Mapping a gene for congenital fibrosis of the extraocular muscles to the centromeric region of chromosome 12

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Congenital Fibrosis of the Extraocular Muscles (CFEOM) is an autosomal dominant, ocular disorder characterized by congenital, nonprogressive, bilateral ptosis and external ophthalmoplegia. The pathophysiology of this disorder is unknown and it is unclear if it has a primary neurogenic or myopathic etiology. We report linkage of this disorder, in two unrelated families, to markers in the pericentromeric region of human chromosome 12. *D12S59* does not recombine with the disease giving a two-point lod score of 12.5 ($\theta = 0.00$). *D12S87* and *D12S85* flank the CFEOM locus with two-point lod scores of 8.9 ($\theta = 0.03$) and 5.4 ($\theta = 0.03$) respectively, defining a region of 8 cM. These data establish a map location for CFEOM and demonstrate that this may be a genetically homogeneous disorder.

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Accurate vision is a complex physiologic process dependent on the proper function of many tissues. One important element is motor control of the globe as mediated by the extraocular muscles (EOMs). Abnormalities of these muscles can lead to severe visual impairment secondary to ptosis (drooping of the eyelid), ophthalmoplegia (inability to move the orbits), strabismus (squint) and amblyopia (defective vision). Although little is known about the normal development and physiology of EOMs, they differ significantly from other skeletal muscles in having a higher innervation ratio, more variation in fibre types and unique physiologic features[1,2]. This uniqueness is also reflected in EOM disease pathology. For example, in Duchenne muscular dystrophy, which results in fibrosis and severe muscle wasting in all major muscle groups, the EOMs are spared from the clinical and pathological consequences of dystrophin loss[3,4]. In contrast, the progressive external ophthalmoplegias selectively involve EOMs, and some cases have been shown to result from defects of mitochondrial DNA[5,6]. To learn more about EOM biology and pathology, we are studying several large families with congenital fibrosis of the extraocular muscles (CFEOM), a disorder selective for EOM.

Clinically, CFEOM (MIM no. 135700) is characterized by congenital, nonprogressive, bilateral ptosis and external ophthalmoplegia, with a compensatory backward tilt of the head. The ophthalmoplegia is virtually complete with the eyes strabismic and fixed at 20 to 30 degrees below the horizontal. Forced ductions demonstrate a marked resistance to passive movement[7,8] and any residual eye movements are often notable for aberrant and jerky convergent or divergent movements on attempted gaze into limited fields of vision[9–11]. Patients often have amblyopia or significant refractive errors[7,8]. General neurological examination is normal in the vast majority of cases.

Autosomal dominant CFEOM has been reported in families of many different ethnic backgrounds and appears to be a completely penetrant disorder[7–9,11–13]. Although there is slight variation among family members with regard to the degree of ptosis and ophthalmoplegia, in general the phenotypic homogeneity, both within and between families is striking. There are also reports of sporadic cases clinically indistinguishable from CFEOM[14–16]. All published chromosomal studies of affected individuals have been normal[17,18]. While there are several reports of sporadic and clinically atypical cases of CFEOM in association with other ocular and systemic disorders[17,18], classic autosomal dominant CFEOM is usually an isolated condition.

The pathophysiology of this disorder is unknown, indeed it is unclear if it has a primary neurogenic or myopathic aetiology. Reports of the pathology of EOMs in autosomal dominant cases of CFEOM are variable, and include muscle replacement by fibrosis or connective tissue[7], "fragile" conjunctiva and adhesions between muscle and Tenon's capsule[19], anomalous muscle insertions with or without selectively absent muscles[13] and, in some cases, virtually normal muscles by light and electron microscopic examination[12]. The congenital and static nature of this disorder contrasts with the progressive course of most other ocular myopathies, many of which are mitochondrial in aetiology[5]. These observations suggest that CFEOM represents a developmental defect of EOMs.

We have employed a linkage mapping approach to

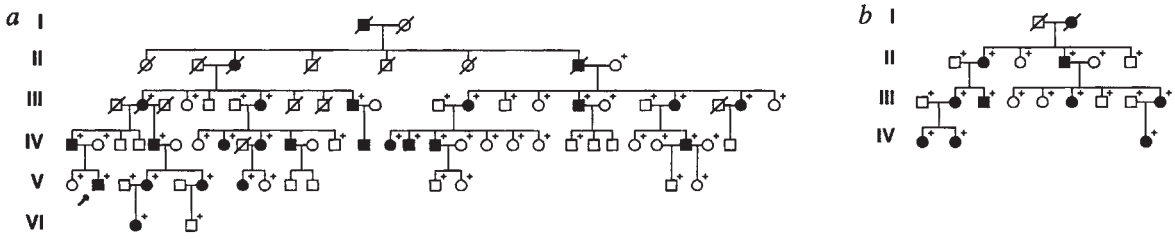

Fig. 1 Pedigrees, as reported by family history, of the two CFEOM families analysed. *a*, family A; *b*, family B. Filled symbols indicate affected with CFEOM. Individuals studied are marked with a plus (+). The proband in pedigree A is indicated with an arrow. References to individuals refer to family (A or B), generation number (Roman numeral) and position number within generation from left to right (Arabic numeral).

place the CFEOM locus in the pericentromeric region of human chromosome 12. This represents the first step toward the positional cloning of this disease gene and a better understanding of EOM development and function.

Clinical description

The proband in pedigree A was identified when he presented to the Children's Hospital neuromuscular clinic at eleven months of age with congenital ptosis and external ophthalmoplegia. After further investigation of his previously unreported New Hampshire family, a total of 20 affected, 18 unaffected family members and ten spouses agreed to participate in the linkage study (Fig. 1*a*). Pedigree B is a southern Ontario family of Scottish descent, of whom all nine affected, five unaffected members and four spouses were available for study (Fig. 1*b*). The inheritance pattern within each family is consistent with autosomal dominant inheritance and complete penetrance as the segregation ratio for these families is 0.49 (33/68 affected offspring with one affected parent). All affected members of each family have congenital and static bilateral ptosis and hypotropic external ophthalmoplegia. There is slight variation in the degree and symmetry of the ptosis, of the hypotropia and strabismus, and of the extent of residual horizontal eye movements within each family. In most of the affected members with residual horizontal movement, convergent or divergent bilateral or unilateral abnormal movements are elicited on attempted upgaze. Some of the affected members of pedigree A have mild facial weakness. Otherwise, they have no abnormalities of tone, strength or reflexes, no cardiac abnormalities, and no fluctuation in ptosis nor ophthalmoplegia with time of day or fatigue. Extensive laboratory, radiologic and surgical pathologic evaluations of several affected members of pedigree A have been consistent with the diagnosis of CFEOM and have shown no abnormalities to suggest mitochondrial dysfunction. Chromosome studies on one affected member of each family were normal.

Linkage of CFEOM to chromosome 12

PCR primers that amplify polymorphic CA repeats at random locations throughout the human genome were employed to identify markers linked to the CFEOM disease locus. Of 37 dinucleotide repeat polymorphisms typed in pedigree A, 34 were found to be unlinked, excluding approximately 1100 cM or one-fifth of the genome (data not shown). This included a dinucleotide marker (*GABRB3*) linked to the Prader-Willi locus[20], which was

Table 1 Pairwise lod scores of chromosome 12 markers with CFEOM

			θ							
Locus		0.00	0.05	0.10	0.20	0.30	0.40	z_{max}	θ_{max}	SI
D12S62	Pedigree A	−∞	−2.0	0.1	1.4	1.4	0.7	1.5	0.25	
	Pedigree B	−∞	1.5	1.5	1.3	0.9	0.4	1.5	0.08	
	Total		−0.5	1.6	2.7	2.3	1.1	2.7	0.21	0.10–0.36
D12S61	Pedigree A	−∞	4.1	3.9	3.2	2.3	1.2	4.1	0.05	
	Pedigree B	−∞	0.1	0.3	0.3	0.2	0.1	0.3	0.16	
	Total		4.2	4.2	3.5	2.5	1.3	4.3	0.07	0.01–0.23
D12S87	Pedigree A	7.3	6.8	6.2	4.8	3.2	1.5	7.3	0.00	
	Pedigree B	−∞	2.0	2.0	1.6	1.1	0.4	2.0	0.07	
	Total		8.8	8.2	6.4	4.3	1.9	8.9	0.03	0.001–0.12
D12S59	Pedigree A	10.2	9.4	8.5	6.7	4.6	2.3	10.2	0.00	
	Pedigree B	2.3	2.1	1.9	1.5	1.0	0.4	2.3	0.00	
	Total	12.5	11.5	10.4	8.1	5.6	2.7	12.5	0.00	+/−0.05
D12S85	Pedigree A	3.7	3.3	2.9	2.0	1.2	0.4	3.7	0.00	
	Pedigree B	−∞	2.0	2.0	1.7	1.1	0.5	2.0	0.07	
	Total		5.3	4.9	3.7	2.3	0.9	5.4	0.03	0.001–0.15
Col2A1	Pedigree A	−∞	3.8	3.9	3.3	2.4	1.3	3.9	0.08	
	Pedigree B	−∞	0.6	0.8	0.9	0.7	0.3	0.9	0.17	
	Total		4.4	4.7	4.2	3.1	1.6	4.7	0.10	0.02–0.26

z_{max}, Maximum likelihood estimate of the lod score; θ_{max}, maximum likelihood estimate of the recombination fraction. SI, Support interval (range of Θs at z_{max} of −1 (refs 35,36).

investigated because of the report of a sporadic case of one child with both disorders[18].

Linkage to chromosome 12 was suggested by three markers with lod scores close to one, at θ ranging from 0.18 to 0.27, within pedigree A. Further analysis of this family using 13 other chromosome 12 markers revealed significant linkage to five of them (Table 1). Three anonymous DNA markers *D12S87*, *D12S59* and *D12S85* were found to completely cosegregate with the disease, giving lod scores of 7.3, 10.2 and 3.7 respectively. The nearest flanking markers, *D12S61* and *Col2A1* demonstrate one and two recombination events respectively between themselves and the CFEOM locus (Table 2), defining a region of approximately 8–9 cM. Pedigree B was tested for linkage to a subset of the chromosome 12 markers (Table 1). *D12S59* was informative for nine meioses and segregated with the disease in all cases, with a lod score of 2.3 at θ = 0.00. *D12S87* and *D12S85* each recombined once in different affected individuals (Table 2) for maximum lod scores of 2.0 at θ = .07. Multipoint analysis of CFEOM versus *D12S87*, *D12S59* and *D12S85* in Pedigree B generated a maximal lod score of 3.6 for placement of the CFEOM locus at *D12S59*. The *D12S59* alleles associated with the disease are different sizes in pedigrees A and B.

Table 2 Key recombination events involving CFEOM locus and selected markers

	Pedigree A			Pedigree B	
Marker	AIV-8	AIV-16	AIV-19	BIII-2	BIV-1
D12S61	ni	–	+	ni	+
D12S87	–	ni	ni	–	+
D12S59	–	–	–	–	–
D12S85	–	ni	ni	+	–
Col2A1	+	+	–	+	–

Individuals, as in Fig. 1.
+, Recombinant event; –, no recombinant event; ni, marker not informative for individual.

The markers used in this analysis are part of two unintegrated linkage maps[21,22]. However, two reports have placed *D12S87* just distal to *D12S59* (refs 23,24), and this is supported by one recombination event in individual BIV-1 (Table 2). At least two offspring of AII-7 and AII-8 are recombinant for *D12S61* but not for *D12S87*, placing *D12S61* distal to *D12S87*. Finally, a recombination event in individual AIV-8 places *Col2A1* distal to *D12S85*. Thus, the integrated map (from 12pter to qter) is *D12S61–D12S87–D12S59–D12S85–Col2A1* with the CFEOM locus at *D12S59*, defining an 8 cM region for the location of the CFEOM gene (Fig. 2). Multipoint analysis of CFEOM versus the map *D12S61–D12S87–D12S59* gave maximal lod scores of 14.9 for placement at *D12S59*, 9.6 for placement in the interval between *D12S61–D12S87* and 10.5 for placement distal to *D12S61*. Similar analysis using the map *D12S59–D12S85–Col2A1* gave maximal lod scores of 14.8 for CFEOM at *D12S59*, 9.0 for CFEOM in the interval *D12S85–Col2A1*, and 9.4 for placement of CFEOM distal to *Col2A1*. Thus, given the assumption that the integrated map is correct, the relative odds for placement of CFEOM in the interval *D12S87–D12S85* are greater than 10,000 to one.

Physical mapping on chromosome 12

Most of the markers used in the linkage study have only been mapped to broad regions of chromosome 12. However, *Col2A1* is mapped to 12q12–q13.2 and *KRAS2* (between *D12S61* and *D12S62*) is mapped to 12p12.1, defining 12p12.1–q13.2 as the physical limits for the CFEOM locus (Fig. 2)[25]. To further refine this localization, relevant markers were amplified from the somatic cell hybrid F11-13 which contains human chromosomes 11 and 12p as its only human content[26]. *D12S62*, *D12S61*, *D12S87* and *D12S59* were all present in the DNA of this hybrid placing them on 12p, while *D12S85*, *Col2A1*, *D12S96* and *D12S103* were absent, suggesting that they are on the long (q) arm (data not shown).

Discussion

Our data demonstrate that a gene for CFEOM resides in the pericentromeric region of human chromosome 12, most likely within the 8 cM interval defined by *D12S87* and *D12S85*. Physical mapping in a somatic cell hybrid suggests that these markers span the centromere with the tightly linked marker, *D12S59*, on the short (p) arm. The nearest physically mapped markers are *Col2A1* at 12q12–q13.2 and *KRAS2* at 12p12.1 (ref. 25), providing outer limits for the physical mapping of this locus on the chromosome.

These data also allow refinement of the physical and genetic maps of chromosome 12. All of the Genethon markers[22] between *D12S87* and *D12S91* are currently mapped to 12p13.2–q24.1 or –q24.3 (Genome Data Base, Baltimore, MD). The refined localization can now be given as 12cen–p12 for *D12S87* and 12cen–q24.1 (or –q24.3) for the remaining loci. Similarly, all loci distal to *D12S59* on the NIH/CEPH collaborative map[21] can be placed unambiguously on 12p. Since our total numbers of meioses

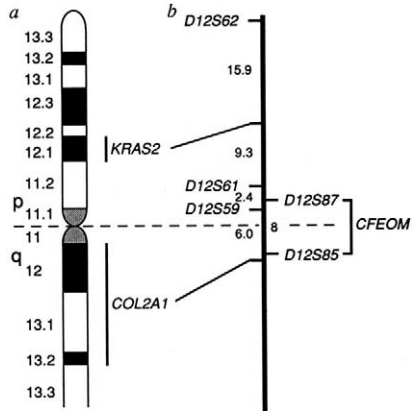

Fig. 2 Map of the centromeric region of human chromosome 12. *a*, physical map showing locations of *KRAS2* and *Col2A1* and their positions on *b*, the integrated genetic map. Markers and distances (in cM) from the NIH/CEPH collabor-ative map[20] are to the left and Genethon linkage markers and dis-tances[21] are to the right. Dashed line indicates approximate position of the centromere. The CFEOM critical region is indicated to the right.

are small and these are not generated from the CEPH mapping families, we have not attempted to derive distances between Généthon and other markers in our integrated map (Fig. 2). However, the relative order provides a useful framework for mapping studies such as ours.

Mapping the CFEOM mutation to human chromosome 12 within two unrelated families is an important step towards positional cloning of the mutant gene, and suggests possible genetic homogeneity for CFEOM. We are currently collecting other pedigrees with this disorder and searching for more genetic markers to further refine the localization and determine if other loci for this disorder exist. The rapid development of improved genetic maps[21,22] and localization of expressed sequence tags[27] and other genes on these maps will make a candidate gene approach feasible. In this regard, it is interesting to note that, among many other genes, the *HOXC* locus has been mapped to 12q12–q13, potentially within our critical region[28]. Given the developmental nature of the CFEOM defect, we are currently developing polymorphic markers around the *HOXC* locus to place this gene cluster on the genetic map.

Elucidation of mutations and the product of the CFEOM gene will lead to a better understanding of the control of development of EOMs in the fetus and may lead to a greater understanding of EOM uniqueness and specificity in pathology. Once mutations causing CFEOM have been identified, it will then be logical to study patients with sporadic but otherwise typical CFEOM, as well as patients with related and sporadic congenital disorders such as strabismus fixus[29], vertical retraction syndrome[30] and unilateral fibrosis with enophthalmos and ptosis[31].

Methodology

Pedigree collection. Each family member was examined by one of the authors (E.C.E.) to confirm status and all living participants were counselled and signed informed consent forms. Photographs and/or video recordings of eye movements were obtained from a majority of affected members.

DNA typing. Blood for DNA extraction was collected from 48 family members of pedigree A and from 18 family members of pedigree B, as indicated by a (+) in Fig. 1. DNA from individual AIII-3 was extracted from postmortem liver. To guard against possible sample mix-ups, each specimen was divided into two and the aliquots were independently processed, as described[32].

Many of the PCR primers for amplifying polymorphic CA repeats were obtained from Val Sheffield and the University of Iowa DNA Marker Cooperative. These were supplemented with markers from the Genethon catalogue[22] purchased from Research Genetics Inc. (Huntsville, Alabama). Other primers were synthesized on an ABI model 380B DNA synthesizer. All primer sequences are available from the Genome Data Base (Johns Hopkins University, Baltimore, MD). Amplification of each dinucleotide repeat polymorphism was performed in an MJ Research thermal cycler using 120 ng of genomic DNA in a 10 µl PCR containing: 1 µl 10X GeneAmp buffer (Perkin Elmer), 200 µM each of dATP, dTTP, dGTP and dCTP, 1 µCi α-^{32}P-dCTP (3,000 Ci mmol^{-1}), 40 ng of each primer and 0.5 U *Taq* polymerase (Perkin Elmer). The Iowa markers were amplified for 30 cycles of: 30 s at 94 °C; 1 min at 55 °C; and 3 min at 72 °C, followed by 10 min at 72 °C. The Genethon markers were amplified for 35 cycles of: 40 s at 94 °C; and 30 s at 55 °C. Markers *D12S59*, *D12S60*, *D12S61* and *D12S62* were amplified for 27 cycles of: 30 s at 94 °C; 75 s at 55 °C; and 15 s at 72 °C, followed by 7 min at 72 °C. PCR products were separated on denaturing polyacrylamide sequencing gels and subjected to autoradiography. The *Col2A1* VNTR was amplified as described[33] using 25 µl reaction volumes and separated on 2.5% NuSieve GTG (FMC BioProducts) agarose gels.

Linkage analysis. Lod scores were calculated using the Linkage 5.1 package of programs[34] assuming an autosomal dominant inheritance, complete penetrance and a disease gene frequency of 0.000005 (that is, disease incidence of 1/100,000 births). Changing the penetrance to 0.9 or varying the incidence by several orders of magnitude had negligible effects on the results. For the random search, each polymorphism was assumed to have ten alleles of equal frequency. Calculations with linked markers in Table 1 used actual allele frequencies determined from 38 different chromosomes in the two families (this refinement, however, had only minimal effects on the results). Two point calculations were done using MLINK and multipoint analysis used LINKMAP, assuming distances of 1, 2, 5 and 1 cM, for the intervals between *D12S61–D12S87–D12S59–D12S85–Col2A1*, respectively.

Physical mapping in somatic cell hybrids. DNA extracted from the somatic cell hybrid F11-13 containing human chromosomes 11 and 12p was obtained from Carol Jones[26]. DNA extracted from somatic cell hybrids containing human chromosome 12 and human chromosome 11 were from the NIGMS human/rodent mapping panel 2 (Coriell Cell Repository, Camden, NJ). Nonradioactive PCR products of these DNAs with primers for markers *D12S61*, *D12S87*, *D12S59*, *D12S85*, *D12S96* and *D12S103* were separated on native 8% polyacrylamide gels. The larger *Col2A1* product was separated on agarose gels.

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