Microphthalmia (OMIM 309700) is an ocular developmental malformation characterized by unusually small eyes (Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/Omim/ National Center for Biotechnology Information [NCBI], Bethesda, MD). The major clinical characteristics include a short axial length (<20 mm), a high degree of hyperopia (+7.00 to +13.00 D), a high lens-to-eye volume ratio, and a high incidence of angle-closure glaucoma after middle age. Some normal adnexal elements and eyelids are usually present. It is also a common symptom in some other ocular abnormalities. Approximately 80% of microphthalmia cases occur as part of syndromes that include other systemic malformations, especially cardiac defects, facial clefts, microcephaly, and hydrocephaly. The reported prevalence of anophthalmia or microphthalmia at birth is 0.66 of 10,000 around the world and 0.3 of 10,000 in China.

Epidemiologic studies have indicated that both heritable and environmental factors cause microphthalmia. Although the precise pathogenesis of microphthalmia is still unknown, studies have demonstrated that it is a genetically heterogeneous disorder. Chromosomal abnormalities may result in syndromic microphthalmia. Studies of different microphthalmia cases and pedigrees have linked it to different chromosomal regions and monogenic causes. Autosomal-dominant microphthalmia pedigrees have been mapped to 2q11–14,5 3q26.3-q27 (chromosome 17, region p12-q12). However, no mutation or CNV (copy number variation) was identified to be responsible for the microphthalmia phenotype of this pedigree.

CONCLUSIONS. A novel suggestive linkage locus for congenital microphthalmia was detected in a Chinese family. This linkage region provides a target for susceptibility gene identification. (Invest Ophthalmol Vis Sci. 2011;52:3425–3429) DOI:10.1167/iovs.10-6747

Subjects and Methods

Subjects

A five-generation Chinese family from Shandong Province in China that had members with diagnosed microphthalmia was involved in the study (Fig. 1). Thirty-four family members underwent general physical and complete ophthalmic examinations. All family members did not have any other physical anomalies. Nine microphthalmia patients expressed the same full phenotype as previously reported. They were affected by isolated microphthalmia in an autosomal dominant transmission manner in both eyes with onset since birth. Detailed information is available in another publication.

Peripheral blood samples from 28 individuals including 9 affected family members (containing all patients) and 19 unaffected members were collected for further analysis. All participants gave written in-
formed consent in accordance with the Declaration of Helsinki before they were enrolled in the study.

**Genotyping**

A 3-ml peripheral blood sample was taken from each individual after informed consent was obtained. Genomic DNA was extracted by using the standard phenol-chloroform method.

The whole-genome scan was performed by using 382 fluorescent microsatellite markers in the 22 pairs of autosomes, with an average spacing of 10-cM scattering on the human genome (Prism Linkage Mapping Set Version 2.0; Applied Biosystems, Inc. [ABI], Foster City, CA). PCR was performed in a 5-μL volume with 50 ng genomic DNA as a template, 0.5 μL PCR 10× buffer, 0.1 μL dNTP mix (2.5 mM), 0.06 μL primers, 0.6 μL MgCl₂ (15 mM), 0.05 U Taq polymerase (AmpliTaq Gold; ABI), and distilled water up to 5 μL. Thermal cycling was performed (GeneAmp 2720; ABI) at 95°C for 12 minutes, then 15 cycles of 94°C for 30 seconds, 65°C for 1 minute, degrading 0.5°C per cycle, and 72°C for 1 minute 50 seconds, followed by 24 cycles of 94°C for 30 seconds, 56°C for 1 minute, and 72°C for 1 minute 50 seconds, with a final extension of 72°C for 15 minutes. PCR products were analyzed on an automated sequencer (model 3100; ABI). A GS400 size standard was used as the internal standard and run in the same lane with the markers. Alleles were then analyzed (GeneScan, ver. 3.0 and GenoTyper ver. 3.7; ABI). In the fine mapping phase, additional fluorescent markers (D17S926, D17S933, D17S1871, D17S783, D17S740, D17S1824, D17S953, D17S1788, D17S799, D17S261, D17S900, D17S1880, D17S1293, D17S1288, D17S839, D17S1872, D17S933, D17S592, and D17S788) were selected from the Marshfield database (http://research.marshfieldclinic.org/ Marshfield Clinic, Marshfield, WI).

**Linkage Analysis**

Two-point LOD scores were calculated by the MLINK program of the LINKAGE package (ver. 5.1; http://linkage.rockefeller.edu/soft/). The allele frequencies of markers were assumed to be equal, as were the recombination frequencies in males and females. The disease was specified to be an autosomal dominant inheritance with 95% penetrance. We assumed a disease allele frequency of 0.0001 and no sex difference in the recombination rates.

**Haplotype Reconstruction**

The haplotype was constructed, using a commercial program (Cyrillc Software, Lake Orion, MI) to define the borders of the cosegregating region and then modifying it by hand.

**Mutation Analysis**

After the whole-genome scan, a candidate approach was used to search for possible candidate genes. The exons of candidate genes were amplified by PCR, and the primers were designed on computer (Premier 5.0; Premier Biosoft, Palo Alto, CA). The PCR reaction included 1 μL (50 ng) genomic DNA, 1 μL (30 ng) each of the primers, 1 μL PCR 10× buffer with MgCl₂ (Roche Diagnostics, USA, Indianapolis, IN), 0.05 μL (5U) Taq polymerase (AmpliTaq Gold; ABI), and 5.85 μL distilled water. Then, PCR products were purified with shrimp alkaline phosphatase (Fermentas International, Glen Burnie, MD) and exonuclease I (Fermentas International) for 85 minutes at 37°C to remove the phosphoryl groups. The samples were then sequenced on an automated sequencer (model 3100; ABI) in both directions.

To explore the possible pathogenetic role of copy number variation (CNV), we performed genome-wide SNP genotyping (Human660W-Quad BeadChip; Illumina, San Diego, CA). Affected individuals III-8 and V-3 were genotyped according to the manufacturer’s guidelines. To call CNVs, we used the PennCNV algorithm (www.openbioinformatics.org, an unaffiliated repository of software), which combines multiple sources of information, including log R ratio (LRR) and B allele frequency (BAF) at each SNP marker, along with SNP spacing and population frequency of the B allele to generate CNV calls.

**RESULTS**

**Linkage Analysis**

A whole-genome scan study was performed, with markers located in four regions—chromosomes 10, region p13; 11, region q23.3; and 17, regions p11.2 and p13.1—demonstrated...
Two-point LOD scores $>1.0$ (Table 1). The maximum LOD score of 4.72 ($\theta = 0$) was obtained at $D17S1857$.

Other surrounding markers were genotyped for fine mapping. Among these microsatellite markers, $D17S740$ failed to amplify, and four markers—$D17S839$, $D17S953$, $D17S2196$, and $D17S1788$—were excluded because of low heterozygosity. Finally, 15 markers remained: $D17S799$, $D17S900$, $D17S261$, $D17S1843$, $D17S1871$, $D17S1872$, $D17S783$, $D17S1824$, $D17S1880$, $D17S2193$, $D17S1872$, $D17S933$, and $D17S927$. The two-point LOD scores of these markers were calculated. A maximum LOD score of 4.97 at recombination 0.00 was obtained at $D17S1880$ and $D17S1293$, and a multipoint LOD score, 4.1, was obtained between $D17S1824$ and $D17S261$, $D17S1834$, $D17S1857$, $D17S1288$, $D17S783$, and $D17S1880$ around $D17S1824$ also obtained a LOD score $>3.0$ at recombination fraction 0.00 (Table 2), which is suggestive of linkage to microphthalmia.

Multipoint linkage analysis resulted in a LOD score $>3.0$ in the region between $D17S900$ and $D17S1880$. The highest multipoint LOD score, 4.1, was obtained between $D17S261$ and $D17S1871$ (Fig. 2).

**Haplotype Analysis**

Ten microsatellite markers for fine mapping were used to construct the haplotypes (Fig. 1). The informative recombination event was present in individual III-3 between markers $D17S900$ and $D17S1843$, placing the disease-causing gene centromeric to the marker $D17S900$. Similarly, recombination events between loci $D17S1293$ and $D17S1872$ occurred in affected individuals III-11, IV-8, and V-3, indicating that the disease gene is telomeric to locus $D17S1293$. In addition, haplotype analysis showed that a cosegregated haplotype expanding from $D17S1843$ to $D17S1293$ was inherited by all nine affected members in the family. Thus, in this family, the disease gene lies within a region of approximately 21.57 cm on chromosome 17, region p12-q12.
significantly positive two-point LOD score was obtained with a maximum 4.97 for marker D17S1824 at a recombination fraction of 0.00. Subsequent haplotype analysis showed that a cosegregated haplotype expanding from D17S1843 to D17S1293 was inherited by all nine affected members in this family.

A total of 153 genes have been mapped to this interval defined by loci D17S900 and D17S1872. Fourteen candidate genes, including UNC119, CRYBA1, RPL23A, NCOR1, COPS3, ALDOC, C17orf39, MED9, NLK, FLII, NUFIP2, CCL8, PROCA1, and SPAG5, were screened on the basis of their high expression and essential function in eyes. Especially, the CRYBA1 protein is the structural constituent of eye lens crystallins. The mutation in the CRYBA4 gene, which is in the same protein family as CRYBA1, is attributed to complex microphthalmia in association with genetic cataracts.19 NCOR1 encodes a protein that mediates ligand-independent transcription repression of thyroid hormone and retinoic acid receptors by promoting chromatin condensation and preventing access of the transcription machinery. ALDOC, COPS3, SPAG5, PROCA1, NLK, RPL23A, and MED9 are at high levels in eyes. Especially, RPL23A, PROCA, and SPAG5 are expressed at high levels in fetal eyes, lens, eye anterior segment, optic nerve, and retina, among other ocular components. The coding regions and intron/exon splicing region of these 14 candidate genes were sequenced, but no pathogenic mutation was found. However, the possibility could not be completely ruled out, because we did not screen the control regions (promoter, 5' and 3' untranslated regions [UTRs]) of these genes, the possibility of a
functional defect in introns has not yet been ruled out. In addition, because the information on genes in this area is limited, the pathogenic gene may not have been identified. With the updated genome and expression studies, however, new eye-related genes will be found that could be our new candidate genes.

In conclusion, the novel localization for an autosomal dominant congenital simple microphthalmia pedigree has been mapped to chromosome 17, region p12q12. Some new genes related to mammalian eye development may be identified from this Chinese family in the future. Our screening is still being undertaken with the hope of identifying the disease gene itself. Further, high-throughput sequencing technology for screening the area will also be taken into account.

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References