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Exclusive gene mapping of congenital microphthalmia in a Chinese family

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Abstract Congenital microphthalmia is a developmental ocular disorder and might be caused by the mutations in the genes involved in eye development. To uncover the genetic cause in a six-generation Chinese pedigree with autosomal dominant congenital microphthalmia, we performed genescan and linkage analysis in this family. Fourteen microsatellite markers on chromosomes 3, 11, 14 and 15 were selected as genetic markers according to the five previously reported loci associated with microphthalmia (*MITF*, *SOX2*, *PAX6*, *MCOP* and *NNO2*). The genomic DNA of each member in the pedigree was amplified with 14 pairs of fluorescence labeled primers. Genome screening and genotyping were conducted on ABI377 DNA sequencer and linkage analysis was performed with Linkage software package. All two-point LOD scores of linkage analysis between the suggested disease genes and microsatellite markers were <–2, which indicated that none of the five genes were responsible for microphthalmia in this Chinese family. Microphthalmia in this family may be caused by mutation in a new gene which is essential in eye development.

Keywords: congenital microphthalmia, gene mapping, microsatellite markers, linkage analysis.

Congenital microphthalmia (CMIC) is an ocular developmental disorder characterized by the small eye (2/3 of the normal volume), short axial length (less than 20 mm), narrow palpebral fissure and small fossa orbitalis. The affected individuals usually develop into farsighted since the shortened axial length causes the focal point to fall behind the retina. Yong microphathalmia patients without being treated in time can develop into irreversible amblyopia. Sometimes, patients also have the phenotype of angle-closure glaucoma resulting from obstruction of the trabecular meshwork, which can produce disturbance of aqueous fluid recirculation and increasing intraocular pressure. Congenital microphthalmia has different clinical manifestations: (1) Nanophthalmos is a relatively rare condition only characterized by a small eye in the absence of any other systemic abnormalities[1]. (2) Most microphthalmia is frequently accompanied with other ocular abnormalities, including anterior segment dysgenesis, congenital cataract, chorioretinal coloboma, retinal dysplasia, optic-nerve coloboma, etc[2]. (3) Anophthalmos is the extremely severe form of microphthalmia. The prevalence of congenital microphthalmia is 1/11077 in China[3]. Although most of the microphthalmia cases are sporadic, some families with autosomal dominant (AD), autosomal recessive (AR) or X-linked recessive inheritance have been reported.

Up to now we still know little about the mechanisms of congenital microphthalmia. However, mutations in different eye-development genes have been reported to be associated with congenital microphthalmia[4,5]. Mutations in the *mitf* gene and *pax6* gene had been proven to cause microphthalmia in mouse[6,7]. Mutations in human *MITF* gene, which maps to chromosome 3p14.1-q12.3, can cause Waardenburg syndrome type 2. Mutations in human *PAX6* gene, which is the homologous gene to *pax6* and maps to chromosome 11p13, can cause aniridia. Furthermore, mutations in the *SOX2* gene, which maps to chromosome 3q26.3-q27, were identified in patients with congenital microphthalmia[8−10]. Othman *et al*.[11] performed a whole genome scan in a large family in which microphthalmia was transmitted in an autosomal dominant manner. Linkage analysis demonstrated tightly linkage between disease-causing gene and microsatellite markers (D11S905, D11S903, D11S1313, D11S4191 and D11S987) and gave a maximum LOD score of 5.92 at a recombination frac-

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tion of 0.00 for the marker D11S903. Then the locus responsible for this microphthalmia pedigree, which was symbolized NNO1(*Nanophthalmos1*, OMIM # 600165), was mapped to chromosome 11p13 in a 14.7 cM interval. In 1998, Bessant *et al*.[12] mapped the disease-causing gene *MCOP* (*Microphthalmos*, OMIM# 251600) to chromosome 14q32 on the basis of a whole-genome linkage analysis in a consanguineous five-generation Pakistan isolated microphthalmia family with autosomal recessive inheritance. Linkage analysis gave a maximum LOD score of 3.55 for the marker D14S65 (at a recombination fraction of 0.00). Surrounding this marker there was a region of homozygosity of 7.3 cM, between markers D14S987 and D14S267, within which the disease gene was predicted to lie. Morle *et al*.[13] studied a five-generation Sephardic Jewish family, in which patients had either unilateral or bilateral microphthalmia of variable severity inherited as an autosomal dominant trait with incomplete penetrance. By a genome scan, they demonstrated the linkage of the disease-causing gene in this pedigree, which was symbolized *NNO2* (*Nanophthalmos2*, OMIM #605738), to chromosome 15q12-q15 in a 13.8 cM interval. A maximum LOD score of 3.77 was obtained at marker D15S1007 (at a recombination fraction of 0.00). Haplotype analyses narrowed the assignment to a 4.5 cM between D15S1002 and D15S1048, and 7.1 cM between D15S1043 and D15S1040. Hitherto, the disease-causing genes of different microphthalmia families have been localized to different intervals in human genome. We performed a study on a six-generation pedigree of autosomal dominant congenital microphthalmia coming from Zhejiang Province in order to investigate the relationship between the gene responsible for this Chinese microphthalmia family and the loci reported previously. This isolated congenital microphthalmia family is the first reported and analyzed Chinese pedigree.

1 Subjects and methods

1.1 Subjects

We collected a six-generation pedigree with autosomal dominant congenital microphthalmia residing in Zhejiang Province (Fig. 1). The autosomal dominant mode of inheritance in this pedigree was confirmed by the following facts: the microphthalmia phenotype was inherited through successive generations independent of sexuality; 50% offsprings would be affected when one of their parents was patient and the other was normal; incidence rate was equal between male and female. Meanwhile, our family accorded with the qualifications used for linkage analysis (viz. little mobility, enough family members and aware of genetic data, such as mode of inheritance, degree of heritability and penetrance), as classified by Liu and He[14].

The affected individuals were evaluated by ophthalmologic examination. A complete ophthalmologic examination showed that affected members had the phenotype of the small eye globe, enophthalmos, narrow palpebral fissure, light ptosis, superficial anterior chamber, small cornea and normal pupil. The proband

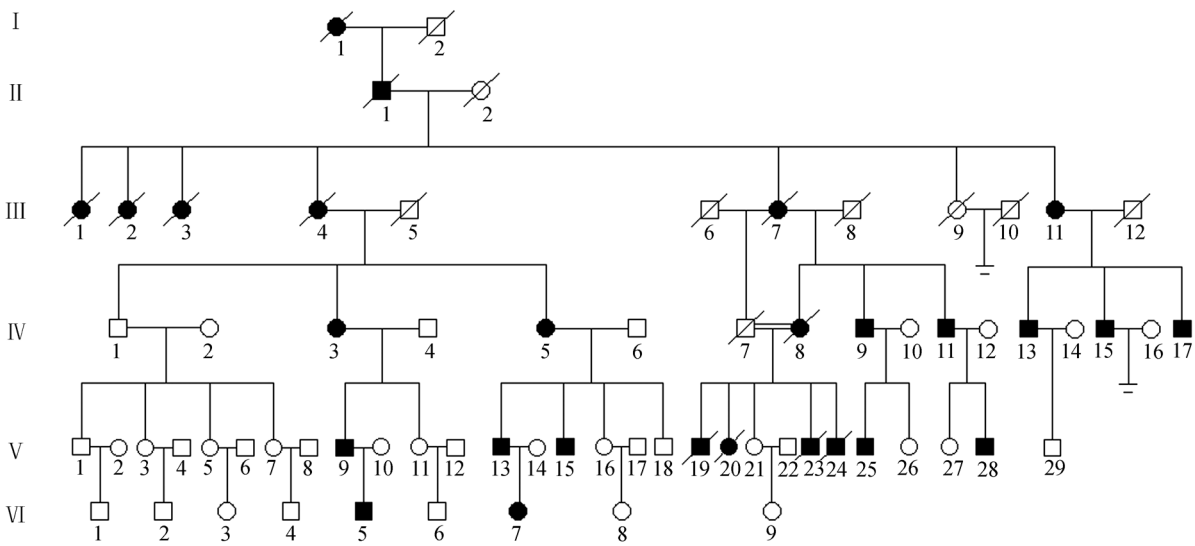

Fig. 1. Pedigree of the family with congenital microphthalmia in our study.

IV 8, the product of a consanguineous marriage, showed the phenotype: bilateral small eye globe, 19.9 mm axial length, narrow palpebral fissure and light cernuous, 9.7 mm horizontal diameter and 9.4mm vertical diameter of cornea, light anterior chamber and normal slit lamp biomicroscopy. Leucocytes culture of peripheral blood indicated that his chromatosome was normal and karyotype was 46, XX. Three sons and one daughter of this proband couple died prematurely[15].

1.2 Extracting genomic DNA

Peripheral blood was collected from each individual with informed consent. Genomic DNA was extracted from peripheral blood by using the standard phenol/chloroform method and was stored at −20℃.

1.3 Amplifying microsatellite marks by PCR

According to the previously reported five loci, 14 microsatellite marks on chromosome 3, 11, 14 and 15 were used as genetic markers (Table 1) and were amplified by PCR with fluorescence labeled primers. PCR was performed in a 25-μL volume containing 10ng genomic DNA, 2.5 μL PCR 10× buffer, 200 μmol/L dNTPs, 0.4 μmol/L of each primer, 1.5 mmol/L $MgCl_2$, 1 U of *Taq* DNA polymerase and 0.1 mg/mL BSA. A three-stage PCR was used and cycling conditions were: 3 min at 94℃, 35 cycles of 30 s at 94℃, 30 s at 53−59℃ and 30 s at 72℃, followed by 5 min extension at 72℃.

Table 1 Candidate loci and the microsatellite markers for linkage analysis

Candidate gene	Locus	Marks	Fragement length (bp)
MITF	3p14.1-p12.3	D3S1300	217−241
		D3S1239	187−193
		D3S3566	218−234
		D3S3698	270−278
		D3S1285	232−242
		D3S3524	225−269
		D3S3551	242−264
SOX2	3q26.3-q27	D3S2314	210−222
PAX6	11p13	D11S904	185−201
		D11S1324	110−128
		D11S914	274−285
		D11S1751	248−310
MCOP	14q32	D14S987	308−320
NNO2	15q12-q15	D15S1007	165−189

1.4 Genescan and genotyping

1.5 μL PCR products mixed with 2.0 μL buffer, which was prepared with Formamid, Polyglucosan and GeneScan-350 ROX^{TM} (8∶4∶3), were denatured at 95℃ for 4 min, put in ice quickly and then size fractionated on a 5% denaturing polyacrylamide gel run on an ABI377 sequencer. GeneScan3.1 and Genotyper2.0 softwares were used to determine the size of alleles. GeneScan-350 ROX^{TM} was used as internal size standards and run in the same lane with the PCR products.

1.5 Linkage analysis

Two-point LOD-scores were calculated by using the MLINK program of the Linkage software package version 5.2. The microphthalmia was assumed to be in an autosomal dominant model with an estimated penetrance of 100%. The gene frequency of disease was set at 0.0001 and the mutation rate was set at zero. The equal recombination frequencies between male and female were assumed. Allele frequencies for all markers were kept equal.

2 Results and discussion

Linkage analysis is an important way for investigating the relative location of a gene according to linkage relationship between gene loci and is popularly used now. LOD-score method is one of the most commonly used approaches in linkage analysis. We get two probabilities from the genetic data about the two loci: one is supposing linkage of the two loci at a recombination fraction; the other is supposing absence of linkage of the two loci at a recombination fraction. Odds of supporting or excluding linkage are obtained by comparing these two probabilities. LOD score just means log of odds. LOD score$\geqslant$1 supports linkage; LOD score$\geqslant$3 confirms linkage; LOD score$\leqslant$−2 excludes linkage. In this research, we found LOD scores consistently $<$−2 at each of these microsatellite markers (Table 2), which demonstrated the absence of linkage between the disease gene in the family and these 14 microsatellite markers, suggesting that the disease-causing gene responsible for the Chinese microphthalmia was not located in any of the reported known *MITF*, *SOX2*, *PAX6*, *MCOP* and *NNO2* loci. Additionally, Yu *et al*.[16] previously performed a preliminary linkage analysis in the same family and excluded several candidate loci (*CHX10*, *MITF*, *RX*, *MCOP*, *NNO1* and *NNO2*). In conclusion, microphthalmia in this family may probably result from defects in a new developmental gene which is essential in eye development.

Table 2 Two-point LOD scores of linkage analysis between the disease gene and microsatellite markers

Microsatellite marker	Recombination fraction						
	0	0.01	0.05	0.10	0.20	0.30	0.40
D3S1300	−∞	0.082	0.646	0.776	0.726	0.537	0.293
D3S1239	−∞	−1.166	−0.504	−0.253	−0.064	−0.009	−0.003
D3S3566	−∞	−0.763	−0.145	0.057	0.168	0.162	0.111
D3S3698	−∞	−0.224	0.356	0.506	0.502	0.375	0.216
D3S1285	−∞	−2.717	−1.392	−0.879	−0.441	−0.231	−0.110
D3S3524	−∞	−2.461	−1.143	−0.642	−0.248	−0.108	−0.060
D3S3551	−∞	−4.406	−2.360	−1.519	−0.747	−0.359	−0.151
D3S2314	−∞	−2.570	−1.255	−0.754	−0.353	−0.185	−0.096
D11S904	−∞	−2.831	−1.458	−0.898	−0.406	−0.182	−0.074
D11S1324	−∞	−4.470	−2.389	−1.517	−0.713	−0.318	−0.118
D11S914	−3.774	−1.658	−0.926	−0.612	−0.321	−0.160	−0.063
D11S1751	−∞	−3.147	−1.200	−0.487	0.020	0.137	0.104
D14S987	−∞	−4.196	−2.119	−1.263	−0.503	−0.167	−0.032
D15S1007	−∞	−5.497	−2.815	−1.739	−0.763	−0.286	−0.068

Congenital microphthalmia is a genetically heterogeneous disorder, so its pathogenesis is complicated. The important period for the occurrence of congenital microphthalmia is the early stage of embryonic and fetal life, which is also critical for human eye development. The optic vesicle begins to develop from the forebrain at the fourth week of gestation. At this period both environmental and genetic factors can lead to obstruction of eye morphogenesis and eventually result in microphthalmia. Maternal vitamin or amino acid deficiency during pregnancy may be the genetic predisposition to the microphthalmia. At the same time, physical and biological factors, e.g. X-ray exposure, virus infection (rubella or cytomegalovirus) and taking medicine could also cause microphthalmia[2]. Genetic factors include mutations in eye-development genes and chromosomal deletion or translocation.

As is well known, microphthalmia is often asymmetric or unilateral. Other ocular abnormalities such as congenital cystic eye or pupil displacement usually accompany microphthalmia in homonymous or opposite eye. The disease-causing gene of different ethnic microphthalmia families have been localized in individual different intervals on chromosome[11−13], indicating the complex aetiology of microphthalmia which may be associated with multiple genes. Moreover, the severity of the disease phenotype often ranges from mild to extreme microphthalmia (anophthalmia) within a single family, suggesting that the environmental factors might have some effects on the penetrance[2,13]. In this context, it is then not strange that the pathogenic gene of the Chinese microphthalmia family falls out of the previously reported loci. In our subsequent work, a genome-scan analysis using 400 microsatellite markers scattering on all the human chromosomes will be performed to localize and clone the disease-causing gene associated with this Chinese microphthalmia family.

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