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Identification of two new mutations in the *GPR98* and the *PDE6B* genes segregating in a Tunisian family

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Autosomal recessive retinitis pigmentosa (ARRP) is a genetically heterogeneous disorder. ARRP could be associated with extraocular manifestations that define specific syndromes such as Usher syndrome (USH) characterized by retinal degeneration and congenital hearing loss (HL). The USH type II (USH2) associates RP and mild-to-moderate HL with preserved vestibular function. At least three genes *USH2A*, the very large G-protein-coupled receptor, *GPR98*, and *DFNB31* are responsible for USH2 syndrome. Here, we report on the segregation of non-syndromic ARRP and USH2 syndrome in a consanguineous Tunisian family, which was previously used to define USH2B locus. With regard to the co-occurrence of these two different pathologies, clinical and genetic reanalysis of the extended family showed (i) phenotypic heterogeneity within USH2 patients and (ii) excluded linkage to USH2B locus. Indeed, linkage analysis disclosed the cosegregation of the USH2 phenotype with the USH2C locus markers, D5S428 and D5S618, whereas the ARRP perfectly segregates with *PDE6B* flanking markers D4S3360 and D4S2930. Molecular analysis revealed two new missense mutations, p.Y6044C and p.W807R, occurring in *GPR98* and *PDE6B* genes, respectively. In conclusion, our results show that the USH2B locus at chromosome 3p23–24.2 does not exist, and we therefore withdraw this locus designation. The combination of molecular findings for *GPR98* and *PDE6B* genes enable us to explain the phenotypic heterogeneity and particularly the severe ocular affection first observed in one USH2 patient. This report presents an illustration of how consanguinity could increase familial clustering of multiple hereditary diseases within the same family.

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Introduction

Hereditary retinal degenerations are a genetically and clinically heterogeneous group of disorders. The most common form of these disorders is retinitis pigmentosa (RP) affecting approximately 1 over 5000 individuals worldwide.^{1,2} RP primarily affects the rod photoreceptors,

whereas the function of the cone receptors is compromised as the disease progresses.³ RP may be inherited as an autosomal recessive, autosomal dominant or as an X-linked recessive trait. Autosomal recessive RP (ARRP) comprises 20–25% of cases of RP. To date, 40 loci have been implicated in non-syndromic RP, of which 30 genes are known, including *PDE6B* gene (<http://www.sph.uth.tmc.edu/Retnet/disease.htm>). This latter gene encodes the β -subunit of retinal rod cGMP phosphodiesterase, a key enzyme involved in photo-transduction in rod photoreceptor cells.⁴ ARRP could also be associated with extraocular manifestations that define specific syndromes such as Usher syndrome (USH) (MIM276900), which represents the most common form of hereditary deafblindness in humans. It has a prevalence of $\sim 1/20\,000$ and represents 50% of the cases with deafblindness.^{5–7} USH is a group of autosomal recessive disorders characterized by RP with sensorineural hearing loss (HL) and variable vestibular function. Three clinically distinguishable USH types have been reported.⁸ People with USH type II (USH2) display congenital moderate-to-severe HL, RP and normal vestibular function. USH2 is genetically heterogeneous with at least three genes so far identified, *USH2A*, the very large G-protein-coupled receptor (GPCR), *GPR98*, and *DFNB31*.^{9–11}

Using a consanguineous Tunisian family, we previously localized the USH2B locus to a 23-cM region on chromosome 3p23–24.2.¹² Here, we report the segregation of missense alleles of *GPR98* and *PDE6B* in the original and the single USH2B family described so far. Interestingly, the overlapping of both mutations underlies severe ocular affection. This report also presents an illustration of how consanguinity could increase familial clustering of multiple diseases within the same family.

Materials and methods

Clinical evaluation

Detailed clinical description of the USH2B family has been reported earlier.^{12,13} Careful clinical re-examination of the USH2B family revealed the segregation of non-syndromic RP within another branch from the USH2B family. A complete historic interview enabled us to draw an extended pedigree from the family and to confirm the segregation of two different diseases, USH2 and non-syndromic ARRP, within the same previously reported USH2B family. A total of 21 additional members were currently ascertained including nine healthy individuals, six USH2 and six non-syndromic ARRP patients. All of them were evaluated for HL by pure-tone audiometry using air and bone conduction (frequencies ranging from 250 to 8000 Hz). Clinical histories were obtained from participating members to rule out obvious environmental causes of HL. Vestibular dysfunction was assessed by caloric tests in patients. All patients from the extended family under-

went an ophthalmological examination including external eye examination, corrected visual acuity and fundoscopic examination. Physical evaluations were undertaken to verify whether the RP was syndromic or non-syndromic. All USH2 family members from the prior studies along with the new ascertained ones were included in this study. The clinical diagnosis of USH2 or ARRP phenotype was established mainly on hearing evaluation and comparison of the onset, severity and progression of the retinal rod-cone dystrophy. Blood samples were collected from the new affected and unaffected family members.

Genotyping and linkage analysis

To confirm linkage to the USH2B locus, additional microsatellite markers were tested within the extended family. These markers were designed from the Généthon human linkage map¹⁴ and from the Marshfield chromosome-3 map (<http://research.marshfieldclinic.org/genetics>). Furthermore, a genome-wide screening was performed with 400 microsatellite markers distributed at average intervals of 10 cM (ABI Prism Linkage Mapping Set 2, Applied Biosystems). Fluorescently labeled alleles were analyzed on an ABI PRISM 3100-Avant automated DNA sequencer (Applied Biosystems, USA). Genetic analysis of non-syndromic ARRP loci was undertaken using fluorescently labeled primers surrounding microsatellite repeats at known RP loci selected from Marshfield chromosome maps (Table 1). Two-point linkage analyses were performed using the FASTLINK version of MLINK from the LINKAGE Program Package.^{15,16} Maximum LOD scores were calculated using ILINK. USH2 syndrome was analyzed as a fully penetrant trait with an estimated frequency of 10^{-3} for the disease-causing allele. Recombination frequencies were assumed to be equal for male and female individuals, and allele frequencies of linkage markers were assumed to be equal.

Mutation screening

Candidate genes in the USH2B interval on chromosome 3p23–24.2 were identified either using the UCSC Genome browser (<http://www.genome.ucsc.edu/>) or suggested by collaborators. Primers used for PCR amplification and subsequent sequencing of USH2B candidate genes were designed from the flanking region of each exon using Primer3 web utility (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/). We also analyzed all the 90 exons of the *GPR98* gene (GeneID: 84059) and the 22 exons of *PDE6B* gene (GeneID: 5158). Primers were synthesized from the consensus intronic sequences except for sets of exonic primers designed to amplify the overlapping parts of exons. To study the c.18131A>G change occurring in exon 85 of the *GPR98* gene, we screened 95 genetically unrelated controls using a PCR/RFLP approach: exon 85 of the *GPR98* gene was amplified with intronic primers resulting in a product of 325 bp. Restriction analysis with *Hpy8I* of the wild-type allele results in two fragments of 265 and 60 bp, whereas

Table 1 ARRP genes tested by linkage analysis and the corresponding microsatellite markers

Genes	<i>ABCA4</i>	<i>CRB1</i>	<i>RPE65</i>	<i>MERTK</i>	<i>SAG</i>	<i>RHO</i>	<i>LRAT</i>	<i>PDE6B</i>
Markers	D1S2779 D1S236 D1S2775	D1S2816 D1S2840 D1S1660	D1S2803 D1S1162 D1S2865	D2S1896 D2S2269	D2S1279 D2S2205	D3S3607 D3S1587	D4S3021 D4S2631	D4S3360 D4S2936 D4S3038 D4S1614
Genes	<i>CNGA1</i>	<i>PDE6A</i>	<i>TULP1</i>	<i>TTPA</i>	<i>RP1</i>	<i>NR2E3</i>	<i>RLBP1</i>	<i>CNGB1</i>
Markers	D4S1536 D4S3002	D5S812 D5S2015	D6S1611 D6S1645	D8S1178 D8S1737	D10S1774 D10S1717 D10S1658 D10S1774 D10S1717 D10S1658	D15S1050 D15S131 D15S204	D15S1045 D15S202 D15S116	D16S3140 D16S3057

the mutated allele is cleaved into three fragments of 239, 60 and 26 bp. The mutation introduces a novel *Hpy8I* site GTNNAC.

Results

Earlier, we had mapped the USH2B gene to a region on chromosome 3p23–24 using a consanguineous Tunisian family named 'Us'.¹² Although the corresponding region was large (23 cM), attractive interesting genes were selected as positional candidates in the USH2B interval (*TMIE*,¹⁷ *SLC26A6* (MIM610068), *NBC3* (MIM603353) and *CELSR3* (MIM604264)). However, no disease-associated alteration was identified in any of them.

In parallel, we had the opportunity to extend the 'Us' pedigree by studying 21 additional healthy and affected individuals as well as a new nuclear-related branch affected by non-syndromic ARRP. The genotyping of the extended 'Us' family with additional microsatellite markers flanking the USH2B interval did not show any homozygous region shared between all USH2 patients, and some of them still show a heterozygous USH2B haplotype (Figure 1; individuals VI-8, VI-9 and VI-10). Moreover, the genotyping of newborn affected USH2 patients VII-10, VII-11 and VII-12 failed to show any linkage and excluded the involvement of the USH2B locus in the family (data not shown). Given the concomitant segregation of USH2 and non-syndromic ARRP in the same family, we undertook a clinical reanalysis on the extended family. All patients and healthy family members in the prior study were included in this study. Clinical examination showed moderate-to-severe non-progressive congenital HL in all USH2-affected members (Table 2 and Figure 2). Vestibular function appeared to be normal in affected individuals and clinical evaluation suggested no skin or renal anomalies. Ophthalmologic examination displayed a progressive night blindness and constriction of the peripheral visual field, with mild-to-severe loss of central vision (Supplementary Figure 1). Compared with younger patients, older subjects showed evidence of deteriorated cone function that indicates the

progressive nature of the retinal dystrophy. All patients underwent dilated indirect funduscopy. Recorded changes included progressive dystrophy of the retina in the retinal periphery, with or without macular involvement, with intraretinal pigment migration, attenuation of the retinal blood vessels and some degree of pallor of the optic disc (Figure 3c). Moreover, in patient VI-10, a 39-year-old woman, fundus photographs showed a more severe RP that had progressed and covered almost the whole retina reaching the center (Figure 3c). When interviewing her relatives, they confirmed the early appearance of night blindness in that woman starting from the beginning of her first decade. Interestingly, this early severe ocular defect was also observed in all patients affected by non-syndromic ARRP. Indeed, those patients share the precocity of the RP onset, which started early at the age of 2 years and subsequently progressed severely until invading the whole retina. We therefore suspected in the 'Us' family, the segregation of two different pathologies non-syndromic ARRP and the USH2 syndrome, which have overlapped in patient VI-10 explaining the severity of her retinal dystrophy.

Genetic findings excluding the USH2B locus, along with the phenotypic heterogeneity observed between the USH2 patients, prompted us to reanalyze the extended family with regard to the concomitant segregation of two different pathologies that is, non-syndromic ARRP and USH2 syndrome. We therefore considered only those patients with both HL and RP as USH2 and all the others as normal but those with RP alone were excluded. We undertook a screening of the whole genome using 400 microsatellite markers at intervals of 10 cM. Surprisingly, a linkage was detected only with the microsatellite marker D5S428 on chromosome 5, corresponding to the USH2C locus. The addition of other markers within the same region and haplotype analysis confirmed the linkage (Figure 1) and disclosed a recombination event occurring in affected individual VI-5 between D5S641 and D5S428. A maximum two-point LOD score of 4.1 was obtained with microsatellite marker D5S618 at a recombination fraction

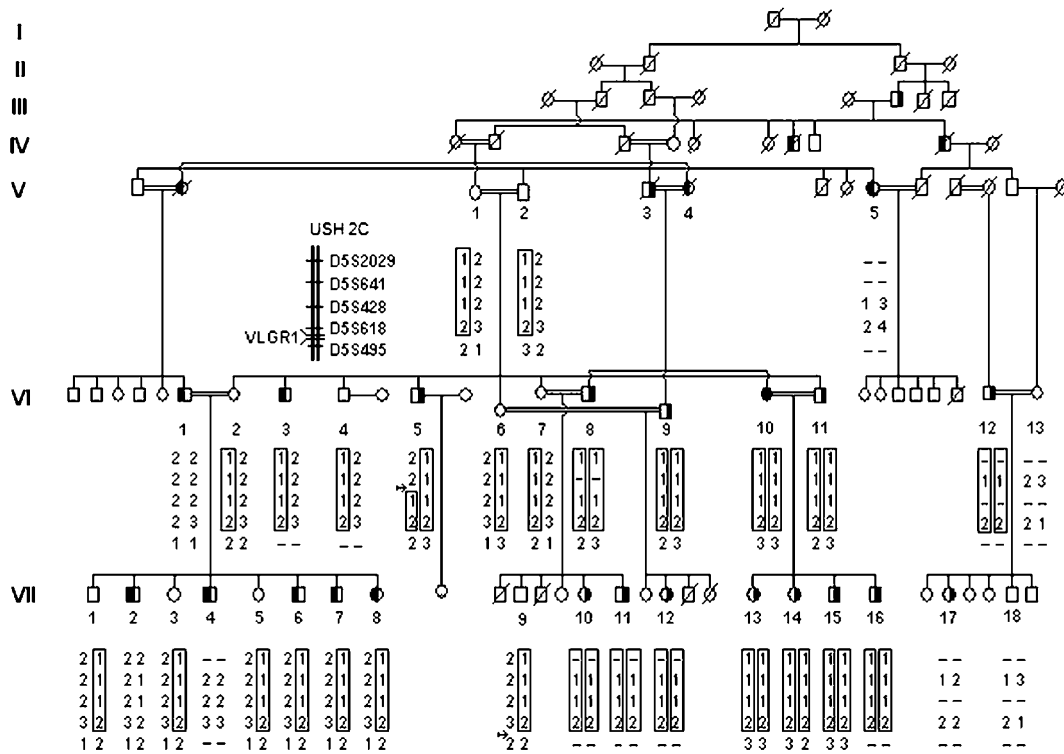


Figure 1 Pedigree and segregation analysis of the extended ‘Us’ family. Haplotypes are shown only for members relevant to the study and represented as columns of numbers, with the ancestral disease-associated haplotype boxed. Individual VI-5 provides a recombination event indicated by an arrow. □, ○ = male and female individuals affected with USH2 syndrome; ■, ● = male and female individuals affected with both USH2 syndrome and ARRPs.

$\theta=0.00$ (Table 3). None of RP patients showed the pathologic USH2C haplotype in two copies, ruling out the involvement of this locus in both phenotypes. We performed, thereby, a genetic analysis of the known ARRPs genes using the corresponding microsatellite markers (Table 1). Linkage analysis showed cosegregation of the RP branch with the telomere of chromosome 4 harboring the *PDE6B* gene. All ARRPs patients were homozygous for both D4S3360 and D4S2936 markers (Figure 3a). Molecular analysis identified a new substitution c.2419T>A, in exon 21 of *PDE6B* gene, that segregates perfectly with the ARRPs phenotype in the family. This change results in the substitution of an evolutionarily conserved tryptophan with a polar arginine at position 807 (Figure 4a).

Sequencing of the *GPR98* coding region in an affected member VI-11 revealed homozygosity for a missense mutation c.18131A>G occurring in exon 85 resulting in the substitution of the residue tyrosine at position 6044 by a cysteine (p.Y6044C). This change perfectly segregates with the USH2 phenotype. Indeed, all patients were homozygous for the c.18131A>G allele and healthy individuals were either heterozygous or homozygous for the wild-type allele. The two parents V-1 and V-2, although aged 82 and 65 years, respectively, did not show any

alteration of the retinal pigmented epithelium or hearing problems despite being double heterozygous for the c.2419T>A and the c.18131A>G mutations (Figure 3a and b). Using a PCR/RFLP approach, we did not find this mutation in 95 genetically unrelated controls. In addition, the c.18131A>G mutation was tested in 24 American USH2 families unlinked to USH2A locus and was found to be in a heterozygous state in one USH2 family. This mutation results in the substitution of a highly conserved tyrosine residue at position 6044 with a cysteine at the second extracellular loop (ECL2) of the *GPR98* (Figure 4b). This newly introduced cysteine residue may disrupt the interloop disulfide bridge between ECL1 and ECL2 leading to an improperly folded loop and consequently to a non-functional receptor. Sequence analysis also identified two new sequence variants, c.16031G>A and c.16279G>A, in the coding exons 74 and 76, respectively, and one nucleotide change (T>C) in intron 25. They were considered as polymorphisms as they have been detected in several unaffected individuals (unpublished data). Interestingly, the combination of *GPR98* and *PDE6B* molecular findings disclosed a double homozygosity involving both genes in the same patient VI-10, which would be expected to be responsible for her severe ocular phenotype (Figure 3c).

Table 2 Audiometric and ophthalmologic features of USH2 patients

Individuals	Code	Age	Sex	Audio (dB)		ORL	Caloric test	Goiter	Ophthalmologic symptoms
				RE	LE				
US24	VI-5	43	M	59.5	55.83	Left hyperflexia	—	Mild peripheric RP	
US13	VI-8	62	M	55.83	56.16	Normal	—	Severe RP	
US9	VI-10	50	F	64.16	63.33	Normal	—	Severe RP	
US15	VI-11	55	M	59	69.16	Normal	—	Mild peripheric RP	
US10	VII-13	23	F	54.5	55.16	Normal	—	Mild peripheric RP	
US11	VII-14	16	F	68.33	63.33	Normal	—	Mild peripheric RP	
US16	VII-15	19	M	55	55	Normal	—	RPE alteration	
US47	VII-17	—	F	63.33	57.5	Normal	—	Mild peripheric RP	

The thresholds values presents an average of different thresholds obtained at frequencies 250, 500, 1000, 2000, 4000 and 8000 Hz. LE, left ear; RE, right ear; RP, retinitis pigmentosa; RPE, retinal pigmentary epithelium.

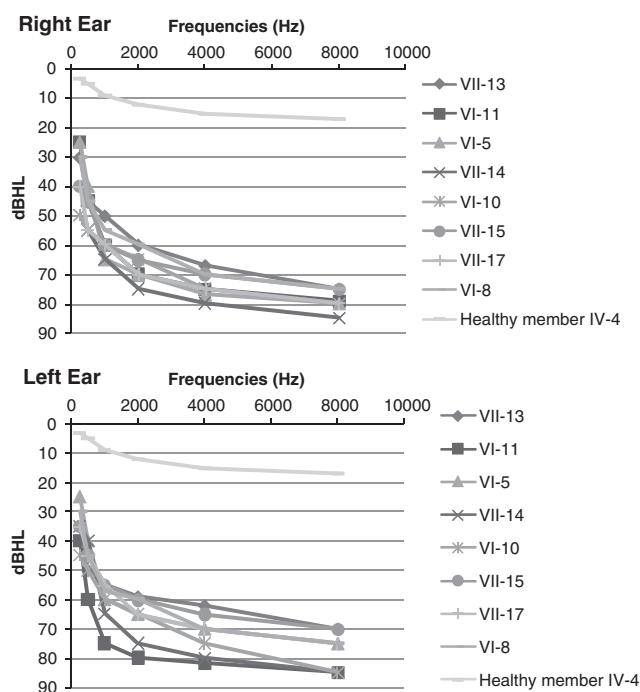
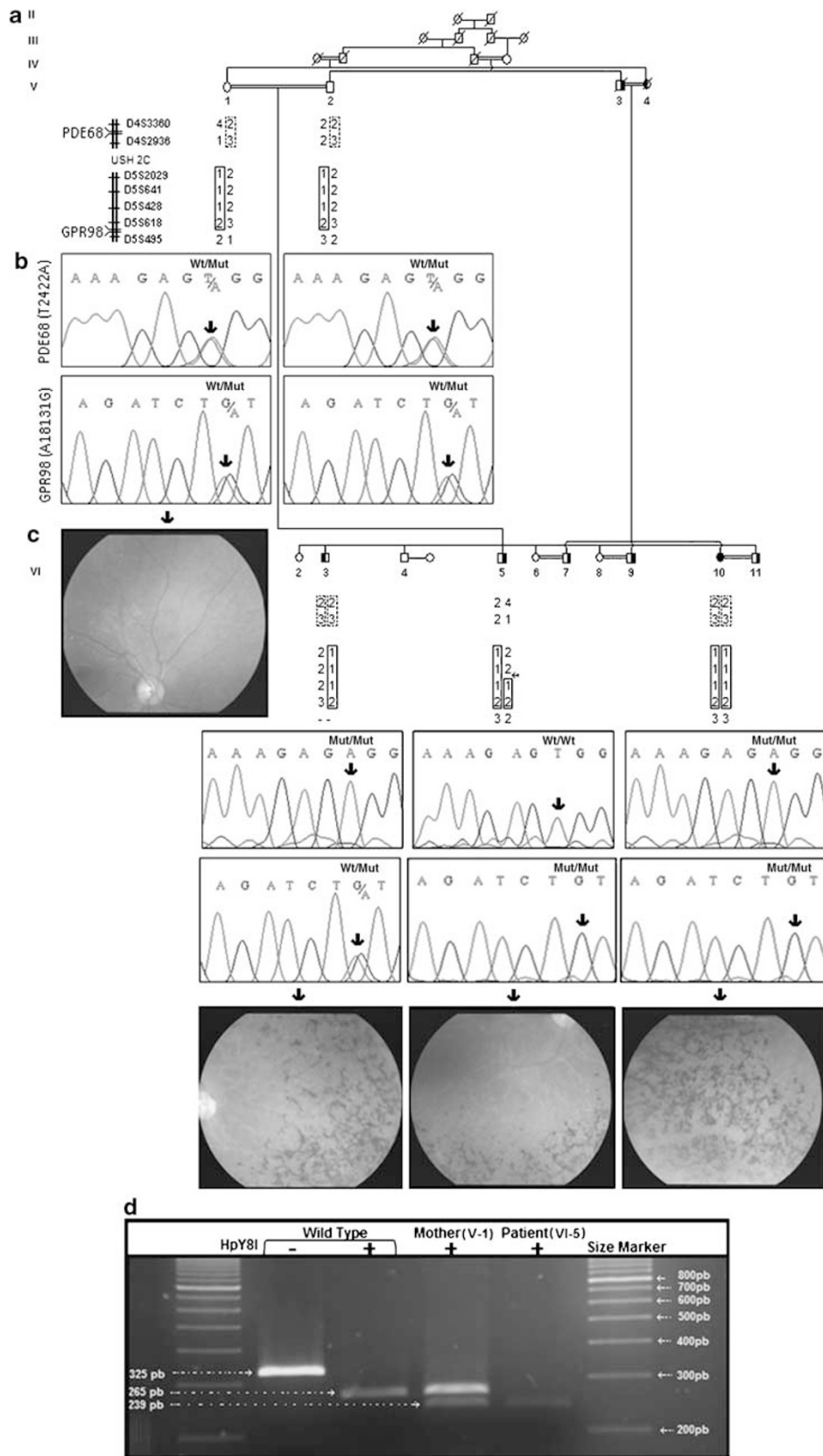


Figure 2 Audiograms from USH2 patients and one healthy individual from 'Us' family. The hearing threshold values were assessed at sound frequencies 250, 500, 1000, 2000, 4000 and 8000 Hz.

Discussion

This study deals with the segregation of two different forms of hereditary retinal degenerations including non-syndromic ARRP and USH2 syndrome within the same consanguineous Tunisian family. Such a familial aggregation of hereditary diseases has been well described in different populations, in particular those with a high consanguinity rate.^{18,19} Indeed, the Tunisian population consists of a mosaic of communities (Phoenicians, Roman, Berber, Arab, Ottoman, etc.). This heterogeneity along with geographic isolation, social traditions and parental involvement in mate selection all contribute to increase consanguinity in these regions. In addition to this aggregation, the consanguinity also seems to be involved in concentrating some diseases, increasing their prevalence in the corresponding population. In fact, although USH2 syndrome seems to be the most common type of USH in western European population,⁹ this form seems to be much less frequent in Tunisian population. It is likely that in certain consanguineous populations, such as Tunisian population, specific types of USH could be relatively frequent because of founder effects.²⁰⁻²² Alternatively, this low frequency of the USH2 type could also be attributed to an under diagnosis of this clinical type justified by its mild ocular and auditive affection.

Figure 3 (a) Pedigree and haplotype of family 'Us' for *VLGR1* and *PDE6B* chromosomal regions. Haplotype analysis revealed that the index patient VI-10 is homozygous for microsatellite markers flanking both the *VLGR1* and the *PDE6B* loci. (b) Mutation analysis revealed two new missense mutations, c.18131G>A and c.2419T>A, occurring in *VLGR1* and *PDE6B* genes, respectively. The parents V-1 and V-2 are double heterozygous for both mutations, and patients showing either ARRP (VI-3) or USH2 (VI-5) alone are homozygous only for c.2419T>A or c.18131A>G, respectively. However, the index patient VI-10 is homozygous for both alterations. (c) Fundus photographs of healthy father, affected individuals presenting with ARRP and USH2 and both forms of retinal dystrophies. There are changes typical of retinitis pigmentosa including attenuation of retinal arteries and bone-spicule pigment deposits in the mid-periphery of the retina. In patient VI-10, note the severe RP that has progressed and covered almost the whole retina reaching the center. (d) Restriction analysis with *Hpy8I*. Exon 85 of the *GPR98* gene was amplified with intronic primers resulting in a product of 325 bp. Digestion of the wild-type allele results in two fragments of 265 and 60 bp. Although the mutated allele is cleaved into three fragments of 239, 60 and 26 bp, the mutation introduces a novel *Hpy8I* site GTNNAC.



We have previously localized the USH2B gene to a 23-cM region on chromosome 3p23–24.2 using the 'Us' Tunisian family.¹² As far as we extended the study on the large family, genetic analysis clearly excluded the involvement of a gene in the USH2B region and thus we withdrew USH2B locus designation. Moreover, clinical findings showed phenotypic heterogeneity in USH2 patients particularly in the ocular affection with regard to the severity and the RP onset. Typically, RP leads to the development of night blindness and loss of peripheral vision. Progressive retinopathy, clinically indistinguishable from classic RP, typically develops in the childhood to adolescent years in all types of USH.^{23–25} However, instances of late-onset RP in cases of USH2 syndrome have been reported.²⁶ Within our family, we noted late-onset RP in the majority of USH2 patients, with the exception of patient VI-10 presenting with an early-onset RP that has already invaded almost the whole retina. Interestingly, this later severe and early-onset phenotype was also present in all ARRP patients, raising the possibility of two overlapping pathologies in patient VI-10.

Genetic and molecular analysis revealed the coexistence of mutations affecting two different genes *GPR98* and

PDE6B, ruling out the involvement of the same gene in both forms of hereditary retinal degeneration. In fact, with the exception of specific USH2A mutations, no change in any of the Usher genes has been implicated thus far as a cause of RP without HL. Indeed, besides as USH2, some mutations in the *USH2A* gene are manifested as atypical USH²⁶ or as non-syndromic recessive RP^{27,28} without hearing or balance deterioration over time in these patients. In addition, each of these USH2A mutations occurred separately in an individual family. Thus, it remains still unknown whether disease mechanisms and dynamics of USH are the same as those underlying non-syndromic RP.

We discovered a missense mutation in exon 21 of *PDE6B* gene at position 2419 where thymidine is replaced by adenine resulting in the substitution of polar arginine with tryptophan. Although the mutation lies outside the catalytic domain of the enzyme, it occurs at an evolutionarily conserved position. It has been postulated through studies on murine and canine models that defective functioning of the phosphodiesterase is detrimental for overall health of rod photoreceptors. Malfunctioning of PDE results in elevated levels of cGMP. Apoptosis is induced due to excess cations flowing in the rod cells leading to retinal degeneration.^{29,30}

Molecular analysis of the coding region of the *GPR98* gene, a member of the GPCR family, showed a homozygous missense mutation c.18131A>G occurring in exon 85 and resulting in the substitution of a highly conserved tyrosine with a cysteine at position 6044 in the ECL2 of the *GPR98* receptor. This mutation was also found in a heterozygous state in one USH2 family unlinked to USH2A locus. Indeed, the majority of family A and family B GPCR contains a conserved pair of extracellular cysteine residues linking

Table 3 Two-point lod scores values calculated for microsatellite markers in the *USH2C* chromosomal region

Markers	Recombination fraction (θ)						
	0	0.01	0.05	0.1	0.2	0.3	0.4
D5S2029	$-\infty$	0.75	1.18	1.14	0.75	0.34	0.08
D5S641	$-\infty$	1.30	1.70	1.61	1.12	0.57	0.15
D5S428	4.03	3.91	3.42	2.87	1.82	0.85	0.16
D5S618	4.09	3.96	3.47	2.91	1.85	0.87	0.17
D5S495	$-\infty$	-3.80	-1.31	-0.46	0.05	0.12	0.05

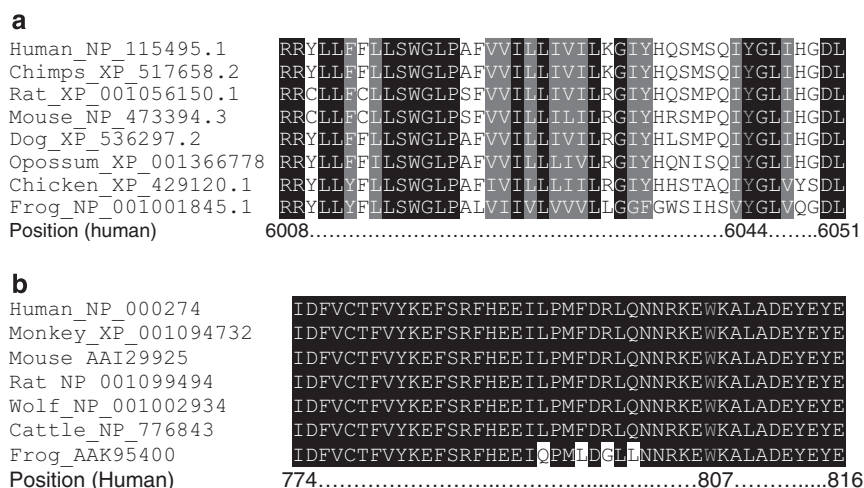


Figure 4 Sequence alignments of *GPR98* (a) and *PDE6B* (b) proteins from vertebrate species for which the sequence was present in *Refseq* database (accession numbers are given after species common name). The mutated residues are highlighted in pink in online version.

ECL1 and ECL2 through a disulfide bond. Numerous functional analyses of GPCR mutants where cysteine residues were replaced by other amino acids have shown that this disulfide bond may be critical for receptor signaling.^{31–33} The structural consequences of the presence of additional cysteine residues within the ECL seem to be complex. It is likely that this newly introduced residue cysteine 6044 may offer alternatives in disulfide bond formation that compete and disrupt the interloop disulfide bridge between ECL1 and ECL2, leading to an improperly folded loop and consequently to a non-functional receptor. Alternatively, this missense mutation may alter the intracellular trafficking of the receptor protein. In fact, more than 80% of small inframe deletion/insertion and missense mutations lead to improperly folded proteins retained intracellularly by the quality control machinery of the endoplasmic reticulum.^{34,35}

Disease-causing mutations of the highly conserved disulfide bond connecting ECL1 and ECL2 have been identified in many GPCRs, including AVPR2,³⁶ RHO^{37,38} and MC2R.³⁹ About 7% of all missense mutations in GPCR are substitutions with cysteine residues found in the putative N-terminal portion and ECL1-3.⁴⁰ Indeed, these extracellular loops of GPCR frequently contain binding sites for peptide ligands. However, the mechanism of receptor activation following ligand binding and the influence of the extracellular loops in aspects of receptor function are poorly understood.

Mutations so far reported in the *GPR98* gene are mostly nonsense mutations occurring either in the large N-terminal ectodomain or in the C-terminal region resulting in a dramatically truncated protein.^{10,41} This is the first report on the occurrence of pathogenic missense mutations in the extracellular domain of *GPR98*, suggesting that this domain could fulfil a range of important roles with respect to GPCR signaling including agonist binding and/or receptor activation. Moreover, unlike the complement factor 5a receptor, C5aR, in which the ECL2 plays a negative regulating role in the receptor activation, ECL2 of *GPR98* seems to have a positive regulator effect. In fact, this amino-acid substitution has disrupted the receptor function instead of generating a ligand-independent activity receptor. Thus, we could suggest a two-site model for ligand binding to *GPR98* supporting other groups, proposing that along with the amino terminus part, the extracellular loops could influence the high-affinity binding of the *GPR98* ligand.

Recent studies have reported that *GPR98* is a component of the ankle link complex,^{42,43} which is one among four distinct types of links that connect stereocilia forming the sensorineural cell hair bundle. This interstereocilial complex, involving also Usherin and likely protocadherin-15,^{44,45} is required for normal development of auditory hair bundles explaining anomalies observed in *USH2* patients.

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