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Physical and transcript map of the autosomal dominant colobomatous microphthalmia locus on chromosome 15q12–q15 and refinement to a 4.4 Mb region

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Congenital microphthalmia is a developmental disorder characterized by shortened axial length of the eye. We have previously mapped the gene responsible for autosomal dominant colobomatous microphthalmia in a 5-generation family to chromosome 15q12–q15. Here, we set up a physical and transcript map of the 13.8 cM critical region, flanked by loci *D15S1002* and *D15S1040*. Physical mapping and genetic linkage analysis using 20 novel polymorphic markers allowed the refinement of the disease locus to two intervals in close vicinity, namely a centromeric interval, bounded by microsatellite DNA markers *m3–m17*, and a telomeric interval, *m76–m24*, encompassing respectively 1.9 and 2.5 Mb. Moreover, we excluded three candidate genes, *CKTSF1B1*, *KLF13* and *CX36*. Finally, although a phenomenon of anticipation was suggested by phenotypic and pedigree data, no abnormal expansion of three trinucleotide repeats mapping to the refine interval was found in affected individuals.

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Introduction

Congenital microphthalmia is an ocular malformation characterized by a small eye (ocular anteroposterior axial length < 20 mm), with a reported prevalence at birth of 1.5 per 10 000.¹ Severity of the disease often ranges from mild to extreme (anophthalmia), even within a single family. Three transcription factor genes have been involved in isolated microphthalmia: *CHX10*, *SOX2* and *RAX*.^{2–5}

Recently, a 24 bp deletion in the Sonic Hedgehog gene was found in a three-generation family.⁶ In addition, four other isolated microphthalmia genes have been mapped.^{7–10} In a 5-generation family, we previously mapped the gene responsible for microphthalmia to a 13.8 cM region that was delimited by two intervals in close vicinity: a centromeric *D15S1002–D15S1048* and a telomeric *D15S1043–D15S1040* intervals.⁹ Here, we report a physical and partial transcript map of the 15q12–q15 chromosomal region and genetic refinement of the critical interval.

Methods

Physical and transcript map was constructed using BLAST homology search. We compared various sequences from

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Figure 1 Genetic, physical and partial transcript map of the autosomal dominant colobomatous microphthalmia critical region on chromosome 15q12–q15. **(a)** Contigs and polymorphic markers. *D15S* markers are from the Genethon map; m: novel polymorphic poly(AC) markers; underlined loci represent boundaries of the critical intervals. **(b)** Genes. Candidate genes are indicated in relief and underlined. Potential genes encoding proteins with unknown function are represented by an asterisk. Trinucleotide repeats are indicated. **(c)** Retinal ESTs. Fetal retinal ESTs are underlined.

the critical 15q12–q15 region, namely known microsatellite loci, STSs, gene sequences and transcripts, to genomic clones and contigs GenBank: <http://www.ncbi.nlm.nih.gov/Genbank>. In an attempt to identify candidate genes, we performed a BLASTx comparison between the sequences of the three contigs encompassing the critical interval and proteins including FGFs and various transcription factors containing either a homeodomain, a paired domain, a bHLH domain, a zinc finger or a leucine zipper domain. Each contig sequence was also compared to dbEST and TIGR databases with the aim of finding candidate transcripts dbEST: <http://www.ncbi.nlm.nih.gov/dbEST>; TIGR : <http://www.tigr.org/tdb/>.

In order to identify novel potentially polymorphic microsatellite DNA markers, we performed a BLAST_n search using poly(AC or GT) sequences against the three overlapping contigs (Figure 1a). Family members were genotyped using the novel microsatellite markers (Figure 1a; Table 1). PCR products were size-fractionated by capillary electrophoresis (MegaBace, Amersham Pharmacia Biotech, France). Allele sizes were determined using the Genetic Profiler software.

Exons and flanking intronic DNA sequences of three candidate genes, namely *CKTSF1B1*, *KLF13* and *CX36*, were PCR-amplified (Table 1) and sequenced in two affected individuals. We identified two CAG and one CGG repeats, mapping within the refined 2.5Mb interval (Figure 1b). Analysis of these three repeats in two severely affected individuals from generation IV and in their parents and

grandparents was performed using PCR-amplification (Table 1) followed by both electrophoresis on a 3% agarose gel and capillary electrophoresis.

Results

BLAST analysis and sequence annotation databases (Map View: <http://www.ncbi.nlm.nih.gov/mapview/build34>; UCSC Browser: <http://genome.ucsc.edu/>) made it possible to focus on three contigs encompassing the linked region (Figure 1). We analysed 34 novel potential polymorphic microsatellite markers, 20 of them were informative in the family studied (Figure 1a). On the basis of haplotype analysis (Figure 2), we refined the critical region, which was previously delimited by two intervals in close vicinity, namely a centromeric *D15S1002–D15S1048* interval and a telomeric *D15S1043–D15S1040* region. A new proximal limit for the centromeric interval was defined by a recombination event which occurred with marker m3 in individuals III:11 and IV:17 (Figure 2), whereas another recombination event in individual V:3 allowed us to place the distal limit at the marker m17 locus. The new centromeric interval *m3–m17* contains large repeated regions, either duplicated or triplicated, thus preventing from using the polymorphic poly(AC or GT) markers mapping within this interval for accurate genotyping. Regarding the telomeric interval, individual V:3 is recombinant with marker m76. A recombination event between loci *D15S1007* and *m24* occurred in individual IV:6. The

Table 1 Primers for PCR amplification of 20 novel informative polymorphic markers (m), 3 novel trinucleotide repeats and coding sequences of the CKTSF1B1, CX36 and KLF13 genes

Name	Forward primer	Reverse primer	Name	Forward primer	Reverse primer
m3	5' AATCCAAATCAGAAGAAATTT 3'	5' AGTGAGCTGAGATCATGACA 3'	m38	5' GTTATTCTAGCCTACAGAGCT 3'	5' ACAGGGATCTGACCTCACAA 3'
m15	5' AAGAGACTGGAGCTCCAATG 3'	5' TAGGCTCAGGAAGTAAGCCA 3'	m45	5' CAGTAGGTAGCTGGTAAGAC 3'	5' TGGTCGCCTCACATTTCTGA 3'
m16	5' CAGCTACATTGTGAGTTATCC 3'	5' TACAGGCATGAGCCAACCAT 3'	m46	5' GACATGTACCCTGTGAACCT 3'	5' AGATGATACCACTGTGCTCC 3'
m17	5' CCTAATGCTACATAGGCAGTT 3'	5' GCCACATTCATATTGAGAGATT 3'	m51	5' TTTCTGGGCTCTCCCATTTCT 3'	5' AAGCCAGGTATGACAGTGTG 3'
m18	5' AGTGTCTTTGAGCATGCAG 3'	5' GGAAGCAATGCAACTCCTCA 3'	m53	5' GAACAGAATGAATGCCACCAT 3'	5' GGACAAACTCAAACCACAGC 3'
m20	5' CCAGCATTATGCTTGCCTGA 3'	5' TTGTAGAATCCTACCTCATCC 3'	m69	5' GACTGTCCTTTCCCACTGT 3'	5' CAGGAGGATCAGCTGAGTCT 3'
m24	5' CATGTTGGTCAGGCTGGTC 3'	5' CACTGCCTACTCTGCCCTGT 3'	m74	5' AAGGATACACCTGAATGCCCTA 3'	5' GCCTCCCAAAGTGCTAGGAT 3'
m25	5' TTGCTGCTTCTCAAGAAGGC 3'	5' TCCATCTCCAAGGGTAGAGA 3'	m76	5' TTTTCAGCAGGCGGAATAT 3'	5' TGATGCATTTTCTTTACAGATGC 3'
m28	5' CCCTGAGACTTCCACTTTGA 3'	5' AGGCAGGGGAATCACTTGAA 3'	m78	5' ACACCCAGAAGCAAAACAGG 3'	5' GCTCTGGTGGAGTCACTACTCTC 3'
m35	5' CAGAGAGTAAGCTCTACAAC 3'	5' CCCATAAGTGACGTCCATCT 3'	m79	5' TGAGATCACCCCAACAAACA 3'	5' CCCCTTTGCAAGATTACAGG 3'
(CAG)n1	5' AAGCGTGTAGAATAGGGCCT 3'	5' TAGGAGTTGGGTCCTCCTAA 3'	(CCG)n	5' GATGATCGGCTACTACCTGA 3'	5' CTCCTCTCCGGTCTGC 3'
(CAG)n2	5' TAGTAGAGACAGGGCTTCAC 3'	5' GATGAGGAATCCAGTTGAG 3'			
	Gene	Exon		Forward primer	Reverse primer
	CKTSF1B1	1st exon		5' TCACTCTCGGTCCCGCTGA 3'	5' TCCGCCCCACTCACATCCCT 3'
		2nd exon		5' GCGTTTAAATGCTAGGTGCTA 3'	5' TGCATTCTAGGACATGCTG 3'
	KLF13	1st exon (1st part)		5' CTGACGACTCGCAGCAAGA 3'	5' GATCCGCGCCACCACGAA 3'
		1st exon (2nd part)		5' TAGCGGACCTCAACCAGCAA 3'	5' CTGTGTGAGTTCTCAGGTGC 3'
		1st exon (3rd part)		5' GCCCCGGCCTCAGACAAAGGGT 3'	5' GGCGTGGTCGGGCAGCTC 3'
		2nd exon		5' TGTGAGAGACCAGGGTGTT 3'	5' GGGTTCAACAACGCTGAATC 3'
	CX36	1st exon		5' GCTGCCCAGTCTTTGTCTG 3'	5' GACTCAGTCCAGGTGTGAG 3'
		2nd exon (1st part)		5' TTCCTGTCAGTGACGCAGC 3'	5' AACCTCTAAACAATCTGGCTC 3'
		2nd exon (2nd part)		5' GTGCTGCAGAACACAGAGAA 3'	5' TACAGCCACGTCTGAGCAG 3'

Figure 2 Pedigree of the colobomatous microphthalmia family. Blackened symbols indicate affected individuals; hatched symbols represent individuals with uncertain status; the disease-associated haplotypes are boxed.

new telomeric interval is flanked by *m76* and *m24* markers. Thus, the refined critical region is composed of a 1.9 Mb centromeric *m3–m17* interval and a 2.5 Mb telomeric *m76–m24* interval.

A total of 57 known genes were located within the three contigs overlapping the critical interval (Figure 1b). Of these, three genes, namely *CKTSF1B1*, *CX36* and *KLF13* encode proteins with a possible role in eye development. However, no change in the coding sequence of these genes could be identified in affected individuals. Moreover, subsequent physical mapping data (Map View: <http://www.ncbi.nlm.nih.gov/mapview/build34>; UCSC Browser: <http://genome.ucsc.edu/>) indicated that the *CX36* gene maps telomeric to the *m76–m24* interval.

Analyses of three trinucleotide repeats in affected individuals and healthy controls did not reveal any modification of the migration pattern, ruling out an abnormal expansion of these repeats as the molecular basis of the disease in this family.

Finally, BLASTn search for homologies using the three candidate contigs against dbEST and TIGR (dbEST: <http://www.ncbi.nlm.nih.gov/dbEST>; TIGR: <http://www.tigr.org/tdb/>) databases identified 9 fetal retinal ESTs and 28 retinal ESTs (Figure 1c).

Discussion

A linkage analysis in a large autosomal dominant colobomatous microphthalmia family using 20 novel polymorphic microsatellite DNA markers allowed us to refine the region 15q12–q15 including the disease-causing gene to two intervals in close vicinity, namely a centromeric interval, flanked by microsatellite DNA markers *m3–m17*, and a telomeric interval, *m76–m24*, encompassing respectively 1.9 and 2.5 Mb. We set up a detailed physical and transcript map, which pointed out three candidate genes: *CKTSF1B1*, *KLF13* and *CX36*, encoding proteins which may be involved in eye development.

First, *CKTSF1B1* maps within the refined interval and its transcripts are expressed at a high level in adult and fetal brains.¹¹ It encodes Gremlin, a protein which belongs to the family of bone morphogenetic protein (BMP) regulators. A targeted inactivation of murine *Bmp7* leads to microphthalmia or anophthalmia.¹² In chick embryos, disruption of the BMP signalling by intraocular overexpression of a BMP antagonist results in microphthalmia.¹³ In mouse *Gli3*^{−/−} mutants, gremlin overexpression causes an optic cup reduction.¹⁴ Thus, gremlin interacts with GLI3, a member of the SHH pathway that has recently been shown to be essential for eye morphogenesis.⁶

Second, the *KLF13* gene codes for a zinc-finger transcription factor, expressed in mouse fetal eye (<http://odn.mdacc.tmc.edu/RetinalExpress>). Finally, the *CX36* gene, which encodes connexin36 belongs to the connexin family, including *Cx50*, a gene whose targeted invalidation in the mouse results in microphthalmia.¹⁵ However, complete sequencing of *CKTSF1B1*, *KLF13* and *CX36* exons and flanking intronic sequences led us to exclude these three candidate genes as the causative gene in this family.

To date, the 4.4 Mb refined interval includes 21 other genes encoding proteins that have no known role in eye development. So, they should be considered as candidate by their position only. A total of 11 retinal ESTs were detected within the critical interval: three correspond to hypothetical proteins and the others have no homology with known proteins. Noteworthy, no mouse mutant with microphthalmia has been mapped yet to the mouse chromosome 2 and 7 regions, which share homologies with human chromosome 15q12–q15.

In the five-generation family studied, the apparently increasing penetrance of the disease through successive generations may suggest anticipation, a feature known to result from trinucleotide repeat expansions. However, no abnormal expansion of the three trinucleotide repeats studied was found in this family. Thus, the apparent anticipation may be due to another yet unidentified trinucleotide repeat expansion or may simply be fortuitous.

Finally, the combined genetic and physical refinement of the disease locus to a 4.4 Mb region, the exclusion of three candidate genes (*CKTSF1B1*, *KLF13* and *CX36*) and the identification of retinal ESTs, represent important steps in the attempt to identify the disease-causing gene itself.

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