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Novel mutations in the long isoform of the USH2A gene in patients with Usher syndrome type II or non-syndromic retinitis pigmentosa

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Abstract

Background—Usher syndrome type II (USH2) is an autosomal recessive disorder characterized by retinitis pigmentosa (RP) and mild to moderate sensorineural hearing loss. Mutations in the USH2A gene are the most common cause of USH2 and are also a cause of some forms of RP without hearing loss (ie non-syndromic RP). The USH2A gene was initially identified as a transcript comprised of 21 exons but subsequently a longer isoform containing 72 exons was identified.

Methods—The 51 exons unique to the long isoform of USH2A were screened for mutations among a core set of 108 patients diagnosed with USH2 and 80 patients with non-syndromic RP who were all included in a previously reported screen of the short isoform of USH2A. For several exons, additional patients were screened.

Results—In total, 35 deleterious mutations were identified including 17 nonsense mutations, 9 frameshift mutations, 5 splice-site mutations, and 4 small in-frame deletions or insertions. Twenty-seven mutations were novel. In addition, 65 rare missense changes were identified. A method of classifying the deleterious effect of the missense changes was developed using the summed results of 4 different mutation assessment algorithms, SIFT, pMUT, PolyPhen, and AGVGD. This system classified 8 of the 65 changes as “likely deleterious” and 9 as “possibly deleterious”.

Conclusion—At least one mutation was identified in 57–63% of USH2 cases and 19–23% of cases of non-syndromic recessive RP (calculated without and including probable/possible deleterious changes) thus supporting that USH2A is the most common known cause of RP in the United States.

Keywords

Usher syndrome; retinitis pigmentosa; mutation; deafness

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INTRODUCTION

Usher syndrome is an autosomal recessive condition where patients have both retinitis pigmentosa and sensorineural hearing loss and, in some cases, vestibular defects. It is the most common cause of deaf-blindness and its prevalence is estimated to be between 3.3 – 6.4 per 100,000 people.[1–5] This syndrome is divided into three clinical subtypes based on the severity and onset of the deafness and the presence or absence of vestibular defects. Usher syndrome type I (USH1) is characterized by RP, profound congenital hearing loss and vestibular dysfunction. Patients with Usher type II (USH2) have RP with partial, early onset hearing loss and no vestibular defect. Usher type III features RP, progressive hearing loss, normally of adult onset and variable vestibular dysfunction. Genetic heterogeneity is evident in Usher syndrome with USHI caused by mutations in any one of 6 genes (MYO7A,[6] USH1C,[7] CDH23,[8,9] PCDH15,[10,11] and USH1G[12]), and the as yet unidentified gene assigned to chromosome 21q21 (USH1E[13]), USH2 caused by three identified genes (USH2A,[14] GPR98 (formerly VLGR1 or MASS1),[15] and DFNB31(Whirlin) [16]) and at least one unidentified gene,[17] and USH3 caused by mutations in the gene CLRN1.[18] USH2 is the most common form of Usher syndrome.[1,3,4] However this may not be the case in all populations where for example, a founder effect may skew the distribution of cases.[5,19,20] Of patients with USH2, it is estimated that between 75% to 90% have pathogenic mutations in the USH2A gene[21–23] making this gene the most common known cause of Usher syndrome.

Mutations in the USH2A gene were first reported in patients with USH2 in 1998.[14] This first study analyzed the 21 exons encoding the originally described USH2A transcript.[24] A longer transcript consisting of 51 additional 3' exons was subsequently identified and mutations causing USH2 were also found in those exons.[25] In a recent study, comprehensive analysis of the newly characterized 51 exons revealed allelic mutations in approximately half of the patients in whom one pathogenic mutation had been identified in a screen of the shorter isoform.[22] More than 70 different null alleles and several different missense mutations have been identified in the USH2A gene in patients with USH2 (for a summary see the UMD-USHbases <http://www.umd.be/USH2A/>[26]). Of these, most are found in only one or a few cases each. The exception to this is the common mutation c. 2299delG (Glu767fs) which has been reported as a frequent mutation in several populations and found to have been derived from a common ancestor.[27] Comprehensive screens of the entire long isoform revealed that approximately 60% of the unique pathogenic mutations occur in the exons specific to the long isoform.[21,23]

One corollary of the fact that many mutations are solitary or found only in a few cases is that inferring the pathogenicity of such sequences can not be confidently accomplished even if the change is not observed among a hundred normal control subjects. For example, a sequence variation would need to be observed in at least 6 out of 200 disease chromosomes if not found in any of 200 normal chromosomes before being considered statistically significantly associated with disease ($p < 0.05$ using a Fisher's exact test). Rare nonsense, frameshift, or splicing mutations can be interpreted as pathogenic null alleles since they are highly likely to be null alleles. However, rare missense variations could be either deleterious or harmless. Computational algorithms for analyzing the consequences of missense substitutions have been developed, and they largely depend on protein sequence alignments across species.[28] In this study the interpretation of identified rare missense changes is aided by a combined analysis of 4 such computational programs.

We previously reported a comprehensive screen of the short isoform in a cohort of patients and found that 79% of the patients with identified USH2A mutations had only one mutant allele detected.[29] One purpose of this current study was to analyze the long isoform to

identify additional patients with mutations in this gene and to identify the second mutation in patients with one previously detected USH2A mutation.

MATERIALS AND METHODS

Patients

This study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Boards of the Massachusetts Eye and Ear Infirmary and Harvard Medical School. Informed consent was obtained from patients included in this study. Over 95% of the patients included in this study were Caucasian which reflects the patient base of the Berman-Gund Laboratory at Massachusetts Eye and Ear Infirmary. The clinical phenotypes of many of these patients have recently been described. [30]

We sequenced the coding exons specific to the long isoform of the *USH2A* gene (exons 22–72) in 108 patients diagnosed with Usher syndrome type II and in an additional 80 patients with non-syndromic retinitis pigmentosa. The diagnosis of Usher syndrome was made based on an ocular examination, electroretinography, and self-reported hearing loss. Hearing tests were not administered to most patients and therefore some of the patients diagnosed as having non-syndromic retinitis pigmentosa may in fact have some hearing deficit. Most (85%) of the patient samples did not yield data for at least one of the exons screened such that 4.7% of the 9776 exon fragments (188 samples $\times$ 52 amplicons) were not screened. Exons 1–21 of the *USH2A* gene were previously sequenced in this set of patients.[29] For select exons additional probands (beyond the 188 core set) were screened as part of the effort to identify more *USH2A* patients. The patients included in this study were also screened for mutations in the *USH3A* gene as part of a concurrent study in a larger set of patients.[31]

DNA sequencing

Oligonucleotide primers designed for each exon and 20 ng of patient DNA were used in the polymerase chain reaction to amplify each exon and nearby flanking intron sequence. Amplified DNA was treated with the enzyme ExoSAP-it (US Biochemical) to eliminate unincorporated PCR primers and dNTPs. The treated sample was then sequenced using the BigDye Terminator v3.1 cycle sequencing reaction and the ABI 3100 Genetic Analyzer (AppliedBiosystems). All sequence variants identified were confirmed by sequencing in both the sense and antisense direction.

Assessment of pathogenicity

For this study, we have classified all nonsense mutations, frameshift mutations, and mutations of the first two bases of canonical intron splice donor or acceptor sites as deleterious. Missense changes with an allele frequency $>4\%$ in patients and normal controls and isocoding changes that were not predicted to alter splicing were classified as benign. Rare missense changes were further analyzed using 4 different computer-generated algorithms (SIFT, PolyPhen, AGVGD, and pMut) designed to predict whether a variation is deleterious. The combined results of these computer analyses were used to develop a classification system for each missense change as follows. First, a score of 0 (benign), 0.5 (possibly deleterious), or 1 (probably deleterious) was assigned based on the results of each of the 4 tests. The scores for all 4 tests were then summed and missense variants with a summed score of 4.0 were classified as “likely deleterious”, those with a summed score of 3.0–3.5 were classified as “possibly deleterious”, those with a total score of 2.0–2.5 were classified as “unknown”, and those with scores <2.0 were classified as likely benign. In addition to the variants identified in the screen of exons 22–72, we used these 4 algorithms to reclassify the missense changes from exons 1–21 that were previously characterized as

variants of uncertain pathogenicity by our group.[29] A brief description of the 4 algorithms used in this analysis is below.

1. *SIFT*: The program, Sort Intolerant From Tolerant (SIFT) (available at <http://sift.jcvi.org>) relies on protein sequence alignments of multiple species and considers the conservation of the affected residue and the type of amino acid substitution to predict whether a change is innocuous or deleterious.[32,33] It has been predicted that limiting the alignment used for the SIFT the analysis to true orthologs may limit false negatives since paralogs may have slightly different sequences that account for their subtle functional differences.[32,34] We created a list of USH2A orthologs through the use of a BLAST search and an ortholog search in the ENSEMBL database and used CLUSTALW to create a homology alignment of the USH2A/ orthologs from human, chimpanzee, horse, dog, vampire bat, armadillo, sloth, opossum, chicken, zebra finch, platypus, zebra fish, stickleback, sea urchin, sea squirt, rat, mouse, and guinea pig. The CLUSTAL alignment was used for the SIFT analysis of the rare missense changes identified in this study and achieved the recommended level of diversity with a median conservation score of ≤ 3 . The output from the SIFT analysis is a binary classification of “tolerated” or “affect”. For our classification, a score of 0 was given for an output of “tolerated” and a score of 1 given for an output of “affect”.
2. *PolyPhen*: The polymorphism phenotyping program, PolyPhen (available at <http://genetics.bwh.harvard.edu/pph/>) is based on an empirical set of rules that combines analyses of a pre-built multiple protein sequence alignment with a number of protein structural and functional attributes to estimate the consequence of an amino acid substitution which is then characterized as benign, possibly deleterious, or deleterious.[35] We assigned a score of 0 to variations characterized as benign, a score of 0.5 for those characterized as possibly deleterious, and a score of 1.0 for those characterized as deleterious.
3. *Pmut*: Pmut (available at <http://mmb2.pcb.ub.es:8080/PMut/>) is a program that uses neural networks trained on known disease-causing human mutations and neutral mutations to provide the binary prediction of “neutral” or “pathologic”. [36] Although the web portal indicates the ability to insert one’s own alignment for analysis, we as well as another group[28] could not get this option to operate successfully so the default alignment was used. A score of 0 was assigned to variants classified as neutral and a score of 1.0 was given to those classified as pathologic for our classification system.
4. *Align-GVGD*: The program Align-GVGD (available at http://agvgd.iarc.fr/agvgd_input.php) combines an assessment of an alignment with the physicochemical characteristics of the amino acid to calculate the Grantham variation (GV) at each position and the distance the substituted base is from the range of variation (GD).[37] We used the same alignment of orthologs used with the SIFT program for this analysis. Based on the GV and GD measurements, a grade of C0-C65 is given where C0 is benign and C65 is most likely pathogenic. For our classification system, a score of 0 was given to grade C0; a score of 0.5 was given to grades C15, C25, or C35; and a score of 1.0 was given for a grade of C45, C55, or C65.

RESULTS

Mutation analyses

Analysis of the sequence of exons 22 through 72 identified 35 mutations interpreted as deleterious (Table 1, Fig. 1). Of these, 17 were nonsense mutations, 9 were frameshift mutations, 4 were mutations of canonical AG or GT splice acceptor or donor sites, 4 were small in-frame deletions or insertions, and one was a missense change, Glu1926Lys, predicted to alter a splice site (prediction score using splice-site prediction software http://www.fruitfly.org/seq_tools/splice.html was reduced from .54 to <.1). These mutations were deemed deleterious since they would substantially alter the encoded protein. Twenty-seven of these mutations were identified in only one patient each and the remaining 8 mutations were identified in 2–7 patients in our study. Twenty-seven of the mutations were novel.

We also identified 65 rare missense changes (<4% allele frequency) (Table 2), 17 of which have been previously reported. Most of the 65 changes were found in only one patient each and, therefore, analyzing ~100 normal controls for these mutations would be unlikely to provide a meaningful assessment of whether the change was disease-causing.

To help assess the possible deleterious effect of the rare missense changes, each was evaluated with the 4 different computer programs SIFT, PolyPhen, Pmut, and AGVGD. The outputs of these individual analyses are summarized in table 2 along with their summed scores (as described in the methods section) which were used to classify the variations. Using this scoring system, 8 missense changes (Cys2128Tyr, Cys2128Phe, Phe2786Ser, Cys3267Arg, Cys3358Tyr, Trp3521Arg, Gly4763Arg, and Cys4808Arg) had scores of 4.0 and were thus initially classified as “likely deleterious”. Two of these changes were previously reported as pathogenic variants (Cys3267Arg and Trp3521Arg).[22,23] The change Phe2786Ser was found in one patient who was later found to have a mutation in the USH3A gene and one patient who had two other clearly deleterious mutations in the USH2A gene, so it is unclear whether the variant Phe2786Ser contributes to Usher syndrome in these 2 patients. Nine missense changes (Pro1978Ser, Tyr1992Cys, Asp2237Tyr, Ser3384Tyr, Leu3606Pro, Asn4094Lys, Pro4269Arg, Pro4818Leu, and Leu4840Pro) had scores of 3.0–3.5 and were therefore classified as “possibly deleterious”. One of the “possibly deleterious” variants (Pro4818Leu) was previously described as pathogenic.[22] The deleterious effects of 12 missense changes were classified as “unknown” with scores of 2.0–2.5. One of these changes (Thr4337Met) was previously reported to be pathogenic[22] and 3 (Glu2238Ala, Glu3088Lys, and Pro3893Thr) were reported previously as nonpathogenic.[22,23] The remaining 36 rare missense variations had scores <2.0 and were classified as “likely benign”. One of these, Ile5126Thr, was recently reported as pathogenic[38] yet had a score of 0.0 using our rating system. The location of the missense changes found to be likely or possibly deleterious is illustrated in Fig. 1.

This same pathogenicity analysis was performed on the 11 missense variations previously reported by our group as being of uncertain pathogenicity.[29] Using this scoring system, none of the missense mutations from the screen of exons 1–21 could be classified as “likely deleterious” (i.e., all scores were < 4.0). The variants Arg464Cys and Arg517Thr were classified as “possibly deleterious” with a score of 3.0; the variants Ser307Ile, Ser391Ile, Gly516Val, Cys575Ser, and Pro1059Leu were classified as “unknown”; and Glu478Asp, Phe739Leu, Thr911Asn, and Leu1470Arg were classified as “likely benign”.

Finally, 33 sequence variants were deemed non-deleterious either because they were isocoding changes and would not be predicted to alter the encoded protein or were missense changes with an allele frequency > 4% and were previously described as polymorphisms in

the NCBI SNP database (Table 3). One notable non-deleterious change was Leu1572Phe. It was found with a high frequency among USH2 patients but was not found among the patients with non-syndromic RP. It was determined that most patients with the Leu1572Phe change also had the common USH2 mutation c.2299delG; Glu767fs, and Leu1572Phe is thus likely over-represented in the USH2 group due to linkage disequilibrium with that mutation.

Of the 108 patients originally diagnosed with USH2 who were included in the screen of the entire gene, 8 were found to carry deleterious mutations of the USH3A gene in a concurrent screen and were therefore re-categorized as USH3.[31] Combining the data from this study with the previous screen of exons 1–21, finds that 32 of the remaining 100 cases of USH2 have 2 clearly deleterious mutations and 25 have 1 clearly deleterious mutation of the USH2A gene. Therefore at least 57% of cases of USH2 carry at least one deleterious mutation of USH2A. If missense variations classified as either “likely deleterious” or “possibly deleterious” are in fact pathogenic mutations, then 37 patients would be counted as having 2 identified mutations and 26 with one mutation, for a total of 63% of patients having at least one detected USH2A mutation. If one assumes that patients with 2 mutations were all compound heterozygotes, then deleterious mutations were identified in 44.5% of the alleles screened. This number would increase to 50% of the alleles screened if all of the likely and possibly deleterious changes are included as mutations.

Among the 80 patients diagnosed with non-syndromic recessive retinitis pigmentosa, 3 had 2 clearly deleterious mutations and 12 had one deleterious mutation (19% total, including exons 1–21). By considering “likely deleterious” and “possibly deleterious” missense changes as mutations, the numbers change to 7 with 2 mutations and 11 with one mutation (23% total).

DISCUSSION

This study confirms earlier findings that mutations are distributed throughout the entire USH2A gene. The identification of 27 novel deleterious changes and 12 novel likely or possibly deleterious missense changes, each found in only one or a few patients, reinforces the idea that many different pathogenic USH2A mutations have arisen and most of which are confined to a few patients or small families. Our findings also corroborate earlier findings that Usher syndrome exhibits both nonallelic heterogeneity and extensive allelic heterogeneity. This intragenic heterogeneity presents difficulties for diagnostic screening of USH2A especially due to the large size of this gene. Efforts have been made recently to increase efficiency and reduce costs of mutation screening for known genes causing Usher syndrome by the development of genotyping microarrays using an arrayed primer extension method (APEX).[40] These arrays are only successful in identifying known mutations, and, since so many patients have unique mutations, they may fail to identify many USH2A mutations. These arrays can be used as a first quick screen of the gene but may require a follow-up detailed analysis of the entire gene in most patients.

We identified 65 rare missense variations most of which were novel to this set of patients. Functional analysis of such a large number of changes is not currently practical and analyses of normal controls will not provide statistically significant evidence for classifying many of them as pathogenic versus nonpathogenic. It is important to find an effective way to differentiate deleterious changes from benign ones. We developed a method of classifying these changes using the summed results of 4 different mutation assessment algorithms. This categorization system enabled us to tentatively define 8 of the 65 as “likely deleterious” and 9 as “possibly deleterious”. We must caution that the approach has not been validated for this gene and its specificity and sensitivity for categorizing missense changes are unknown.

However recent studies of cancer genes have showed that when the output of several of these algorithms are in agreement the predictive value increases to 88.1% compared to 72.9%–82% when taken alone.[34] Even with combining the outcomes of 4 algorithms, the predictive value is not 100% as is exemplified by one of the changes identified in this study. The novel change, Phe2786Ser was classified as “likely deleterious” using our system, yet it was unlikely to be a cause of Usher syndrome since it was found in one patient who had 2 clearly pathogenic mutations elsewhere in the USH2A gene and another patient who had a mutation in the USH3A gene. Authors of some of these algorithms have cautioned that the predictions yielded by these programs should not be used for diagnostic purposes;[33] instead, the programs may help to select mutations that could be the focus of further analysis.

As with almost all screens for mutations in humans, many mutations are overlooked because the assays do not include most of the intron sequences, the 5' and 3' untranslated regions, the promoter region, and distant enhancer sequences. In addition, the assays do not detect large deletions, insertions, or rearrangements, and the sequencing of some exons of some samples fails for obscure technical reasons. Thus, our results can provide only an estimate of the prevalence of USH2A mutations in patients. With this in mind, the USH2A gene appears to be the major cause of USH2 with at least one mutation found in 57–63% of USH2 cases (percentages calculated without and including probable and possibly deleterious changes). Furthermore, USH2A causes a substantial number of cases of nonsyndromic retinitis pigmentosa (19–23% of cases). However since audiograms were not obtained, some of these patients may have un-reported hearing loss and may in fact, be USH2 patients. Given that the recessive form of RP accounts for 50–60% all cases of RP, that Usher syndrome represents 20–40% of the recessive cases,[41] and that Usher type II represents 50–75% of all Usher cases,[1,3,4] mutations in the USH2A gene likely account for 12 to 25% of all cases of RP (lower prevalence among USH2 {i.e. $0.57 \times 0.5 \times 0.2 \times .5 = 2.9\%$ } + lower prevalence among ARRP {i.e. $0.19 \times 0.5 = 9.5\%$ } combined = 12% to higher prevalence among USH2 {i.e. $0.63 \times 0.6 \times 0.4 \times .75 = 11.3\%$ } + higher prevalence among ARRP {i.e. $0.23 \times 0.6 = 13.8\%$ } combined = 25%). Since rhodopsin mutations account for 10% of cases of RP and RPGR account for 8% of cases, mutations in USH2A appear to be the most common known cause of RP in the United States.

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Figure 1.

Schematic illustration of the USH2A gene and the encoded usherin protein. The location of all deleterious, probably deleterious, and possibly deleterious mutations found in our patients are indicated. The locations of amino acids altered by the missense mutations are also indicated on the schematic representation of the usherin protein.

Deleterious mutations identified in exons 22–72 of the USH2A gene and their distribution among patients with USH2 or recessive non-syndromic retinitis pigmentosa (ARRP).

Table 1

exon	Deleterious mutations identified in exons 22–72		protein change	distribution among all Usher type II patients screened			distribution among all ARRP patients screened		
	nucleotide change			homoz	hetero	wild type	homoz	hetero	wild type
22	c.4645C>T		Arg1549X[21]	0	4	206	0	0	145
22	c.4758+1G>A		n/a	0	0	210	0	1	144
26	c.5191_5192delAT		Met1731ValfsX8*	0	1	217	0	0	150
27	c.5514T>A		Tyr1838X[38]	0	1	194	0	0	139
28	c.5776G>A		Glu1926Lys**	0	1	216	0	0	149
28	c.5836C>T		Arg1946X	0	1	216	0	0	149
int28	c.5775+1G>A		n/a[23]	0	1	216	0	0	149
int29	c.5857+1G>C		n/a*	0	1	216	0	0	149
int29	c.5857+2T>C		n/a*	0	2	215	0	0	149
30	c.5933_5940delCTGTTGTC		Pro1978GlnfsX5*	0	1	207	0	0	141
32	c.6169C>T		Gln2057X*	0	1	192	0	0	130
32	c.6289_6302delATCTATTCAGGCAG		Ile2097IleX1	0	1	192	0	0	130
40	c.7493delG		Ser2498MetfsX30*	0	1	99	0	0	67
41	c.8167C>T		Arg2723X*	1	2	101	0	0	73
42	c.8557A>T		Arg2853X[23]	0	1	107	0	0	76
44	c.8740C>T		Arg2914X	0	1	75	0	0	57
44	c.8834G>A		Trp2945X*	0	1	75	0	0	57
47	c.9270C>A		Cys3090X	0	1	104	0	0	76
47	c.9336_9359delTTATACCAACACCCACAAATTCGTGG		Ile3113_Gly3120del	0	1	104	0	0	76
48	c.9424G>T		Gly3142X[21]	0	2	105	0	0	80
50	c.9787_9807delATTGGTGATTCTCTGCTGTGGC		Ile3263_Gly3269del	0	1	107	0	0	80
52	c.10190_10191delAA		Lys3397ArgfsX20*	0	1	107	0	0	80
59	c.11533C>T		Gln3845X*	1	0	80	0	0	53

exon	Deleterious mutations identified in exons 22-72		protein change	distribution among all Usher type II patients screened			distribution among all ARRP patients screened		
	nucleotide change			homoz	hetero	wild type	homoz	hetero	wild type
61	c.11864G>A		Trp3955X[25]	0	2	184	0	5	140
61	c.12006C>A		Tyr4002X	0	1	185	0	0	145
int61	c.12067-2A>G		n/a[39]	1	1	103	0	0	82
63	c.12490delC		His4164ThrfsX3	0	1	203	0	0	141
63	c.12954C>A		Tyr4318X	0	1	106	0	0	79
63	c.13335_13347delGAACATGGACTCTinsCTTG		Glu4445_Ser449del-insAspLeu	0	0	108	0	2	79
63	c.13499_13500insTACTCTCAC		Thr4500_Pro4501insThrLeuThr	0	1	107	0	0	80
64	c.14010_14062del		Glu4671AsnfsX4*	0	0	98	0	1	78
64	c.14131C>T		Gln4711X*	0	1	97	0	0	79
67	c.14787delA		Glu4930AsnfsX20	0	0	104	0	1	64
68	c.14803C>T		Arg4935X[21]	0	3	103	0	1	79
68	c.14879_c.14880delAAins45		Gln4960Leufs*	0	1	105	0	0	80

References for previously reported mutations are indicated in the column labeled "protein change".

* mutations for which the clinical findings of patients have recently been reported[30]

** a missense change that is predicted to destroy an intron splice site (splice site probability reduced from .54 to <.1- see results) and is thus classified as deleterious, Homoz = number of patients homozygous for rare allele, Hetero = number of patients heterozygous for the rare allele, Wild-type = number of patients homozygous for the wild type, or normal sequence at the respective position. Total numbers are not equal as some samples did not yield data for some exons and some exons were screened in additional sets of patients (see methods).

Table 2

Classification of rare missense variations identified in exons 22–72 of USH2A and their distribution among patients with USH2 or recessive non-syndromic retinitis pigmentosa (ARRP).

Rare missense changes			Protein prediction – deleterious effect					Distribution among USH2			distribution among ARRP		
exon	nucleotide change	protein change	Sift	PolyPhen	pMut	AGVGD	Score	homoz	Hetero	wild type	homoz	hetero	wild type
27	c.5506C>A	Pro1836Thr	tolerated/.17	poss/1.951	neutral/8	C0	.5	0	1	194	0	0	139
30	c.5858C>G	Ala1953Gly	affect/.05	poss/1.505	neutral/8	C0	1.5	0	1	207	0	1	140
30	c.5932C>T	Pro1978Ser	affect/0.0	prob/2.396	neutral/7	C65	3	0	0	208	0	1	140
30	c.5975A>G	Tyr1992Cys	affect/0.0	prob/2.758	path/6	C35	3.5	0	2	206	0	0	141
32	c.6240G>T	Lys2080Asn ^b [23]	tolerated/.1	benign	neutral/7	C0	0	0	1	192	0	1	129
33	c.6347A>G	His2116Arg	tolerated/.06	prob/2.413	neutral/3	C0	1	0	1	180	0	0	83
33	c.6383G>A	Cys2128Tyr	affect/0.0	prob/3.346	path/8	C65	4	0	1	180	0	0	83
33	c.6383G>T	Cys2128Phe	affect/0.0	prob/3.346	path/2	C65	4	0	1	180	0	0	83
34	c.6587G>C	Ser2196Thr	tolerated/.74	benign/.192	neutral/8	C0	0	0	2	202	0	2	136
35	c.6709G>T	Asp2237Tyr	affect/.01	prob/2.104	path/3	C15	3.5	0	0	108	0	1	77
35	c.6713A>C	Glu2238Ala ^b [22]	affect/.01	prob/2.059	neutral/7	C15	2.5	0	3	105	0	1	77
35	c.6778T>C	Ser2260Pro	affect/.01	poss/1.729	neutral/1	C0	1.5	0	1	107	0	0	78
36	c.6875 G>A	Arg2292His ^b [23]	tolerated/.65	prob/2.312	neutral/1	C0	1	0	7	176	0	3	79
41	c.7685T>C	Val2562Ala ^b [23]	tolerated/.26	benign/.496	neutral/7	C0	0	0	1	102	0	4	69
41	c.7718G>A	Arg2573His	tolerated/.24	benign/.95	neutral/2	C0	0	0	0	104	0	1	72
41	c.7915T>C	Ser2639Pro	affect/.01	poss/1.71	neutral/0	C0	1.5	0	2	102	0	0	73
42	c.8357T>C	Phe2786Ser	affect/0.0	prob/2.208	path/1	C55	4 [†]	0	2	106	0	0	76
43	c.8624G>A	Arg2875Gln ^b [22]	affect/.03	benign/0.0	neutral/1	C0	1	0	4	89	0	6	69
43	c.8656C>T	Leu2886Phe ^b [22]	tolerated/1.0	poss/1.96	neutral/7	C0	0.5	0	8	85	0	4	71
44	c.8790T>G	Asn2930Lys	tolerated/.13	benign/1.41	neutral/4	C0	0	0	0	76	0	2	55
47	c.9262G>A	Glu3088Lys ^b [23]	affect/0.0	poss/1.609	neutral/4	C55	2.5	0	2	103	0	1	75
47	c.9343A>G	Thr3115Ala ^b [23]	tolerated/1.0	benign/.238	neutral/7	C0	0	0	9	96	0	4	72

Rare missense changes			Protein prediction – deleterious effect							Distribution among USH2				distribution among ARRP			
exon	nucleotide change	protein change	Sift	PolyPhen	pMut	AGVGD	Score	homoz	Hetero	wild type	homoz	hetero	wild type				
49	c.9595A>G	Asn3199Asp ^b [23]	tolerated/1.0	benign/.421	neutral/9	C0	0	0	9	94	0	3	70				
50	c.9799T>C	Cys3267Arg ^d [22]	affect/0.0	prob/3.571	path/5	C65	4	0	1	107	0	0	80				
51	c.10073G>A	Cys3358Tyr	affect/0.0	prob/3.346	path/8	C65	4	0	0	84	0	2	74				
51	c.10151C>A	Ser3384Tyr	affect/.00	poss/1.954	path/5	C15	3	0	0	84	0	1	75				
52	c.10342G>A	Glu3448Lys	tolerated/.21	poss/1.52	neutral/5	C0	0.5	0	1	107	0	0	80				
52	c.10385C>T	Thr3462Ile	tolerated/.59	benign/.22	path/1	C0	1	0	1	107	0	0	80				
53	c.10437G>T	Trp3479Cys	tolerated/.07	prob/3.308	path/5	C15	2.5	0	1	103	0	0	78				
53	c.10561T>C	Trp3521Arg ^d [23]	affect/0.0	prob/3.902	path/8	C65	4	0	3	101	0	0	78				
53	c.10575G>A	Gly3529Ser	affect/0.0	poss/1.824	neutral/5	C55	2.5	0	1	103	0	0	78				
55	c.10817T>C	Leu3606Pro	affect/.0	prob/2.076	path/1	C25	3.5	0	0	99	0	1	72				
55	c.10852G>A	Gly3618Ser	affect/.03	poss/1.555	neutral/6	C0	0	0	0	99	0	1	72				
57	c.11156G>A	Arg3719His	affect/.0	poss/1.979	neutral/3	C25	2	0	0	99	0	1	70				
59	c.11504C>T	Thr3835Ile ^b [23]	tolerated/.1	poss/1.567	path/0	C0	0	5	1	74	1	2	50				
59	c.11532A>G	Ile3844Met	affect/.01	benign/1.425	neutral/9	C0	1	1	0	80	0	0	53				
60	c.11677C>A	Pro3893Thr ^b [23]	affect/0.0	prob/2.396	neutral/6	C35	2.5	0	5	102	1	2	77				
60	c.11711G>A	Arg3904Lys	affect/0.0	poss/1.529	neutral/9	C25	2	0	1	106	0	0	80				
62	c.12282T>G	Asn4094Lys	affect/0.0	prob/2.063	neutral/6	C65	3	0	0	105	0	1	81				
63	c.12522C>G	Ser4174Arg	tolerated/.53	benign/.063	path/0	C0	1	0	1	203	0	0	141				
63	c.12575G>A	Arg4192His	tolerated/.23	benign/.424	neutral/2	C0	0	0	0	204	1	1	139				
63	c.12742C>A	His4248Asn	tolerated/	prob/2.073	neutral/8	C0	1	0	0	204	0	1	140				
63	c.12806C>G	Pro4269Arg	affect/0.0	prob/2.621	path0	C65	3	1	0	106	0	0	79				
63	c.13010C>T	Thr4337Met ^d [22]	affect/0.0	poss/1.835	neutral/1	C15	2	0	1	106	0	0	79				
63	c.13297G>T	Val4433Leu	tolerated/.30	benign/.969	neutral/6	C0	0	0	3	104	0	2	77				
63	c.13339A>G	Met4447Val	affect/.02	prob/2.266	neutral/5	C0	2	0	0	108	0	1	79				
63	c.13709G>A	Arg4570His	tolerated/.20	benign/.32	neutral/0	C0	0	0	1	107	0	1	79				
64	c.13984C>G	Gln4662Glu	Tolerated/.36	poss/1.546	neutral/9	C0	0.5	0	1	97	0	0	79				

Rare missense changes			Protein prediction – deleterious effect							Distribution among USH2			distribution among ARRP		
exon	nucleotide change	protein change	Sift	PolyPhen	pMut	AGVGD	Score	homoz	Hetero	wild type	homoz	hetero	wild type		
64	c.14074G>A	Gly4692Arg	affect/.02	poss/1.913	path/2	C0	2.5	0	1	97	0	0	79		
65	c.14287G>A	Gly4763Arg	affect/.00	prob/2.274	path/3	C65	4	0	1	107	0	1	79		
65	c.14333C>A	Ala4778Asp	tolerated/.07	benign/1.237	neutral/0	C0	0	0	1	107	0	0	80		
66	c.14422T>C	Cys4808Arg	affect/0.0	prob/3.571	path/6	C65	4	0	1	137	0	0	114		
66	c.14449G>A	Gly4817Arg	affect/.00	poss/1.198	path/3	C25	3.5	0	1	137	0	0	114		
66	c.14453C>T	Pro4818Leu ^{d[22]}	affect/.02	prob/2.567	path/5	C0	3	0	1	137	0	0	114		
66	c.14513G>A	Gly4838Glu	tolerated/1.0	benign/.773	path/2	C0	0	0	0	138	0	1	113		
66	c.14519T>C	Leu4840Pro	affect/02	poss-1.58	path/0	C0	2.5	0	0	138	0	1	113		
66	c.14531C>T	Thr4844Met	tolerated/.08	benign/.34	neutral/1	C0	0	0	0	138	0	1	113		
66	c.14543G>A	Arg4848Gln	tolerated/1.0	benign/.396	neutral/3	C0	0	0	0	138	0	2	112		
67	c.14753C>T	Thr4918Met	affect/.01	benign/1.421	path/1	C0	2	0	1	103	0	0	65		
70	c.15076A>G	Lys5026Glu	tolerated/1.0	benign/1.382	neutral/4	C0	0	0	0	108	0	2	78		
70	c.15091C>T	Arg5031Trp ^{b[23]}	tolerated/.07	benign/.298	path/9	C0	1	0	1	107	1	3	76		
71	c.15377T>C	Ile5126Thr[38]	tolerated/.09	benign/1.434	neutral/5	C0	0	0	3	100	0	1	80		
71	c.15428G>A	Arg5143His	affect/.03	poss/1.707	neutral/1	C0	1.5	0	0	103	0	2	79		
71	c.15433G>A	Val5145Ile	tolerated/.58	benign/.992	neutral/9	C0	0	0	0	103	0	1	80		
72	c.15562A>G	Ser5188Gly	tolerated/.46	poss/1.636	neutral/7	C0	0.5	0	0	107	0	2	76		

The output from 4 different sequence analysis programs used to predict pathogenicity are shown along with a summed score. Variations with a score of 4.0 were classified as “likely deleterious”, those with a score of 3.0–3.5 were classified as “possibly deleterious”, those with a score of 2.0–2.5 were classified as “unknown”, and those with a score <2.0 were classified as “benign”. References of previously published changes are indicated in the column labeled “protein change”.

^a previously reported as pathogenic

^b previously reported as non-pathogenic

^c patient with this change already has two other clearly pathogenic mutations in USH2A

^d patient with this change has a deleterious mutation in USH3A gene, Homoz = number of patients homozygous for rare allele, Hetero = number of patients heterozygous for the rare allele, Wild-type = number of patients homozygous for the wild type sequence at the respective position. Total numbers are not equal as some samples did not yield data for some exons and some exons were screened in additional sets of patients (see methods).

Table 3

Isocoding changes and missense polymorphisms found in the coding region of exons 22–72 of the USH2A gene and their distribution among patients with USH2 or recessive non-syndromic retinitis pigmentosa.

exon	nucleotide change	protein change	distribution among all Usher type II patients screened			distribution among all ARRP patients screened		
			homoz	hetero	wild type	homoz	hetero	wild type
22	c.4714 C>T	Leu1572Phe	2	38	170	0	0	145
25	c.4994T>C	Ile1665Thr	2	24	68	3	18	52
25	c.5013C>A	Gly1671Gly	5	35	54	4	16	53
27	c.5367A>G	Leu1789Leu	0	1	165	0	0	140
27	c.5409C>G	Val1803Val	0	1	165	0	1	139
28	c.2751C>T	Tyr1917Tyr	0	1	216	0	0	149
31	c.6141 G>A	Leu2047Leu	0	0	180	0	0	151
32	c.6186A>C	Pro2062Pro	0	0	193	0	1	129
32	c.6270 G>A	Leu2090Leu	0	1	192	0	0	130
32	c.6317C>T	Thr2106Ile	22	68	103	15	55	60
33	c.6369 C>T	Cys2123Cys	0	0	181	0	3	80
34	c.6506T>C	Ile2169Thr	51	95	58	35	66	37
38	c.7131C>T	Asn2377Asn	0	1	208	0	0	142
40	c.7506G>A	Pro2502Pro	0	14	86	0	5	62
45	c.8937 A>G	Val2979Val	0	0	106	0	1	79
46	c.9213 G>A	Ser3071Ser	1	1	179	0	0	82
47	c.9296 A>G	Asn3099Ser	0	6	99	0	12	64
48	c.9430G>A	Asp3144Asn	0	6	99	0	11	69
52	c.10232 A>C	Glu3411Ala	28	51	29	18	43	19
55	c.10836 C>A	Val3612Val	0	0	99	0	1	72
59	c.11520T>C	Tyr3840Tyr	0	1	80	0	0	53
60	c.11602A>G	Met3868Val	11	25	71	3	22	55
61	c.11736G>A	Glu3912Glu	0	0	186	0	2	143
61	c.11907 A>T	Pro3969Pro	0	1	185	0	2	143

exon	nucleotide change	protein change	distribution among all Usher type II patients screened			distribution among all ARRP patients screened		
			homoz	hetero	wild type	homoz	hetero	wild type
61	c.11946G>A	Leu3982Leu	9	23	154	4	22	119
62	c.12093C>T	Tyr4031Tyr	1	2	105	0	0	80
63	c.12597 T>C	Ala4199Ala	0	0	204	0	1	140
63	c.12612A>G	Thr4204Thr	27	56	121	12	38	91
63	c.12666A>G	Thr4222Thr	21	97	86	23	55	63
63	c.13191G>A	Glu4397Glu	6	24	77	6	17	56
63	c.13440 G>A	Arg4480Arg	0	2	103	0	1	81
66	c.14481C>T	Ala4827Ala	0	1	137	0	1	113
72	c.15522 T>C	Tyr5174Tyr	0	0	107	0	2	76

The change Leu1572Phe was found to be in linkage disequilibrium with the common USH2 mutation c.2299delG; Glu767fs and is thus over-represented in the USH2 group. Homoz = number of patients homozygous for rare allele, Hetero = number of patients heterozygous for the rare allele, Wild-type = number of patients homozygous for the wild type, or normal sequence at the respective position. Total numbers are not equal as some samples did not yield data for some exons and some exons were screened in additional sets of patients (see methods).