Confirmation of the Role of DHX38 in the Etiology of Early-Onset Retinitis Pigmentosa

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PURPOSE. Retinitis pigmentosa (RP) is a genetically heterogeneous trait with autosomal-recessive (ar) inheritance underlying 50% of genetic disease cases. Sixty-one arRP genes have been identified, and recently, DHX38 has been reported as a potential candidate gene for arRP with only a single family reported with a variant of unknown significance. We identified a missense variant in DHX38 that co-segregates with the arRP phenotype in two Pakistani families confirming the involvement of DHX38 in the etiology of early-onset RP.

METHODS. Exome sequencing was performed using two DNA samples from affected members of Pakistani families (MA88 and MA157) with early onset arRP. Sanger sequencing of DNA samples from all family members confirmed the segregation of candidate variant within both families.

RESULTS. A novel missense DHX38 variant c.971G>A; p.(Arg324Gln) was identified which segregates with the arRP phenotype and yielded a logarithm of the odds (LOD) score of 5.0 and 4.3 for families MA88 and MA157, respectively. This variant is predicted to be conserved and deleterious by several bioinformatics tools.

CONCLUSIONS. We identified a second deleterious DHX38 variant that segregates with arRP in two families, providing additional evidence that DHX38 is involved in RP etiology. DHX38 encodes for pre-mRNA splicing factor PRP16, which is important in catalyzing pre-mRNA splicing.

Keywords: DHX38, early onset autosomal recessive retinitis pigmentosa, cataract, exome sequencing, linkage analysis, mendelian disease, retinal dystrophy
We identified a second missense \textit{DHX38} variant, c.971G>A; p.(Arg324Gln), through genome-wide genotyping, homozygosity mapping, linkage analysis, and exome and Sanger sequencing using DNA samples from two arRP consanguineous Pakistani families (MA88 and MA157). This conserved and deleterious missense variant segregated with the arRP phenotype and produced LOD scores of 5.0 and 4.3 for families MA88 and MA157, respectively, when parametric linkage analysis was performed.

**MATERIALS AND METHODS**

**Patients**

The institutional review boards of the Quaid-i-Azam University and the Baylor College of Medicine and Affiliated Hospitals approved the study. The study protocol was conducted according to the Tenets of the Declaration of Helsinki. Written informed consent was obtained from all participating members of the MA88 and MA157 families affected with arRP that were ascertained from two different villages of Bagh district in Azad Kashmir of Pakistan.

Family history and clinical information were obtained from both MA88 and MA157 family members (Fig. A and Table). Fundoscopy was performed for every affected family member who participated in the study. Peripheral blood samples from pedigrees MA88 and study from MA157 and unaffected family member (MA157-6; Figs. A–C). Vision tests and examinations were also performed on unaffected members from both families, MA88-2, MA88-7, MA88-10, MA157-2, and MA157-6. Peripheral blood samples were collected from MA88 (N = 11) and MA157 (N = 8) family members. DNA was isolated from the collected blood samples using a standard phenol-chloroform extraction protocol.

**Homozygosity Mapping and Linkage Analysis**

Genome-wide genotyping using the Infinium HumanCoreExome Chip (Illumina, San Diego, CA, USA), which contains approximately 550-K single-nucleotide polymorphism (SNP) markers with a mean intermarker distance of 5.5 kb, was performed using 10 DNA samples (4 affected and 6 unaffected) from MA88 family. One additional affected family member (MA88-5) was collected after genome-wide genotyping was completed. The SNP genotype data were analyzed using homozygosity mapping and linkage analysis. Homozygosity mapping was performed using affected and unaffected family members using HomozygosityMapper. Two-point and multipoint linkage analyses were performed using Superlink assuming a completely penetrant autosomal recessive mode of inheritance with a disease allele frequency of 5.0 \( \times 10^{-6} \). Marker allele frequencies were estimated from founders and reconstructed founder genotypes from pedigree MA88 and other Pakistani pedigrees, which were genotyped at the same time. To obtain genetic map distances, for multipoint linkage analysis, interpolation was performed using the Rutgers-Combined Linkage maps. For family MA157 whole-genome genotyping was not performed, but an affected family member was selected for exome sequencing. For both families two-point linkage analysis was performed for the \textit{DHX38} variant, c.971G>A; p.(Arg324Gln) by analyzing data from the 11 and eight members from family MA88 and MA157, respectively, using a minor allele frequency of 5.0 \( \times 10^{-5} \) for the alternative allele of c.971G>A.

**Exome Sequencing, Filtering, and Analysis**

DNA samples from one affected male from each family (MA88-3 and MA157-7) were selected for exome sequencing. Sequence capture was performed using the Roche NimbleGen SeqCap EZ Human Exome Library v.2.0 (~37-Mb target, Basel, Switzerland). Sequencing to a median read depth of 77x for MA88-3 and 90x for MA157 was performed on a HiSeq4000 (Illumina). Fastq files were aligned to the hg19 human reference sequence using Burrows-Wheeler Aligner (BWA) to generate demultiplexed BAM file. Variant detection and calling were performed using the Genome Analysis Toolkit (GATK). The variant call file (VCF) was annotated using ANNOVAR and Variant Effect Predictor (VEP). Variant filtering was performed using Gemini, prioritizing variants with a minor allele frequency less than 0.001 in every ancestry group within Genome Aggregation Database (gnomAD) and are also predicted to be deleterious and conserved by multiple bioinformatics tools (Polyphen2, Harvard Medical School, Boston, MA, USA; Provian, J. Craig Venter Institute, La Jolla, CA, USA; SIFT, Fred Hutchinson Cancer Research Center, Seattle, WA, USA; Mutation taster, Universitätsmedizin, Berlin, Germany; GERP++) RS, Stanford University, Stanford, CA, USA; and phyloP, Universitätsmedizin, Berlin, Germany). Variants that lie within regions of linkage and homozygosity mapped in MA88 family were prioritized. The exome data from MA88-3 and MA157-7 were also analyzed by examining all variants regardless of frequency within and surrounding the 16q22 region, which contains \textit{DHX38}, to determine if they shared a haplotype in common.

**Segregation Validation by Sanger Sequencing**

To verify co-segregation of \textit{DHX38} variant c.971G>A; p.(Arg324Gln) in families MA88 and MA157, forward and reverse PCR primers were designed. Standard conditions were used to perform PCR reactions. The amplified PCR products were treated with ExoSAP-IT PCR Product Cleanup Reagent (ThermoFisher Scientific, Sugar Land, TX, USA). Sequencing was run on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye-Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sanger sequencing was performed using available DNA samples from families MA88 (N = 11) and MA157 (N = 8).

**RESULTS**

**Clinical Findings**

Members from both arRP families developed night blindness between 3 (MA88-5 and MA88-11) and 4 (MA88-3, MA88-4, MA88-8, MA157-3, MA157-4, MA157-5, MA157-7, and MA157-8) years of age and developed complete blindness between the ages of 7 and 8 (Table). Currently, all affected family members are older than 20 years of age. Affected members of MA88 are blind but retain light perception and affected MA157 members are blind with no light perception. The three affected males from family MA88 were diagnosed with bilateral cataracts at 6 years of age. Affected female family member MA88-8, displayed cataracts at 19 years of age; however, her affected female cousin MA88-11, did not have any sign of cataracts at 20 years of age. All affected MA157 family members (4 males and 1 female) started developing cataracts at approximately 10 years of age. For the three affected females (MA88-8, MA88-11, and MA157-8) fundoscopy displayed macular atrophy, attenuation of arteries, and clustered areas of intraretinal pigment on the periphery. Fundoscopy was also performed on the affected males from both families (MA88-3, MA88-4, MA88-5, and MA157-7) but due to bilateral cataracts it was not possible to view the retina, optic disc, or macula. Fundoscopy performed on the unaffected MA157-6 at 21 years of age showed a healthy
Genetic and clinical findings for arRP Pakistani families, MA88 and MA157. All individuals from family MA88 with a DNA sample underwent whole-genome genotyping except MA88-5 who was ascertained after genotyping was completed. MA88-3 and MA157-7 were selected to undergo exome sequencing. (A) Fundoscopy images obtained from MA88-11 (20 years old), MA88-8 (19 years old), and MA157-8 (17 years old) display clustered areas of intraretinal pigment on the periphery, macular-atrophy, and attenuation of arteries, while fundoscopy for unaffected MA157-6 (21 years old) was normal. Fundoscopy images for the males who all had bilateral cataracts are not displayed, because it was not possible to view the retina, optic disc, or macula. Pedigree drawing (B) of family MA88 and (C) family MA157. Squares represent males and circles females with filled
The DHX38 c.971G>A variant found in family MA88 and MA157 was previously reported in gnomAD at a very low frequency \(4.1 \times 10^{-9}\), with only one alternative allele that has been reported (Latino population) in a total of 246,008 haplotypes. This variant has a CADD C-score\(^1\) of 34 and was predicted as probably damaging and deleterious by several additional bioinformatic tools (Polyphen2 HVAR score 0.999, PROVEAN score = -3.78, SIFT score = 0.01, Mutation Taster = disease). The amino acid residue is also highly conserved in 95 vertebrate species (GERP\(^+\) RS score = 4.69 and phyloP score = 9.24).

**DISCUSSION**

In this report, we identified a novel missense variant c.971G>A: p.(Arg324Gln) within DHX38 that segregates within two Pakistani families MA88 and MA157 with early-onset arRP. The novel arRP missense variant was identified using genome-wide genotyping, linkage analysis, homozygosity mapping, and exome and Sanger sequencing using DNA exome data from individual 3 (MA88) contained 25,961 variants with alternative alleles in 12,502 genes and the exome data from individual 7 (MA157) had 32,462 variants with alternative alleles in 12,619 genes.

**Filtering and Segregation Analysis**

The DHX38 c.971G>A variant mapped to the 16q21-q23.1, region of linkage and homozygosity, which was identified by analyzing family MA88 genotyping data. Additionally, of the candidate variants identified for families MA88 and MA157 only the DHX38 NM_014003.3: g.72133641G\(>\)A; p.(Arg324Gln) variant, which is in exon 8 was found in both exomes. Furthermore, the DHX38 variant had previously been reported to be a gene of uncertain significance for arRP. To ensure that the variant within this gene was not a sequencing or an alignment error, the respective genomic region was examined in the exome data from MA88-3 and MA157-7 individuals by using the Integrative Genome Viewer (IGV).\(^{15}\) The variant had a high reads quality in both aligned exome data. Sanger sequencing was used to further validate the variant and established that it co-segregated with the arRP phenotype for both families MA88 and MA157. Affected arRP individuals are homozygous for the alternative allele and unaffected family members either heterozygous or wild-type genotype (Figs. B, C).

Analysis of single nucleotide variations (SNVs), using the exome data from MA88-3 and MA157-7, surrounding the causal variant DHX38 chr16:72133641G\(>\)A identified a 15.7-Mb (based on 96 SNVs; chr16: 65038674-80718879) shared haplotype. The size of the haplotype is a strong indication that families MA88 and MA157 likely have a quite recent common ancestor.

**Homozygosity Mapping and Linkage Analysis**

Analysis of the genotype array data for the four MA88 family members revealed a homozygous region of 10.76 Mb flanked by rs12931803 and rs17679567 (chr16: 66122316-7689012) on chromosome 16q21-q23.1. Multipoint linkage analysis with the markers in this region produced a LOD score of 4.8. Two-point linkage analysis with the DHX38 variant, c.971G\(>\)A; p.(Arg324Gln) produced a LOD score of 5.0 and 4.3 at theta = 0 for MA88 and MA157 families, respectively.

**Bioinformatic Evaluation of DHX38 c.971G>A: p.(Arg324Gln)**

The DHX38 c.971G>A: p.(Arg324Gln) variant found in family MA88 was reported in gnomAD at a very low frequency \(4.1 \times 10^{-9}\), with only one alternative allele that has been reported (Latino population) in a total of 246,008 haplotypes. This variant has a CADD C-score\(^1\) of 34 and was predicted as probably damaging and deleterious by several additional bioinformatic tools (Polyphen2 HVAR score = 0.999, PROVEAN score = -3.78, SIFT score = 0.01, Mutation Taster = disease). The amino acid residue is also highly conserved in 95 vertebrate species (GERP\(+\) RS score = 4.69 and phyloP score = 9.24).

**Table.** Phenotype Details and the Age of Onset for the Affected Family Members From MA88 and MA157

| Family Members | Sex | Night Blindness Onset Age | Complete Blindness Age of Onset | Cataract Onset Age | Cataract Age at Last Exam | Current Cataract Status | Fundoscopy |
|----------------|-----|---------------------------|---------------------------------|-------------------|--------------------------|------------------------|------------|
| MA88-3         | Male| 4                         | 7-8                             | 6                 | 25                       | Bilateral/dense        | Uninformative* |
| MA88-4         | Male| 4                         | 7-8                             | 6                 | 27                       | Bilateral/dense        | Uninformative* |
| MA88-5         | Male| 3                         | 7-8                             | 6                 | 29                       | Bilateral/dense        | Uninformative* |
| MA88-8         | Female| 4                       | 7-8                             | 19                | 19                       | Mild                   | Reported†    |
| MA88-11        | Female| 3                       | 7-8                             | No                | 20                       | No                     | Reported†    |
| MA157-3        | Male| 4                         | 7-8                             | 10                | 28                       | Bilateral/dense        | Uninformative* |
| MA157-4        | Male| 4                         | 7-8                             | 10                | 27                       | Bilateral/dense        | Uninformative* |
| MA157-5        | Male| 4                         | 7-8                             | 10                | 22                       | Bilateral/dense        | Uninformative* |
| MA157-7        | Male| 4                         | 7-8                             | No                | 25                       | Bilateral/dense        | Uninformative* |
| MA157-8        | Female| 4                       | 7-8                             | No                | 17                       | Mild                   | Reported†    |

*LP, Light perception.

  * Uninformative fundoscopy due to cataracts.

† Figure A.
samples from family MA88 and by exome and Sanger sequencing of DNA samples from MA157. In silico prediction tools indicate that this missense variant is likely pathogenic. This variant segregates perfectly with the arRP phenotype in families MA88 and MA157 and was not reported in 30,766 controls or one million individuals in gnomAD. This is the second arRP variant reported in DHX38, which is classified as likely pathogenic, suggesting that DHX38 is a novel gene underlying severe early-onset arRP.

DHX38 encodes PRP16, which belongs to the subfamily of DEAH box proteins. DEAH box proteins are involved in a diversity of cellular processes essential in the modification of RNA secondary structure, pre-mRNA splicing, and spliceosome assembly. The PRP16 is an RNA-dependent ATPase that interacts with spliceosome during the second splicing step. The cryo-EM structure of the spliceosome after the first catalytic step reveals a restructurting of several RNA and protein domains mediated by PRP16. The movement of the branched intron away from the catalytic center is initiated by PRP16. This conformational rearrangement, induced by the ATPase activity, may influence the conformational rearrangement of the spliceosome. Although PRP16 is suggested to be crucial in the fidelity of splicing, DHX38 encodes PRP16, which belongs to the subfamily of DEAH box proteins. DEAH box proteins are involved in a diversity of cellular processes essential in the modification of RNA secondary structure, pre-mRNA splicing, and spliceosome assembly. The PRP16 is an RNA-dependent ATPase that interacts with spliceosome during the second splicing step. The cryo-EM structure of the spliceosome after the first catalytic step reveals a restructurering of several RNA and protein domains mediated by PRP16. The movement of the branched intron away from the catalytic center is initiated by PRP16. This conformational rearrangement, induced by the ATPase activity, may influence the conformational rearrangement of the spliceosome. Although PRP16 is suggested to be crucial in the fidelity of splicing, this variant segregates perfectly with the arRP phenotype in two Pakistani families. The arRP families provide additional evidence for the involvement of DHX38 in arRP. Further genetic, functional, and structural studies are needed to reveal the function of the N-terminus region of PRP16 protein and its implication in excising the lariat intron and joining the exons during the second step of the splicing process.

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APPENDIX

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