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Refined Mapping of a Gene Responsible for Fukuyama-Type Congenital Muscular Dystrophy: Evidence for Strong Linkage Disequilibrium

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Summary

Fukuyama-type congenital muscular dystrophy (FCMD), the second most common form of childhood muscular dystrophy in Japan, is an autosomal recessive severe muscular dystrophy associated with an anomaly of the brain. After our initial mapping of the FCMD locus to chromosome 9q31-33, we further defined the locus within a region of ~5 cM between loci D9S127 and CA246, by homozygosity mapping in patients born to consanguineous marriages and by recombination analyses in other families. We also found evidence for strong linkage disequilibrium between FCMD and a polymorphic microsatellite marker, mfd220, which showed no recombination and a lod score of (Z) 17.49. A "111-bp" allele for the mfd220 locus was observed in 22 (34%) of 64 FCMD chromosomes, but it was present in only 1 of 120 normal chromosomes. This allelic association with FCMD was highly significant (χ^2 =50.7; P<.0001). Hence, we suspect that the FCMD gene could lie within a few hundred kilobases of the mfd220 locus.

Introduction

Since the discovery of the Duchenne muscular dystrophy (DMD) gene product, "dystrophin" (Hoffman et al. 1987), by the "positional cloning" approach, intensive investigations have been under way toward both resolution of the pathophysiology of muscular dystrophy and improvement of therapeutic approaches. Another muscular dystrophy, Fukuyama-type congenital muscular dystrophy (FCMD), is an autosomal recessive disorder characterized by severe congenital muscular dystrophy associated with CNS involvement. The syndrome was first described in 1960 (Fukuyama et al. 1960). The phenotype consists of muscular dystrophy combined with brain anomalies due to a defect in the migration of neurons (Fukuyama et al. 1981). It is the second most common form of childhood muscular dystrophy in Japan; the incidence is 7–12/100,000. One in 100 persons is presumed to be a heterozygous carrier (Osawa 1978; Fukuyama and Ohsawa 1984).

Patients with FCMD manifest weakness of facial and limb muscles and general hypotonia that usually appears before 9 mo of age. Functional disabilities are more serious in patients with FCMD than in DMD patients; usually the maximum motor function is shuffling, and most patients are never able to walk. Simultaneously they exhibit severe mental and speech retardation, and they require careful nursing, since there is no effective therapy. Patients usually become bedridden before 10 years of age, because of generalized muscle atrophies and joint contractures, and most of them die by the age of 20 years (Fukuyama et al. 1981).

The cause of this pathology is unknown. No strong hypothesis has emerged with respect to the biochemical defect responsible for FCMD, and no cytogenetic defects are obvious. Dystrophin is known to be associated with a large oligomeric complex of sarcolemmal glycoproteins (dystrophin-associated proteins/[DAPs]) (Ervasti and Campbell 1991). Matsumura et al. (1993) have reported that expression of DAPs, especially 43DAG, is abnormally low in FCMD patients, and others have noted a significant reduction in immunostaining of a laminin isoform (merosin), an extracellular matrix linked with DAPs (Hayashi et al. 1993). Genes encoding these proteins are located on chromosomes 3p21 (Ibraghimov-Beskrovnaya et al. 1993) and 6q22-23 (Tryggvason 1993), respectively.

However, it was around D9S58 on chromosome 9q31-33 that we recently localized the FCMD locus, using genetic linkage analysis and homozygosity mapping (Toda et al. 1993). Subsequently we used additional markers in this region to refine the FCMD locus. We here describe mapping of the FCMD locus within a region of ~5 cM and

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present evidence for strong linkage disequilibrium between FCMD alleles and a polymorphic microsatellite marker, mfd220.

Subjects, Material, and Methods

FCMD Families

Twenty-one of the FCMD families providing DNA for the genetic linkage analyses in the present study had formed the basis of a previous study (Toda et al. 1993). An additional 10 families permitted linkage analysis of DNA samples from a total of 99 individuals, 42 of whom were affected. Seventeen affected individuals in 15 of these families were the offspring of consanguineous marriages. For disequilibrium analysis, four families with single affected individuals were added. FCMD was diagnosed on the basis of standard clinical criteria (Fukuyama et al. 1981).

DNA Typing

DNA was extracted from whole blood, biopsied skeletal muscle, and formalin-fixed and paraffin-embedded autopsy specimens, according to standard techniques (Goelz et al. 1985; Sambrook et al. 1989). Individuals were genotyped with polymorphic microsatellite markers CA246 (authors' unpublished data), D9S58 and D9S59 (Kwiatkowski et al. 1992), D9S105 and D9S106 (Wilkie et al. 1992), HXB (Ozelius et al. 1992), D9S109 (Furlong et al. 1992), D9S127 (Lyall et al. 1992), D9S176 (Weissenbach et al. 1992) and mfd220 (Weber 1993). PCR was performed in 25μl reaction volumes containing 20 ng of genomic DNA, 20 pmol of one unlabeled primer and 20 pmol of one primer end-labeled with 1.0 mCi [γ-^{32}P]ATP by using T4 polynucleotide kinase, 1 $\times$ PCR buffer (16.6 mM NH_4SO_4, 67 mM Tris-HCl pH 8.8, 10 mM β-mercaptoethanol, and 6.7 mM EDTA), 10% (v/v) dimethyl sulfoxide, 1.5 mM each dNTP, 5 mM $MgCl_2$, and 1.25 units of *Taq* DNA polymerase. Samples were incubated in a DNA thermocycler (Nippon Genetics) for 36 cycles under the following conditions: 94°C for 2 min, 55°C for 3 min, and 72°C for 2 min. The first denaturation and final elongation steps were extended to 5 min and 10 min, respectively. The PCR products were resolved on 6% polyacrylamide gels containing 7 M urea and 32% formamide.

Linkage Analysis

The FCMD families were analyzed for genetic linkage by using the LINKAGE program, version 5.2 (Lathrop et al. 1984). All complex matings were dealt with by breaking the loops and inserting a genetically identical person (double individual) into the pedigree. The gene frequency for the FCMD allele was assumed to be .0052, as reported elsewhere (Osawa 1978). Allele frequencies for each marker were determined in 120 chromosomes from unrelated Japanese individuals. Recombination frequencies (θ) were assumed to be equal between males and females. The MLINK program was used for pairwise analysis. The significance of linkage disequilibrium was tested using standard χ^2 formulas, with the Yates correction if the size of any of the expected classes was fewer than five.

Results

Linkage Analysis

The results of two-point linkage analyses in FCMD families, with each of seven markers (CA246, D9S105, D9S109, D9S127, D9S176, mfd220, and D9S58) are presented in table 1. The order of these loci was estimated as follows: centromere–D9S176–3 cM–(D9S109,D9S127)–1.7 cM–mfd220–(CA246)–8.5 cM–D9S58–2.8 cM–D9S105–telomere (Blumenfeld et al. 1993; Kwiatkowski et al. 1993*a*, 1993*b*; Attwood et al. 1994; Buetow et al. 1994). CA246 was ordered tentatively on the basis of recombination events observed in the FCMD families, homozygosity in inbred patients, and pairwise linkage analysis. Significant Z values to the FCMD locus were obtained at all of the seven loci examined. It was noteworthy that there were no obligate recombinants between FCMD and mfd220, where $Z = 17.49$.

Recombination and Homozygosity Mapping

To further investigate the region cosegregating with FCMD, we analyzed recombination events in FCMD families. Haplotypes were constructed assuming the most parsimonious linkage phase. Seven families exhibited recombination between FCMD and some marker loci. Figure 1 shows examples of recombination events detected in the FCMD pedigrees. Crossing-over was evident between D9S127 and more centromeric loci in families 11 and 30. Recombinations at more distal loci, D9S105 and/or D9S58, were observed in families 26 and 27.

In autosomal recessive disorders, affected individuals from consanguineous families may be homozygous by descent, at the region surrounding the disease locus (Lander and Botstein 1987). We examined affected members of the inbred FCMD families for homozygosity at seven 9q microsatellite loci (fig. 2). Most of the patients were homozygous at the mfd220 locus, in spite of remarkably high heterozygosity in the general population. The patients in families 2, 7, 19, and 21 were heterozygous at D9S176, and those in families 1, 7, and 14 were heterozygous at CA246 and D9S58. The results of the homozygosity mapping suggest that the FCMD gene lies within a region distal to D9S176 and proximal to CA246. Taking both analyses into consideration, we concluded that the most likely location of the FCMD gene is between two loci, D9S127 and CA246.

Linkage Disequilibrium

The prevalence of FCMD in Japan suggests the possibility of a founder effect, i.e., a common origin of most

Table 1

Pairwise Z, at Various θ Values, of Chromosome 9 Markers with FCMD

	Z AT θ =									
MARKER	.00	.01	.05	.10	.15	.20	.30	.40	Z_{max}	θ_{max}
D9S176	−∞	6.00	6.95	6.32	5.31	4.22	2.26	.84	6.95	.05
D9S109	−∞	9.47	8.61	7.07	5.57	4.20	2.02	.63	9.47	.01
D9S127	−∞	10.34	9.65	8.27	6.84	5.46	3.04	1.20	10.34	.01
mfd220	17.49	16.97	14.93	12.46	10.14	7.98	4.32	1.65	17.49	.00
CA246	6.34	7.20	7.22	6.25	5.09	3.95	2.01	.69	7.42	.03
D9S58	−∞	6.31	8.00	7.61	6.51	5.27	2.75	.92	8.04	.06
D9S105	−∞	3.10	4.91	4.81	4.15	3.32	1.72	.57	4.99	.07

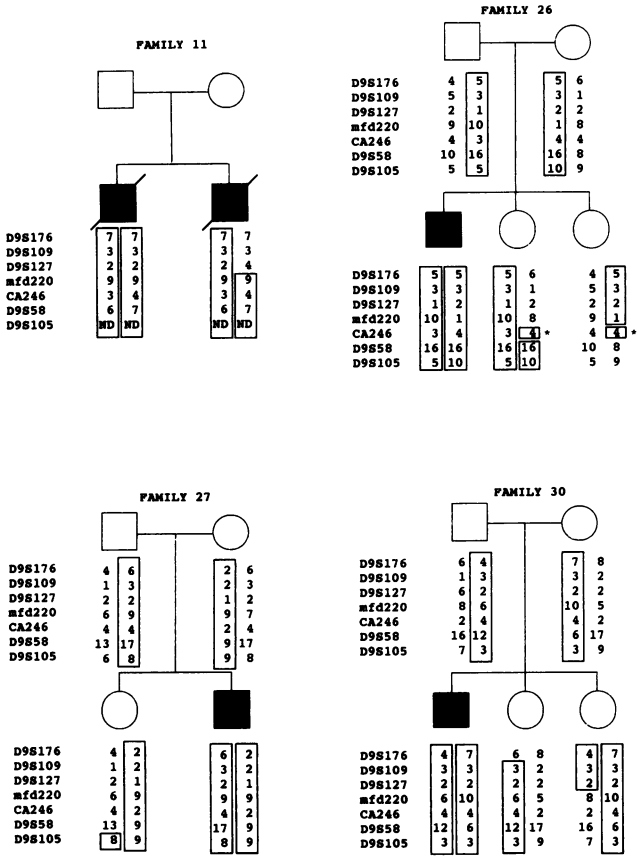

Figure 1 Recombination mapping. Genotypes are indicated for seven polymorphic microsatellite loci in families demonstrating crossovers near the FCMD gene. The haplotype carrying the FCMD allele is boxed. Asterisks under pedigree 26 indicate uncertainty with respect to the precise positions of crossovers, because of the uninformativeness of CA246 in the mother of this family. ND = not determined, because of PCR failure in formalin-fixed samples. The observed recombination events in these families place the FCMD gene proximal to D9S58 and distal to D9S127.

affected alleles. We could type the 64 chromosomes of 32 FCMD patients (one from each family) at the mfd220 locus; 12 mfd220 alleles had been revealed among 60 unrelated Japanese individuals, 90% of whom were heterozygous. Among these 12 alleles, 8 were present in FCMD chromosomes. However, a "111-bp" allele was significantly overrepresented (table 2): the "111-bp" allele at the mfd220 locus was seen in 22 (34%) of the 64 FCMD chromosomes, but this allele was observed in only 1 of the 120 normal chromosomes ($\chi^2 = 50.7$; $P < .0001$). No other marker locus yielded mathematically significant evidence for linkage disequilibrium.

Discussion

The salient features of FCMD are dystrophic muscles with elevated levels of serum creatine kinase and severe mental retardation. The involvement of multiple siblings of both sexes, as well as consanguinity of parents of some affected individuals, imply autosomal recessive inheritance of the FCMD allele (Fukuyama et al. 1981; Fukuyama and Ohsawa 1984).

Since no strong hypothesis with respect to the biochemistry of FCMD has been put forward, and since no cytogenetic defects have been reported, we considered that the

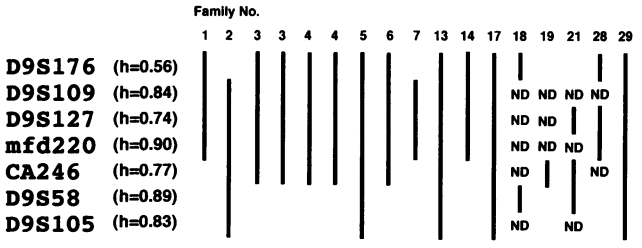

Figure 2 Homozygosity mapping. Solid lines denote portions of the chromosomes that are homozygous in each inbred patient. ND = not determined, because of PCR failure in formalin-fixed samples; and h = observed heterozygosity. The results of this procedure support placement of the FCMD gene distal to D9S176 and proximal to CA246.

Table 2

Linkage Disequilibrium of FCMD with mfd220

PCR Product Size (bp)	No. of Chromosomes Observed	
	Control	FCMD
129	1	1
127	4	0
125	3	0
123	4	2
121	5	0
119	24	11
117	8	0
115	11	1
113	34	18
111	1	22
103	3	2
101	22	7
Total	120	64

NOTE.—$\chi^2 = 50.7$; 6 df; $P < .0001$.

best approach toward the isolation of the FCMD gene was "positional cloning" by linkage analysis. Consequently, we localized the FCMD locus to chromosome 9q31-33, using genetic linkage analysis (Toda et al. 1993).

By extending our study with additional markers and more families, we have been able to define the FCMD locus within the ~5 cM of genomic DNA between D9S127 and CA246, a region that includes the mfd220 locus. The markers reported here will provide useful resources for accurate prenatal and carrier diagnosis of members in families carrying FCMD. The close proximity of mfd220 to FCMD is strongly supported by detection of tight linkage disequilibrium.

Linkage disequilibrium between polymorphic DNA marker loci and disease loci has been described for a number of genetic diseases. In cystic fibrosis (CF), strong allelic associations existed between the loci lying within the >300-kb interval and the disease locus, and the loci defined by DNA markers XV2c and CS.7 were proved to lie in a region 150 kb away from the CF gene (Farrall et al. 1987; Kerem et al. 1989). Similarly, two loci (D4S95 and D4S127) showed linkage disequilibrium with the Huntington disease (HD) locus (MacDonald et al. 1992); D4S95 was also proved to lie ~200 kb away from the 5′ end of the HD gene (Huntington's Disease Collaborative Research Group 1993). Furthermore, strong linkage disequilibrium has been reported between polymorphic alleles of the colony-stimulating factor 1 receptor (CSF1R) gene on chromosome 5 and diastrophic dysplasia (DTD) in Finland, and that study predicted that the DTD gene should lie within a region ~60 kb proximal to the CSF1R gene (Hästbacka et al. 1992).

We have shown here that 22 (34%) of 64 FCMD chromosomes carried a "111-bp" allele at the mfd220 locus, even though this allele was observed in only 1 of 120 normal chromosomes. Since the carrier frequency for FCMD is estimated to be ~1% in the Japanese population, the majority of the "111-bp" chromosomes present in the normal population might reflect FCMD carriers, although such a conclusion is highly speculative. Since the higher mutation rates and the large number of alleles typical of microsatellite loci (Kwiatkowski et al. 1993*b*) can affect allelic frequencies significantly, it is conceivable that detection of linkage disequilibrium may be obscured. However, our results demonstrate that not only is disequilibrium analysis based on microsatellite markers feasible, but that it can lead to a reduction in the size of a candidate region.

On the basis of evidence in the other studies described above, we strongly suspect that the FCMD gene lies within a few hundred kilobases of mfd220. Identification of additional polymorphic markers very close to mfd220 by using YAC clones, as well as analysis of linkage disequilibrium with these new markers, would further clarify the region likely to contain the FCMD gene.

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