Identification of an **HMGB3** Frameshift Mutation in a Family With an X-linked Colobomatous Microphthalmia Syndrome Using Whole-Genome and X-Exome Sequencing

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**IMPORTANCE** Microphthalmias are rare disorders whose genetic bases are not fully understood. **HMGB3** is a new candidate gene for X-linked forms of this disease.

**OBJECTIVE** To identify the causative gene in a pedigree with an X-linked colobomatous microphthalmos phenotype.

**DESIGN, SETTING, AND PARTICIPANTS** Whole-genome sequencing and chromosome X-exome–targeted sequencing were performed at the High Throughput Sequencing Laboratory of the Genetic Resources Core Facility at the Johns Hopkins University School of Medicine on the DNA of the male proband and informatically filtered to identify rare variants. Polymerase chain reaction and Sanger sequencing were used to confirm the variant in the proband and the carrier status of his mother. Thirteen unrelated male patients with a similar phenotype were also screened.

**MAIN OUTCOMES AND MEASURES** Whole-genome and X-exome sequencing to identify a frameshift variant in **HMGB3**.

**RESULTS** A 2–base pair frameshift insertion (c.477_478insTA, coding for p.Lys161Ilefs*54) in the **HMGB3** gene was found in the proband and his carrier mother but not in the unrelated patients. The mutation, confirmed by 3 orthogonal methods, alters an evolutionarily conserved region of the **HMGB3** protein from a negatively charged polyglutamic acid tract to a positively charged arginine-rich motif that is likely to interfere with normal protein function.

**CONCLUSIONS AND RELEVANCE** In this family, microphthalmia, microcephaly, intellectual disability, and short stature are associated with a mutation on the X chromosome in the **HMGB3** gene. **HMGB3** should be considered when performing genetic studies of patients with similar phenotypes.

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Microphthalmia can be classified into 2 major forms. In simple microphthalmia, the eye is anatomically normal except for decreased axial length. Complex microphthalmia is characterized by short axial length and anterior and/or posterior segment dysgenesis. Microphthalmia is a heterogeneous condition and both forms may be sporadic or inherited. Microphthalmia may be isolated or occur as one of several other features in various syndromes. The prevalence of microphthalmia has been estimated at 0.2 to 3.0 per 10,000 births.1–2 There are at least 6 X-linked loci in the Online Mendelian Inheritance in Man database (http://omim.org) that include microphthalmia in the phenotype description.

In 1971, Goldberg and McKusick3 reported a kindred from Maine with X-linked microphthalmos, microcephaly, intellectual disability, and kyphoscoliosis similar to the phenotype (MCOPS1 [OMIM 309800]) first described by Lenz4 but lacking digit or urogenital abnormalities. Forrester et al5 mapped the MCOPS1 locus to Xq27-28 in a different family described as having Lenz microphthalmia, and Esmaeilpour et al6 identified the mutation in the NAA10 gene (OMIM 300013). We were able to study the original family described by Goldberg and McKusick7 and identify the genetic basis as an X-linked gene not previously associated with this phenotype. The proband’s DNA was analyzed using 2 independent next-generation sequencing technologies, whole-genome sequencing and exome capture of X-linked genes followed by next-generation sequencing. Confirmatory polymerase chain reaction (PCR) and Sanger sequencing were performed on the proband and his mother. In addition, PCR sequencing was used to evaluate the identified candidate gene in unrelated male patients with a similar phenotype.

Methods

Patients

The Johns Hopkins University School of Medicine Institutional Review Board approved this study. Written informed consent was provided for genetic studies from the family described by Goldberg and McKusick.3 DNA was isolated (Qiagen) from Scope mouthwash collections obtained from the proband and his mother. In addition, DNA was also obtained from 13 unrelated male patients with undiagnosed syndromes of microphthalmia, intellectual disability, and/or additional malformations. Six had colobomatous microphthalmia, with 4 also having intellectual disability. Two patients had bilateral microphthalmia with intellectual disability. Two had microphthalmia and congenital cataracts, 1 of whom also had intellectual disability. One patient each had sclerocornea, bilateral anterior segment dysgenesis, or bilateral anophthalmia. Other malformations included congenital heart or kidney disease in 5 patients and cleft palate and thumb anomalies in 1 patient.

Sequencing

Whole-genome sequencing was performed by Complete Genomics Inc using 10 μg of DNA from the proband. X-exome sequencing was performed by the High Throughput Sequencing Laboratory of the Genetic Resources Core Facility (http://grcf.jhmi.edu) at the Johns Hopkins University School of Medicine. A total of 3 μg of DNA was used to create an Illumina library using standard methods. Genomic DNA was sheared to a size of 150 to 200 base pairs (bp) using a Covaris E210 system (Covaris Inc). End repair and addition of an overhanging A base were performed with an NEBNext DNA Laboratory prep kit (New England Biolabs). DNA fragments were ligated to library adapters (Illumina). The ligated fragments were then size-selected through purification using solid-phase reversible immobilization beads and PCR amplified to prepare the libraries. The Bioanalyzer DNA1000 kit (Agilent Technologies) was used for quality control of the libraries to ensure adequate concentration and appropriate fragment size. Target capture was performed using the SureSelect X-exome demo kit (Agilent Technologies). Under standard procedures, biotinylated RNA oligonucleotides were hybridized with 500 ng of the library. Magnetic bead selection was used to capture the resulting RNA-DNA hybrids. RNA baits were removed with ribonuclease and the remaining DNA products PCR amplified. Sample indexing was introduced at this step. The High Sensitivity DNA Analysis kit (Agilent Technologies) was used for quality control of adequate fragment sizing and quantity of DNA capture.

Paired-end 100-bp reads were produced on an Illumina Genome Analyzer IIx. Raw data were processed through Illumina software, generating base calls and corresponding base-call quality scores. These data were then processed with CIDsRSeqSuite 2.0 software,7 an analysis suite that uses open-source programs. Reads were aligned to hg19 using the Burrows-Wheeler Aligner (BWA), resulting in a binary alignment map (BAM) file. Postprocessing of the aligned data included flagging of molecular and optical duplicates using Picard (http://sourceforge.net/projects/picard/), and local realignment and base-call quality score recalibration using GATK 1.6.8 Variant calling was performed by SAMtools (http://samtools.sourceforge.net) and output in VCF format v4.0 (http://www.1000genomes.org/node/101). Complete Genomics variant files were converted to VCF format using Complete Genomics Analysis Tools (http://cgatools.sourceforge.net). For whole genome and exome data, we focused our efforts on nonsynonymous coding substitutions and frameshift mutations on chromosome X and ranked variants that were predicted to be damaging based on conservation,9 SIFT,10 and Polyphen211 scores. For insertions and deletions (indels), all calls in exonic regions were manually reviewed in the event that the different calling methods might have placed the same indel at different genomic positions.

After identification of the suspect variant in the proband, we used PCR and Sanger sequencing of the exon of interest to confirm the mutation in him and his mother. Both PCR and Sanger sequencing were performed for each of the exons of HMGB3 from the 13 unrelated male patients. The PCR primer sequences for the 4 HMGB3 exons are provided in the eTable in the Supplement.
Results

Case History and Clinical Findings

At the time of initial examination by Goldberg and McKusick,3 the proband (IV-3) (Figure 1) was an 8-year-old boy who was born at term, weighing approximately 1800 g, below the third percentile. His head circumference was 45.5 cm, which is below the 50th percentile for a child 3 years old. He had intellectual disability, diastema of the upper incisors, and anteverted pinnae with minimal convolutions. He had reported congenital pes varus corrected by special shoes.

The proband also exhibited bilateral ptosis, pendular nystagmus, and right esotropia. There was no light perception in the right eye, and best-corrected visual acuity was 12/400 OS with refraction of +1.00 + 5.00 × 065. Corneal diameters were 3 mm horizontally and vertically in the right eye and 4 mm horizontally and 6 mm vertically in the left eye. Both corneas were clear, and each eye had inferonasal iris coloboma. A posterior segment examination could not be performed in the right eye. However, dilated examination of the left eye revealed a large chorioretinal coloboma that involved the optic nerve.

Three other male family members were noted to have a similar phenotype with colobomatous microphthalmia, microcephaly, slow development, and short stature (III-3, III-4, and III-18) (Figure 1) but were unavailable for molecular analysis. The proband's mother had left esotropia and a prominent limbal dermoid temporally in the left eye.

High-Throughput Sequencing Results

Complete Genomics sequencing was compared with Illumina data because of insufficient depth, largely at regions that were near the ends of targeted regions. Nine positions in the Illumina data were not called because of poor mappability. The region of overlap between the whole-genome sequence from Complete Genomics was compared with sequence data obtained in our laboratory using the Agilent X-exome capture reagent and Illumina sequencing. The filtering cascades for whole-genome and X-exome sequences are listed in Table 1. Whole-genome sequencing and X-exome capture identified 2 possible candidates (Table 2). The first mutation, PORCN (OMIM 300651), is the human homologue of Drosophila porcupine, mutations in which cause focal dermal hypoplasia (FDH; OMIM 305600). Focal dermal hypoplasia is described as an X-linked dominant trait that affects female carriers. Ninety percent of patients are female, suggesting that it is generally an embryonic lethal for male fetuses. In addition, 95% of cases are sporadic. Microphthalmia is reported as a feature in approximately 15% of cases.

The second mutation was a 2-base pair TA insertion that is predicted to cause a frameshift mutation in the fourth exon of a gene (HMGB3; OMIM 300193) that codes for the evolutionarily conserved high-mobility-group protein HMGB3. The mutation had a PhastCons9 score of 1 and a PolyPhen2 classification of damaging. The insertion was visualized using the Integrative Genomics Viewer12 (eFigure 1 in the Supplement) and confirmed by Sanger sequencing of exon 4 (Figure 2). The variant c.477_478insTA produces the predicted protein change p.Lys161Ilefs*54. Sanger sequencing of the mother confirmed her carrier status (eFigure 2 in the Supplement). We also carefully examined the NAA10 gene for sequence abnormalities that might have been missed by our filtering algorithms; none were found. No other X-linked variants from the whole-genome or X-exome sequencing were strong candidates in terms of potential pathogenicity.

Targeted HMGB3 Sequencing in Male Patients With Related Phenotypes

Thirteen unrelated male patients diagnosed as having microphthalmic conditions of unknown genetic basis were screened for HMGB3 mutations by Sanger sequencing. No variants were identified.

Discussion

We describe a mutation in a kindred with a syndrome with the features of microphthalmia, microcephaly, intellectual disability, and short stature. Using a combination of whole-
genome sequencing, targeted chromosome X-exome sequencing, and confirmatory Sanger sequencing, we found a mutation in the HMG3 gene. By combining orthogonal sequencing methods, our ability to identify pathogenic variants was significantly improved over the use of a single technique. As noted above, no other variants with predicted damaging effects were found in the whole-genome or targeted exon sequencing data from the proband.

We suggest that the mutation arose in or was passed from the great grandmother (I-1), who had 8 daughters and 1 son. The skewed female to male ratio of offspring may have resulted from the loss of affected males during pregnancy. We have been unable to identify HMG3 mutations in other patients with microphthalmia phenotypes, and thus the condition reported here is rare. Consequently, we propose that this particular disorder be named Maine microphthalmia (reclassified OMIM 300915) to distinguish it from related phenotypes and as originally suggested by Goldberg and McKusick.

HMG3 is a member of a subfamily of genes thought to play a key role in DNA replication, nucleosome assembly, and transcription. For the related protein HMG1, the acidic tail was found to interact with histone H3 in the nuclease to target HMG1 to nucleosome linker DNA, a binding site for HMG1 on chromatin. Morpholino knockdown of the Xenopus ortholog, Xhmgb3, was reported to have a significant role in retinal progenitor proliferation during eye development and to produce reduced eye and brain sizes in developing embryos. As can be seen in Figure 3, the carboxyl terminus of the HMG3 protein is remarkably conserved in vertebrates. The predicted substitution of the acidic polyglutamic tail with a basic polyarginine domain is expected to have a significant effect on HMG3 function. Although we cannot entirely rule out that the variant seen in PORCN may be causative and segregate as an X-linked recessive, we believe that the milder phenotype seen in our patient, the neutral Condel score, and the mode of inheritance make it significantly less likely than the clearly deleterious frameshift in HMG3.

There are 110 clinical synopses in the OMIM database that include the matching term microphthalmia, of which at least 46 have known mutations that cause more than 50 clinical entities with microphthalmia. Among them, several phenotypes include clinical features seen in Maine microphthalmia. MCOPS2 (OMIM 300166) is characterized with microphthalmia, intellectual disability, external ear malformations, scoliosis, and genitourinary malformations caused by abnormalities in the BCO3 gene (OMIM 300485) at chromosome Xp11.4. MCOPS3 (OMIM 206900) is characterized by microphthalmia, external ear malformations, genitourinary malformations, and tracheoesophageal malformations caused by mutations in SOX2 (OMIM 184429) at 3q26.2. MCOPS9 (OMIM 601186) is characterized by microphthalmia, intellectual disability, and genitourinary, cardiac, and pulmonary malformations caused by mutations in STRA6 (OMIM 610745) at 15q24. BCO3 is a repressor of BCL6 (OMIM 109565), a transcription repressor required for germinal center formation. SOX2 is a member of the SRY-related HMG-box family of transcription factors required for gene regu-

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Table 1. Filtering Results From Whole-Genome and X-Exome Sequencing of the Proband

<table>
<thead>
<tr>
<th>Identified Variants</th>
<th>Complete Genomics Whole Genome</th>
<th>Agilent/Illumina X-Exome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total variants</td>
<td>726 238</td>
<td>858</td>
</tr>
<tr>
<td>Chromosome X only</td>
<td>17 684</td>
<td>858</td>
</tr>
<tr>
<td>Not in dbSNP 138</td>
<td>10 032</td>
<td>70</td>
</tr>
<tr>
<td>Frameshift or missense</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Highly conserved</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Final manual review</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>


Table 2. The 2 Genes Identified by Each Filtering Cascade on Chromosome X

<table>
<thead>
<tr>
<th>Position</th>
<th>Ref</th>
<th>Alter</th>
<th>Type</th>
<th>Gene</th>
<th>Change</th>
<th>Condel Scorea</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 370 741</td>
<td>C</td>
<td>A</td>
<td>SNV</td>
<td>PORCN</td>
<td>Missense Ala&gt;Glu</td>
<td>0.36–0.46</td>
<td>Condel neutral</td>
</tr>
<tr>
<td>150 156 265</td>
<td>NA</td>
<td>TA</td>
<td>Indel</td>
<td>HMG3</td>
<td>Frameshift</td>
<td>NA</td>
<td>See text</td>
</tr>
</tbody>
</table>

Abbreviations: Alter, alternative nucleotide; Indel, insertion/deletion; NA, not applicable; Ref, human reference sequence; SNV, single-nucleotide variant.

a Condel scores were calculated using the online web tool at http://bg.upf.edu/condel/analysis.

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Figure 2. Alignment of Sanger Sequence From the Proband Against the Human HMG3 Reference cDNA NM_005342.2

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Figure 3. Alignment of Sanger Sequence From the Proband Against the Human HMG1 Reference cDNA NM_005342.2

Exon 4 was amplified by polymerase chain reaction and sequenced. The resulting sequence was in agreement with the insertion/deletion identified from the Illumina X-exome and Complete Genomics whole-genome sequences.
ation in the central nervous system and in many other tissues throughout development. Additional functional and animal studies of these and other genes will be necessary to fully understand the developmental pathways and networks involved in microphthalmia. On the basis of this study, we believe that HMGB3 should be added to the list of candidate genes to be investigated in future studies of this and other inherited ocular disorders, especially in male patients.

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Author Contributions: Dr Scott had full access to all the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Scott, Mohr, Barton, Pittiglio, Roderick, Chassaing, Calvas, Prabhu, Jabs.

Acquisition, analysis, or interpretation of data: Scott, Mohr, Kass, Barton, Pittiglio, Ingersoll, Craig, Marosy, Doheny, Bromley, Chassaing, Calvas, Prabhu, Jabs.

Drafting of the manuscript: Scott, Mohr, Barton, Pittiglio, Roderick, Chassaing, Calvas, Prabhu, Jabs.

Critical revision of the manuscript for important intellectual content: Mohr, Kass, Barton, Pittiglio, Ingersoll, Craig, Marosy, Doheny, Bromley, Chassaing, Calvas, Prabhu, Jabs.

Statistical analysis: Mohr.

Administrative, technical, or material support: Scott, Mohr, Kass, Barton, Pittiglio, Ingersoll, Craig, Marosy, Bromley, Chassaing, Calvas, Prabhu, Jabs.

Study supervision: Scott, Doheny, Jabs.

Conflict of Interest Disclosures: None reported.

Additional Information: This work is dedicated to the memory of Victor A. McKusick, MD, the Johns Hopkins University School of Medicine, and Thomas H. Rodenick, PhD, The Jackson Laboratory. Center for Human Genetics. We thank the family who provided samples and have waited so many years to learn the cause of their disorder.

Additional Contributions: Drs Marla O’Neill, MD, MPH, and Joanna Amberger, BA, the Johns Hopkins University School of Medicine, discussed the phenotype and reviewed the manuscript. They were not compensated for their work.

REFERENCES


OPHTHALMIC IMAGES

Multiple Serous Retinal Detachments Seen on Wide-Field Imaging in a Patient With Sympathetic Ophthalmia

Bryn M. Burkholder, MD; James P. Dunn, MD

A 20-year-old healthy patient with a history of corneal perforation from chronic herpetic disease in the left eye presented with decreased vision in the right eye. Wide-field fluorescein angiography showed punctate areas of leakage and multiple lobular serous retinal detachments in the posterior pole of the right eye (A), consistent with a diagnosis of sympathetic ophthalmia; the subretinal fluid had resolved 2 months later, after treatment with oral prednisone and initiation of mycophenolate mofetil (B).