In the human genome, null mutations in protein-coding genes can lead to a wide range of phenotypic effects, ranging from invisible to severe phenotypes \[1\]. Null mutations are typically responsible for recessive diseases such as Duchenne muscular dystrophy \[2\] and erythropoietic protoporphyria \[3\] in which approximately 70% to 80% of the detected mutations are null mutations. Analyzing null mutations specific to certain diseases among the millions of genomic variants captured by whole exome sequencing is crucial for the identification of novel disease genes.

We previously analyzed several known myopia genes and myopia-associated genes based on whole exome sequencing data obtained from samples of 298 probands with early-onset high myopia \[4,5\]. However, we identified one null mutation in one proband that was associated with high myopia as well as other variants in a small proportion of probands, which had undetermined pathogenicity \[4,5\]. The cause of the remaining majority of this cohort is unknown. These findings suggest that variants in novel genes might cause this disease.

METHODS

Subjects: This study is part of a project established to investigate genetic defects associated with eoHM. Our aim was to identify novel genes responsible for eoHM using the same eoHM cohort that was used in our previous study \[5\]. Briefly, probands were recruited from the clinic at the Zhongshan Ophthalmic Center according to the following inclusion criteria: 1) spherical refraction in each meridian of ≤–6.00 D in both eyes, 2) development of high myopia before the age of 7 years, and 3) no other known ocular or related systemic diseases. The 507 controls were unrelated probands with genetic eye diseases other than myopia, including retinal degeneration and glaucoma. The 480 healthy controls had bilateral refraction of between −0.50 and +1.0 D spherical equivalents without a family history of high myopia and had a best unaided visual acuity of 1.0 or better without another known eye or systemic disease. Written informed consent was obtained from the participants or their guardians following the tenets of the Declaration of Helsinki. This

Therefore, in the current study, our aim was to identify null mutations in novel genes associated with eoHM using whole exome sequencing.

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Whole exome sequencing was performed with an Agilent SureSelect Human All Exon Enrichment Kit V4 array (51,189,318 bp; Agilent, Santa Clara, CA) that covered more than 20,000 genes (approximately 334,000 exons). DNA fragments were sequenced using an Illumina HiSeq 2000 system (Illumina, San Diego, CA). The average sequencing depth was 125-fold. Reads were mapped against UCSC hg19 (GenomeUCSC) using Burrows-Wheeler Aligner (BWA). The parameters used for whole exome sequencing have been previously described [5].

Null mutations, including homozygous and compound heterozygous truncation variants, were selected from whole exome sequencing data on the 298 probands with eoHM. These data were compared with those of the 507 probands with other forms of eye disease. Null mutations specific to eoHM were considered potential candidates. The minor allele frequency of each variant was obtained from public databases, including dbSNP, 1000 Genomes, Exome Variant Server, and Exome Aggregation Consortium (EXAC). Null variants with a minor allele frequency of >0.01 were excluded, and the remaining variants were further confirmed using Sanger sequencing and subsequently validated in available family members and 480 healthy controls. Primers were designed using the Primer3 online tool and are listed in Appendix 1. The methods used to perform Sanger sequencing, including amplification, sequencing, and analysis of the target fragments, have been previously described [4]. The variants are described according to the Human Genome Variation Society (HGVS).

RESULTS

Evaluation of the whole exome sequencing data on 298 probands with eoHM revealed the presence of millions of variants targeting approximately 20,000 genes and null mutations in a few genes, LRPAPI (Gene ID 4043; OMIM 104225) and LOXL3 (Gene ID 84695; OMIM 607163), that appeared to associate with high myopia after a series of bioinformatic filters. Null mutations in LRPAPI have been associated with high myopia in humans [6]. Previously, we identified an additional null mutation (c.199delC) in LRPAPI in a consanguineous family that has been reported in our previous study of known myopia genes [4]. Here, the null mutations detected in LOXL3 included a homozygous frameshift mutation (c.39dup; p.L14Afs*21) and a compound heterozygous frameshift variant (c.39dup; p.L14Afs*21 and c.594delG; p.Q199Kfs*35), which were identified in two of the 298 probands with eoHM (Table 1, Figure 1A). These mutations in LOXL3 were confirmed with Sanger sequencing and were absent in 1,974 alleles of ethnicity-matched controls from the same region (507 individuals with other conditions and 480 healthy control individuals; Table 1). These null mutations were also not present in the 1000 Genomes, Exome Variant Server, and Exome Aggregation Consortium databases. The two probands were singleton cases, and their parents carried only heterozygous mutations (Figure 1A). These null mutations in LOXL3 were predicted to result in degradation of the transcript by nonsense-mediated mRNA decay [7,8]. The mutation frequencies and spectra in different types of variants of LOXL3 are shown in Appendix 2. Other less likely pathogenic heterozygous variants in LOXL3 are listed in Appendix 3.

The two probands with a LOXL3 mutation developed high myopia before reaching 7 years of age. One proband had a refractive error of −18.50 DS for the right eye and −18.00 DS for the left eye, and the other had a refractive error of −23.00 DS for the left eye and retinal detachment in the right eye (Table 2). Examination with an opthalmoscope revealed myopic fundus with crescent and tigroid forms in the two probands (Figure 1B-D).

DISCUSSION

In the current study, we revealed homozygous frameshift (c.39dup, p.L14Afs*21) and compound heterozygous frameshift (c.39dup, p.L14Afs*21; c.594delG, p.Q199Kfs*35) mutations in LOXL3 in two of the 298 probands with eoHM. These null mutations cosegregated with high myopia and were absent in the 1,974 alleles of the controls.

High myopia is a leading cause of visual impairment worldwide. Several lines of evidence indicated that excess elongation of eye size and axial length is due to abnormal extracellular matrix (ECM) remodeling in the sclera mediated by the transforming growth factor (TGF)-beta pathway [9-14]. LOXL3, a member of the lysyl oxidase gene family, encodes an extracellular copper-dependent amine oxidase. Loxl3 expression is enriched in the retina and the central nervous system [15,16]. The encoded protein is induced through the TGF-beta pathway [17,18] and plays a critical role in the covalent cross-linking of collagen and elastin in the ECM, which is essential for ECM integrity in connective tissues [15,16,19-21].

Abnormal LOXL3 function has been reported to be the cause of multiple types of defects in humans as well as in animals. A recent study identified a homozygous missense mutation (c.2027G>A, p.C676Y) in exon 12 of the LOXL3 gene as the cause of autosomal recessive Stickler syndrome in a consanguineous family [22], with high myopia a
### Table 1. LOXL3 Mutations Identified in Families with Early-Onset High Myopia.

| Family | Exon | Position   | DNA change | Protein change | Status | Co-segregation | Note  | Allele frequency |
|--------|------|------------|------------|----------------|--------|----------------|-------|------------------|
|        |      |            |            |                |        |                |       | Normal control   |
|        |      |            |            |                |        |                |       | Others\textsuperscript{§} |
|        |      |            |            |                |        |                |       | Databases\textsuperscript{#} |

|        | HM293 | E2        | 74779723    | c.39dup        | p.L14Afs*21 | Homo          | Yes   | Novel            | 0/960 | 0/1014 | None   |
|        | HM407 | E2        | 74779723    | c.39dup        | p.L14Afs*21 | Hetero        | Yes   | Novel            | 0/960 | 0/1014 | None   |
|        | HM407 | E4        | 74776594    | c.594delG      | p.Q199Kfs*35 | Hetero        | Yes   | Novel            | 0/960 | 0/1014 | None   |

Note: Homo, homozygous; Hetero, heterozygous. \textsuperscript{§}, Samples from patients with other eye diseases, including glaucoma and retinal degeneration. \#, Databases including 1000G-\textsuperscript{nomes}, Exome Variant Server, dbSNP, and Exome Aggregation Consortium.
constant feature in this family [22]. This missense mutation was located in an evolutionarily conserved region and was predicted to be pathogenic [22]. In animal studies, knockdown of lox3b in zebrafish led to craniofacial abnormalities [21], and Loxl3−/− mice demonstrated craniofacial and spinal defects and smaller lungs at the embryonic stage (E18.5) [23]. The structure and axial length of the eyes in Loxl3-knockout mice were hard to determine, as all the knockout mice showed perinatal lethality [23]. We have generated a heterozygous Loxl3-knockout mouse model but we were unable to get any homozygous Loxl3-knockout mouse (unpublished data), also suggesting embryonic lethal in mice on complete absence of Loxl3. In the current study, the two unrelated patients with null mutations in LOXL3 exhibited high myopia without other known ocular or related systemic diseases, representing milder phenotypes than observed in previous studies in zebrafish or mouse [21,23]. Although the mechanism by which different LOXL3 mutations cause variable phenotypes is unclear, high myopia is a common symptom present in syndromic diseases such as congenital

Figure 1. Null mutations in LOXL3 identified in two probands with early-onset high myopia. A: Sequence chromatography and pedigrees of HM293 and HM407. Sequence changes detected in the patients with early-onset high myopia are presented in the left column, whereas healthy sequences appear in the right column. The sample from the mother in family HM407 was not available. M1, c.39dup; M2, c.594delG; +, wild-type. B, C, D: Fundus photos for both eyes of HM293II1 (B, C) and the left eye of HM427II1 (D) revealed myopic fundus with crescent and tigroid forms. The fundus photo for the right eye of HM427II1 is not available.
Table 2. Clinical Information for Patients with LOXL3 Mutations.

| Patient | Gender | Age at exam (years) | First symptom | BCVA | Refraction | Axial length (mm) | Fundus |
|---------|--------|---------------------|---------------|------|-------------|------------------|--------|
| HM293II1 | Male   | 3                   | PV            | NA/NA | −18.50DS-1.25DC | −18.00DS-2.00DC | NA/NA  | Myopic/Myopic |
| HM407II1 | Male   | 15                  | PV            | HM/0.04 | NA*        | −23.00DS-3.50DC | 27.15/33.58 | RD/Myopic |

Note: PV, poor vision; NA, not available; HM, hand move; RD, retinal detachment; #, refraction was not available due to retinal detachment.
night blindness, caused by mutations in \textit{NYX} \cite{24}, and Bornholm eye disease, caused by \textit{OPNILW} \cite{25}. Mutations in \textit{NYX} and \textit{OPNILW} have also been reported to cause high myopia alone due to mutations in different locations \cite{26-28}. The null mutations in \textit{LOXL3} were determined to be located in exons 2 and 4, which is distinct from the location of the previously reported mutation in Stickler syndrome. Because of the lack of follow-up visits to further confirm the phenotypic information, we can only assume that mutations located in different locations in \textit{LOXL3} might have independent effects on patient phenotypes.

In conclusion, our results reveal the presence of null mutations in \textit{LOXL3} in families with eoHM. Due to limited phenotypic information and a lack of functional studies, these findings only indicate that null mutations in \textit{LOXL3} are likely to be associated with autosomal recessive eoHM. Meanwhile, our current approach may miss other types of variants if they are associated with high myopia. Our upcoming study will be designed to solve this issue by examining all other variants across the whole exome between cases and controls. The molecular mechanism underlying the role of \textit{LOXL3} in high myopia, as well as in Stickler syndrome, will be the subject of further study.

**APPENDIX 1. PRIMERS USED FOR POLYMERASE CHAIN REACTION**

To access the data, click or select the words “Appendix 1.”

**APPENDIX 2. THE VARIANT FREQUENCIES AND THE PROPORTION OF VARIANTS TYPES OF \textit{LOXL3} IN THE 298 PATIENTS WITH EARLY-ONSET HIGH MYOPIA.**

To access the data, click or select the words “Appendix 2.”

In this study, 1.68% (5/298) of patients with eoHM harbored variants in \textit{LOXL3}, in which 0.67% (2/298) of patients carried two null variants, 0.67% (2/298) of patients carried two heterozygous missense variants, and 0.34% (1/298) of patients carried one splicing change.

**APPENDIX 3. RARE VARIANTS IDENTIFIED IN 298 PATIENTS WITH EOHM AND 507 CONTROLS**

To access the data, click or select the words “Appendix 3.”

Note: NA, not available; Hetero, heterozygous; §, Samples from patients with other eye diseases, including glaucoma and retinal degeneration; #, Databases including 1000Genomes, Exome Variant Server, dbSNP, and Exome Aggregation Consortium. †, Allele frequency found in Exome Aggregation Consortium database but not found in any other databases.

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