (QIAGEN®) according to manufacturer recommendations. Total RNA samples quantification and integrity evaluation based on the RNA integrity number (RIN),

was determined with the Bioanalyzer (Agilent Technologies) according to manufacturer recommendations.

Reverse Transcriptase (RT)-PCR: The total RNA samples were used to synthetize cDNA by RT-PCR with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to manufacturer recommendations and using a Veriti® Thermal Cycler (Applied Biosystems).

PCR for cDNA samples: To control de cDNA quality, PCR with *B2M* (used to control the presence of cDNA) and *MYO7A* Exon 40 intronic primers (used to control the absence of genomic DNA) were performed on a Veriti® Thermal Cycler (Applied Biosystems), following a protocol similar to the described above for DNA. The region surrounding the mutation in *MYO7A* transcript was amplified by PCR and the PCR products underwent an electrophoresis, similar to the described above.

Purification of PCR products for Sequencing reaction: The amplified cDNA samples, later used for Sequencing reaction, were purified accordingly to the protocol previously described.

Sequencing reaction: Sequencing reactions were performed accordingly to the protocol previously described.

Capillary electrophoresis: Automated capillary DNA Sequencing was performed accordingly to the protocol previously described.

Results

Genetic studies

USH1 patient sample was collected after a complete ophthalmological, audiological and vestibular evaluation that established the diagnosis. After patient's DNA Sequencing and PCR-RFLP analyses (Figure 1A-B), the presence of c.4489G>C variant was confirmed in patient's *MYO7A* gene exon 34.

For evaluation of the genotypic and allelic frequencies in a normal population 250 samples from individuals without USH were analysed with the same PCR-RFLP analysis. None of the 250 normal samples presented the c.4489G>C variant, all showing a homozygous wild type genotype (Figure 1C).

In silico studies

Bioinformatics analysis with SIFT software determined that *MYO7A* gene c.4489G>C variant was damaging (score 0.00) (Figure 2A), PolyPhen-2 software concluded that the variant was probably damaging (score 1.000) (Figure2B), PROVEAN software showed that the variant was deleterious (score -7,216) (Figure 2C) and Mutation Taster defined this variant as disease causing (Figure 2D).

It was also performed an evolutionary conservation study of the region surrounding the c.4489G>C variant using both *MYO7A* gene nucleotide and Myo7a protein amino acid sequences, with a primate and a non-primate groups, as showed in Figure 2E. It was found that Guanine nucleotide in position 4489 and Glycine (Gly) amino acid in position 1497 are invariably present in all analysed species.

Transcript analyses

In order to study the expression of c.4489G>C variant allele in *MYO7A* gene transcripts, total RNA extraction from the patient and two normal individuals without USH nasal epithelium samples was performed, followed by RNA integrity number (RIN) determination with the Bioanalyser (Agilent Technologies) aiming to quantify and evaluate the integrity of the extracted total RNA samples. All samples presented a RIN superior to 5 in a scale of 0 to 10 revealing a good level of integrity of total RNA samples. In detail, patient's total RNA sample had a RIN of 6.6, while normal individuals without USH samples showed a RIN of 6.8 and 6.7 (Figure 3A-C).

After RT-PCR of total RNA samples, amplification and sequencing of *B2M* gene transcripts was performed aiming to confirm cDNA synthesis (Figure 3D-E). Additionally, contamination with genomic DNA was discarded after failing to amplify a fragment using *MYO7A* gene intronic primers (Figure 3D).

The patient and the normal individuals without USH cDNA samples were used to amplify and Sequencing *MYO7A* gene transcripts surrounding the c.4489G>C variant regions. As showed in Figure 3F-G, it was possible to identify the expression of c.4489G>C variant in the patient cDNA sample and its absence in the normal individuals cDNA samples.

Discussion

USH is a rare, complex and genetically heterogeneous disease that can be highly incapacitating. USH affects vision, hearing and sometimes balance, if not early recognized and managed carefully it may lead to poor social skills and quotidian life, being responsible for a very low life quality. This way, a lot of effort is made to slow down the degenerative processes, treat complications and help patients cope with the social impact of the sensory deficits [40]. Although this effort, the therapeutic options are limited and it is nevertheless important continue studying, trying to achieve an early diagnosis, adequate managing and genetic counselling for patients and families. Accordingly, this study proposed to find the genetic mutation that is responsible for USH1 in a Portuguese patient.

After TR analysis of 9 of the 10 known USH associated genes in the USH1 patient DNA sample, a new homozygous variant was found. The c.4489G>C variant is a guanine to cytosine substitution at position 4489 that is predicted to be a missense variant, which have been widely described as responsible for USH phenotype [41], since it may cause a glycine to arginine change at the codon 1497 (Gly1497Arg) of Myo7a protein. Moreover, the effect of c.4489G>C mutation in the FERM domain it is unclear, even considering that glycine amino acid is a conformation breaker while arginine is an indifferent former, possibly causing a different secondary structure to the Myo7a. Therefore, Sequencing of *MYO7A* gene exon 34 and PCR-RFLP analysis of patient's DNA sample allowed confirming the presence of the new c.4489G>C variant (Figure 1A-C). Then, aiming to determine the frequency of the possible causing disease variant in the Portuguese population, the same PCR-RFLP protocol was used to analyse 250 samples from individuals without USH. The c.4489G>C variant was not found in any of such samples, allowing to determine a genotypic and allelic frequency of 0, and supporting the hypothesis that this variant is a rare event in the Portuguese population, probably USH disease causing. Nonetheless, since the patient's family was not available, the

variant segregation analysis in the USH1 patient family was not accomplished and this important test for variant pathogenicity determination will be taken into consideration as future work.

In silico studies were also performed to predict the implications of the new variant. Such studies allowed to determine that this may be a damaging variant according to the SIFT software, probably damaging using the PolyPhen-2 software, deleterious using the PROVEAN software and disease causing by Mutation Taster software, further supporting the possibility of the identified variant be a mutation (Figure 2A-D).

Additionally, evolutionary conservation studies were performed with nucleotide and amino acid sequences for c.4489G>C (Gly1497Arg) variant using two groups of species, a group with four primate species (*P. troglodytes, G. gorilla, M. mulatta* and *P. abelii*) and a group with five non-primate species (*M. musculus, R. norvegicus, D. rerio, D. melanogaster* and *C. elegans*) (Figure 2E). Both groups were compared with the normal *H. sapiens* and patient's nucleotide and amino acid sequences surrounding variant. With these studies, it is possible to evaluate how conserved are the analysed *loci* in the species evolution and differentiation, with a special emphasis in as more conserved are the *loci* among phylogenetically distant species as significant they are for the protein structure or function and as significant they are for cellular, tissue and organism homeostasis [42]. An extremely high conservation region was observed among all analysed primate and non-primate species both in nucleotide and amino acid sequences (Figure 2E), meaning that one alteration in the *MYO7A* gene 4489 *locus* can cause significant changes on the protein structure and function and be likely responsible for the USH aetiology in the studied patient.

Altogether, these data support the hypothesis that *MYO7A* gene c.4489G>C variant is indeed a mutation, responsible for patient's USH1 phenotype.

Moreover, it is important to study the gene expression at the transcript level for further understanding the cellular mechanisms affected by the c.4489G>C mutation. With this in mind, nasal epithelium samples, a tissue previously reported as appropriate for study USH transcripts [43], were collected from the patient and two individuals without USH and total RNA was extracted. As RNA can be rapidly digested in the presence of the nearly ubiquitous RNase enzymes and can compromise expression assays [44], in order to control the RNA integrity, it was used an algorithm created by Agilent Technologies that is an user-independent, automated and reliable procedure for standardization of RNA quality control allowing the calculation of an RNA integrity number (RIN) [45]. This algorithm is based on the evaluation of the ratio 28S:18S by electrophoresis combined with microfluidics technology that provides electrophoretic separations in an automated and reproducible manner, classifying the RNA integrity in a range of 1 to 10 and considering as acceptable for further use samples with a RIN superior to 5. All three extracted total RNA samples fulfilled this requirement (Figure 3A-C).

After RT-PCR of the total RNA extracted, it was performed a PCR amplification with primers overlapping the *B2M* gene transcripts end of an exon and the beginning of the next using the three cDNA samples previously synthetized and aiming to control the efficacy of the procedure. Then, such *B2M* gene amplified cDNA was sequenced to assure the cDNA analysis only (Figure 3D-E). *B2M* gene codes for β2-microglobulin, a serum protein found in association with the major histocompatibility complex class I heavy chain on the surface of nearly all nucleated cells and generally found in all tissues, being described as a good housekeeping gene for expression studies [46]. Besides, to exclude the contamination with genomic DNA, PCR amplification with intronic primers for *MYO7A* gene exon 40 was attempted but not observed (Figure 3D). This way, it was possible to proceed for *MYO7A*

transcripts analysis since the RT-PCR procedure synthetized cDNA without genomic DNA contamination.

The *MYO7A* transcripts analysis through PCR amplification and PCR product sequencing showed the presence of the c.4489G>C homozygous variant sample (Figure 3F-G), and it is likely that it will be translated into protein probably influencing the normal function of the Myo7a protein and, more precisely, the FERM domain of the first MyTH4-FERM tandem domain.

Nevertheless, to further understand the molecular consequences caused by the c.4489G>C mutation, it is essential to study the mutated Myo7a protein structure and function, mainly taking into account if the interactions between Myo7a and other proteins are compromised (for example with myrip), possibly disturbing the complex usher interactome. Consequently, such better understanding of the disease molecular mechanisms may allow the development of genetic therapies similar to the already used in some retinal dysfunctions. It is likely that an important future milestone for USH research will be to provide better and more efficient therapeutic tools to improve patients' life quality.

Conclusion

In conclusion, this study revealed that the novel c.4489G>C *MYO7A* gene variant is a homozygous missense mutation, which is likely responsible for the USH1 Portuguese patient phenotype Additionally, it was shown that this mutation is expressed in patient's nasal epithelium transcripts, suggesting that it is translated into Myo7a protein possibly affecting normal protein structure and/or function.

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Figures legends

Figure 1 – Genetic studies. A: Electropherogram of *MYO7A* gene Exon 34 region surrounding the c.4489G>C variant of WT sample; B: Electropherogram of *MYO7A* gene Exon 34 region surrounding the c.4489G>C variant of patient sample; C: Photography of variant detection by PCR-RFLP analysis. N – NZY DNA Ladder VI (NZYTECH), Ht – Heterozygous sample obtained after mixing the DNA of a patient with the c.4489G>C variant and a wild-type sample without the c.4489G>C, M – Homozygous mutated patient, W – Controls wild-type, U – Undigested sample.

Figure 2 – *In silico* studies of c.4489G>C (Gly1497Arg) *MYO7A* gene variant. A: SIFT software prediction; B: PolyPhen-2 software prediction; C: PROVEAN software prediction; D: MutationTaster software prediction; E: Evolutionary conservation study of nucleotide and amino acid sequences for *MYO7A* gene surrounding c.4489G>C variant. Conserved nucleotide and amino acid highlighted with grey shading. Nucleotides corresponding to the 4489G allele are yellow shading, amino acids corresponding to 1497Gly are green shading and mutant nucleotide and amino acid are red shading.

Figure 3 – Transcripts analyses. A: Quantification and RIN determination of patient´s total RNA sample; B: Quantification and RIN determination of normal individual 1 without USH total RNA sample; C: Quantification and RIN determination of normal individual 2 without USH total RNA sample; D: Photography of a) cDNA quality assessment and b) genomic DNA contamination assessment. N – NZYDNA Ladder VI (NZYTECH), P – patient sample, C1 – normal individual without USH samples, Ctr+ – positive control, Ctr- – negative control; E: Electropherogram of *B2M* gene transcript; F: Electropherogram of *MYO7A* gene transcript

region surrounding the c.4489G>C variant of WT sample; G: Electropherogram of *MYO7A* gene transcript region surrounding the c.4489G>C variant of patient sample.

Figure 1

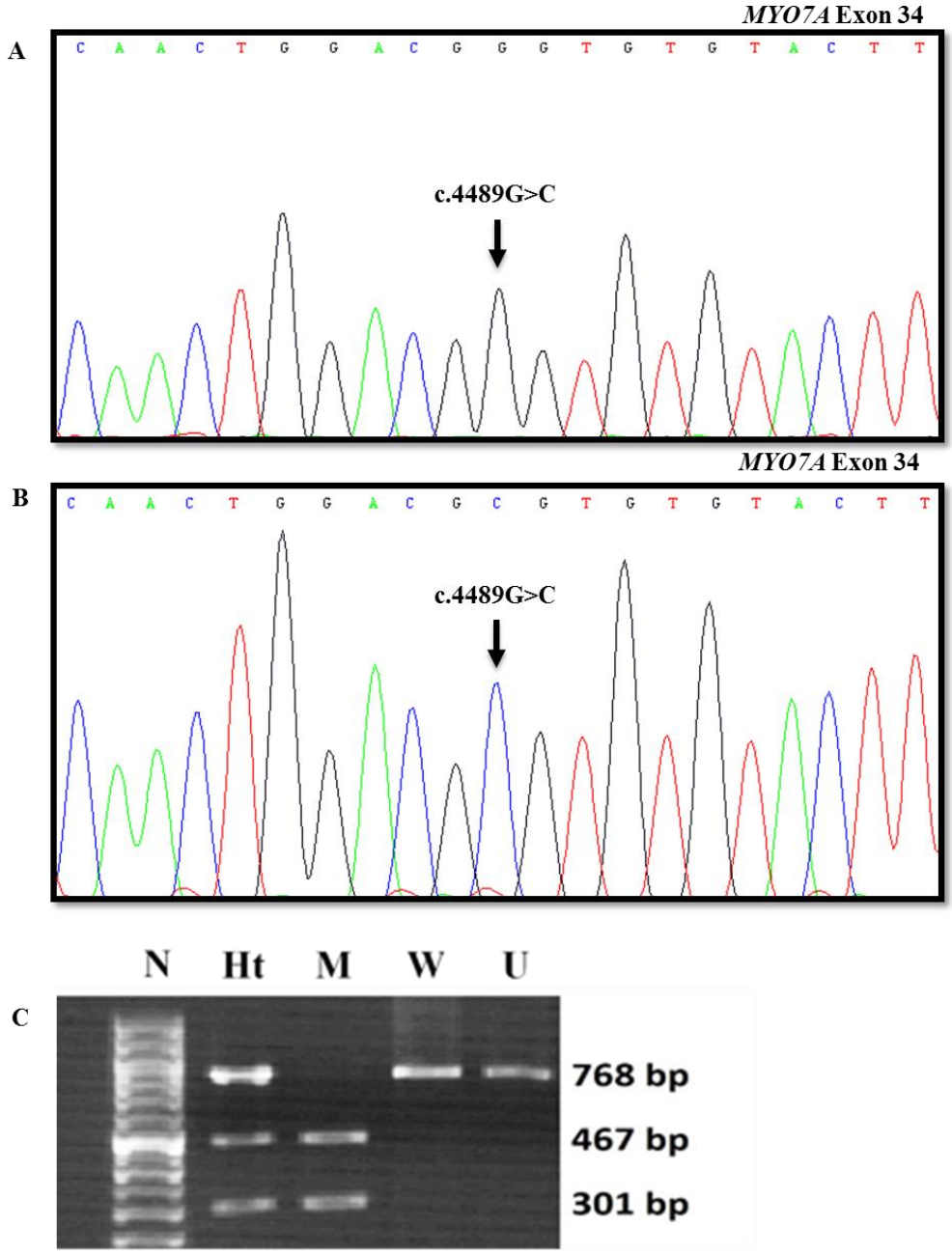

A
MYO7A Exon 34
C A A C T G G A C G G G T G T G T A C T T
c.4489G>C
B
MYO7A Exon 34
C A A C T G G A C G C G T G T G T A C T T
c.4489G>C
C
N
Ht
M
W
U
768 bp
467 bp
301 bp

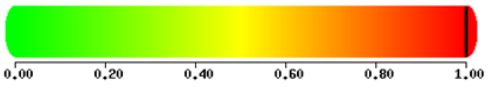
Figure 2

A

ENSP	Pos	Ref	Subst	Prediction	SIFT Score	Median Information Content	# Seqs
ENSP00000386331	1497	G	R	DAMAGING	0	2.78	100

B

This mutation is predicted to be **PROBABLY DAMAGING** with a score of **1.000** (sensitivity: **0.00**; specificity: **1.00**)



C

Variant	PROVEAN score	Prediction (cutoff= -2.5)
G1497R	-7.216	Deleterious

D

Prediction **disease causing** Model: *simple_aae*, prob: 0.999999999990132

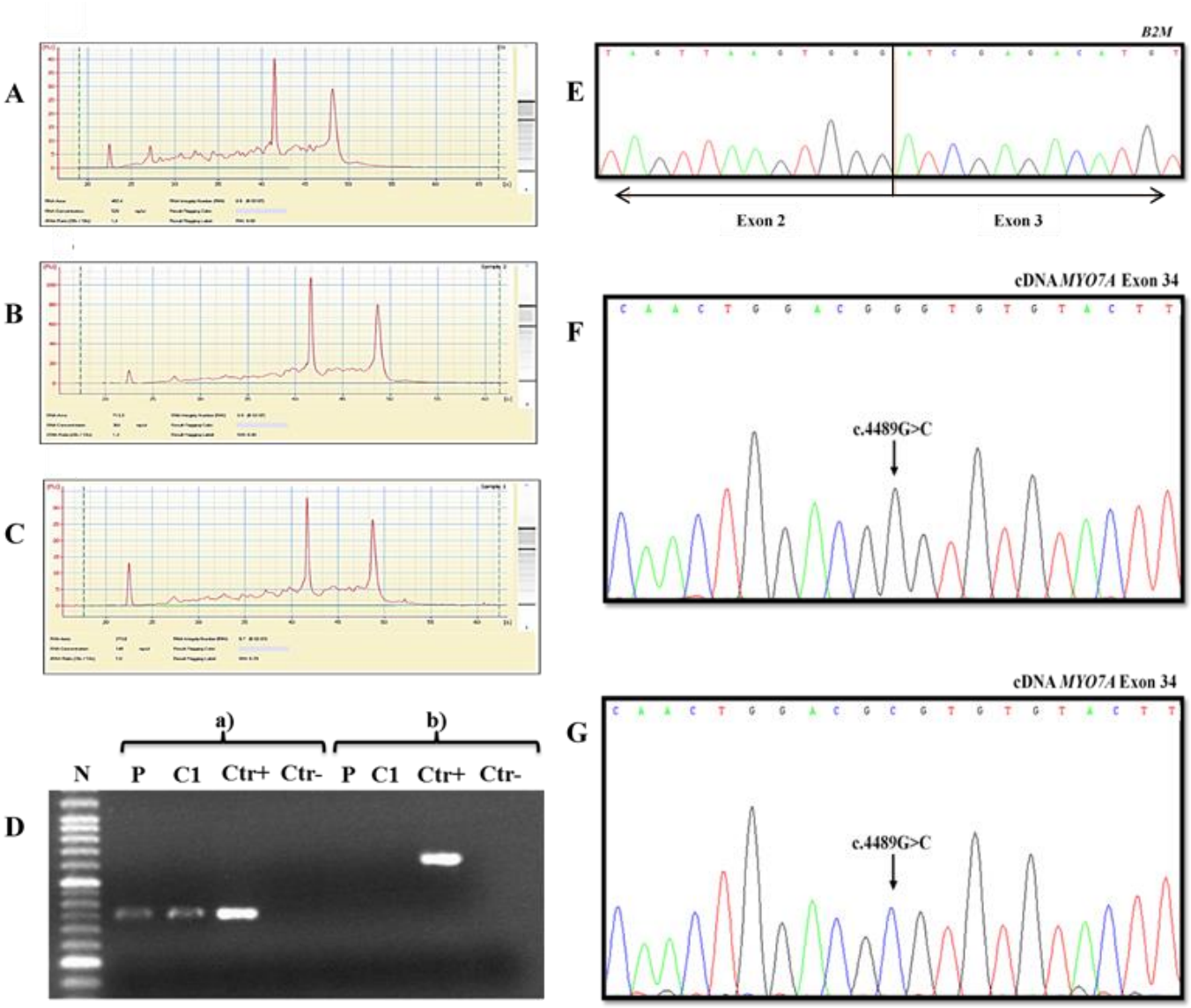
Summary

- amino acid sequence changed
- protein features (might be) affected
- splice site changes

E

Primates	Nucleotide Sequence	Amino acid Sequence
Homo sapiens	GCC GTC AAC TGG ACG GGT GTG TAC TTT GTG GAT GAG	AVNWTGVYFVDE
Pan troglodytes	GCC GTC AAC TGG ACG GGT GTG TAC TTT GTG GAT GAG	AVNWTGVYFVDE
Gorilla gorilla gorilla	GCC GTC AAC TGG ACG GGT GTG TAC TTC GTG GAT GAG	AVNWTGVYFVDE
Macaca mulatta	GCT GTC AAC TGG ACA GGC GTG TAC TTT GTG GAT GAG	AVNWTGVYFVDE
Pongo abelii	GCC GTC AAC TGG ACG GGC GTG TAC TTT GTG GAT GAG	AVNWTGVYFVDE
USH1 Patient	GCC GTC AAC TGG ACG CGT GTG TAC TTT GTG GAT GAG	AVNWTRVYFVDE
Non Primates		
Homo sapiens	GCC GTC AAC TGG ACG GGT GTG TAC TTT GTG GAT GAG	AVNWTGVYFVDE
Mus musculus	GCT GTC AAC TGG ACG GGT GTG TAC TTC GTG GAC GAG	AVNWTGVYFVDE
Rattus norvegicus	GCT GTG AAC TGG ACG GGC GTC TAC TTC GTG GAC GAG	AVNWTGVYFVDE
Danio rerio	GCT GTA AAC TGG ACC GGT GTG TAT TTT GTG GAC GAG	AVNWTGVYFVDE
Drosophila melanogaster	GCG GTG AAC TGG ACC GGC GTG TAT GTG GTT GAT GAT	AVNWTGVYVVDD
Caenorhabditis elegans	GCT GTG AAT TGG ACT GGT GTC TAT GTG GTT GAT GAT	AVNWTGVYVVDD
USH1 Patient	GCC GTC AAC TGG ACG CGT GTG TAC TTT GTG GAT GAG	AVNWTRVYFVDE

Figure 3


A
B
C
D
a)
b)
N
P
C1
Ctr+
Ctr-
P
C1
Ctr+
Ctr-
E
B2M
Exon 2
Exon 3
F
cDNA MYO7A Exon 34
c.4489G>C
G
cDNA MYO7A Exon 34
c.4489G>C