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Assignment of a Form of Congenital Muscular Dystrophy with Secondary Merosin Deficiency to Chromosome 1q42

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Summary

We have previously reported an autosomal recessive form of congenital muscular dystrophy, characterized by proximal girdle weakness, generalized muscle hypertrophy, rigidity of the spine, and contractures of the tendo Achilles, in a consanguineous family from the United Arab Emirates. Early respiratory failure resulting from severe diaphragmatic involvement was present. Intellect and the results of brain imaging were normal. Serum creatine kinase levels were grossly elevated, and muscle-biopsy samples showed dystrophic changes. The expression of the laminin-α2 chain of merosin was reduced on several fibers, but linkage analysis excluded the *LAMA2* locus on chromosome 6q22-23. Here, we report the results of genomewide linkage analysis of this family, by use of homozygosity mapping. In all four affected children, an identical homozygous region was identified on chromosome 1q42, spanning 6–15 cM between flanking markers D1S2860 and D1S2800. We have identified a second German family with two affected children having similar clinical and histopathological features; they are consistent with linkage to the same locus. The cumulative LOD score was 3.57 ($\theta = .00$) at marker D1S213. This represents a novel locus for congenital muscular dystrophy. We suggest calling this disorder "CMD1B." The expression of three functional candidate genes in the CMD1B critical region was investigated, and no detectable changes in their level of expression were observed. The secondary reduction in laminin-α2 chain in these families suggests that the primary genetic defect resides in a gene coding for a protein involved in basal lamina assembly.

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Introduction

In the past few years, there has been remarkable progress in the understanding of the genetic and biochemical basis of the muscular dystrophies. The majority of these conditions are caused by mutations in genes that encode sarcolemmal or extracellular-matrix proteins. A deficiency of the sarcolemmal protein dystrophin is the hallmark of Duchenne and Becker muscular dystrophies (MIM 310200) (Koenig et al. 1987), whereas the sarcoglycans are involved in the limb-girdle muscular dystrophies (LGMDs) (Campbell 1995). The laminin-α2 chain is deficient in merosin-deficient congenital muscular dystrophy (CMD) (Tomé et al. 1994; Dubowitz 1995). A congenital myopathy with some clinical and histopathological similarities to CMD (Hayashi et al. 1998) is caused by mutations in the integrin α7 gene. Bethlem myopathy (MIM 158810), a condition that can mimic CMD (Jobsis et al. 1999), is the result of defects in collagen VI (Jobsis et al. 1996; Pan et al. 1998). It has also been reported that some patients with LGMD have partial merosin deficiency (Herrmann et al. 1996; Mora et al. 1996; Naom et al. 1997; Sewry et al. 1997; Cohn et al. 1998; Muntoni et al. 1998*a*). The results of mutational analysis of the laminin-α2 gene (*LAMA2*) on chromosome 6q22-23 demonstrated that this group of patients represents a milder allelic variant of merosin-deficient CMD (Allamand et al. 1997; Naom et al. 1998, 2000).

A deficiency of extracellular-matrix proteins can also be found in other muscular dystrophies. A reduced expression of the laminin chains of merosin has been reported in Fukuyama muscular dystrophy (FCMD [MIM 253800]), a form of CMD with a severe involvement of the CNS (Hayashi et al. 1993). Since the defective gene in FCMD has been mapped to chromosome 9, the reduction in merosin expression represents a secondary phenomenon and raises the possibility of the basal lamina playing a major role in the disease. The recent observation that the FCMD gene product is secreted ex-

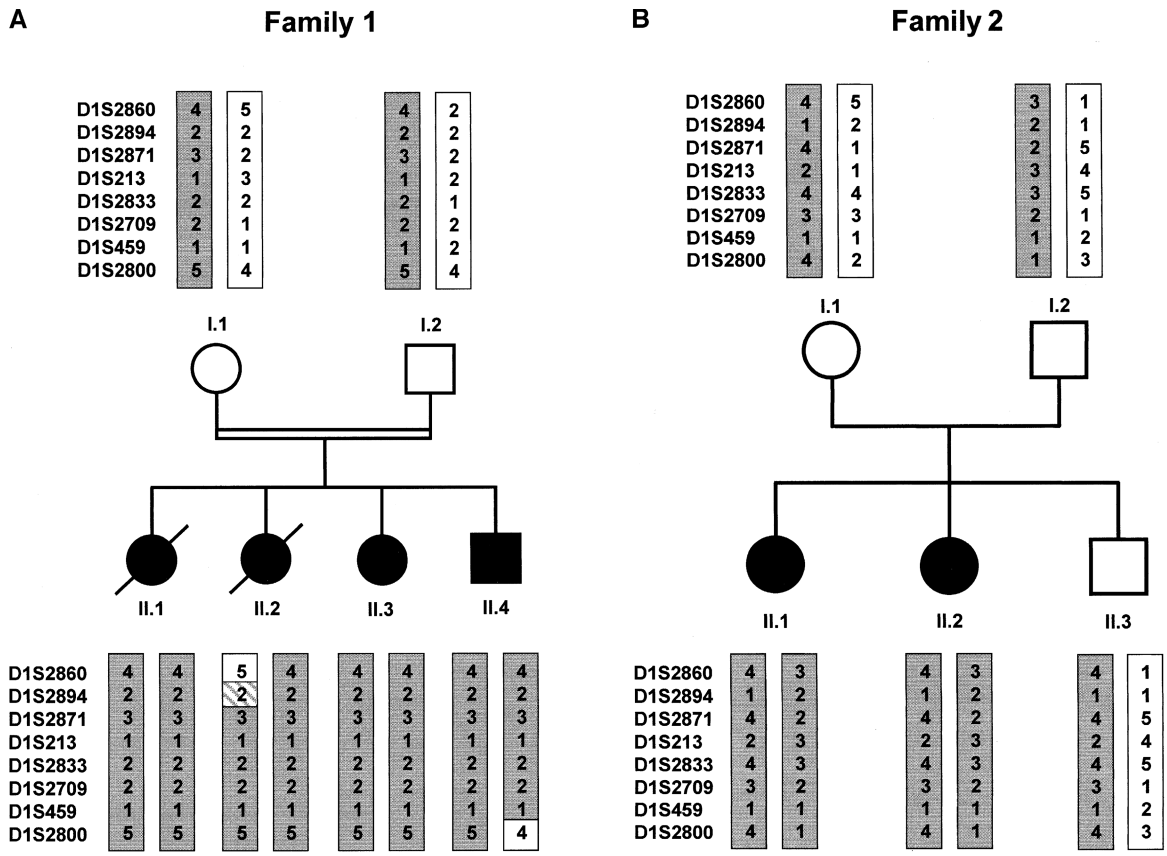

Figure 1 Pedigrees of families 1 (*A*) and 2 (*B*), showing the haplotypes for chromosome 1q42 markers. The disease haplotype is denoted by the gray-shaded bars. The diagonally hatched section of a bar denotes an allele that could not be unequivocally assigned. The CMD1B critical region is defined, in family 1, by recombination events in patients II.2 and II.4.

tracellularly substantiates this prediction (Kobayashi et al. 1998). A reduction in the expression of the laminin-α2 chain of merosin has also been reported in muscle-eye-brain disease (MEB [MIM 253280]), a form of CMD characterized by severe mental retardation and by ocular and brain abnormalities (Haltia et al. 1997). The recent mapping of this condition to chromosome 1p32-34 again suggests that the reduction in merosin expression is a secondary phenomenon (Cormand et al. 1999).

We recently described a form of CMD characterized by proximal muscle weakness, muscle hypertrophy, and early respiratory failure (Muntoni et al. 1998*b*). Affected individuals were demonstrated to have a deficiency of laminin α2 in muscle, but this was a secondary phenomenon, since linkage to the *LAMA2* locus on chromosome 6q22-23 was excluded (Muntoni et al. 1998*b*). Genomewide linkage analysis of this family has allowed us to map the locus responsible for this form of CMD to chromosome 1q42. A second family with similar clinical and histopathological features has been identified. The family data are consistent with linkage to the same locus, thereby providing further evidence that this phenotype represents a separate form of muscular dystrophy.

Patients, Material, and Methods

Case Histories

Family 1.—The clinical details of this consanguineous family from the United Arab Emirates (fig. 1*A*) have been reported elsewhere (Muntoni et al. 1998*b*) and are summarized in table 1. Early motor milestones were delayed in the four affected children; they were able to walk but could not run, and they rose from the floor with the use of a Gower maneuver. Facial weakness, generalized muscle hypertrophy, and wasting of the sternocleidomastoids and pectoralis muscle were consistent features. For >9 years, there was no significant progression of the muscle weakness; however, the two oldest girls died of respiratory complications, one at age 4 years and the other at age 7 years. The two remaining children, ages 8 and 5 years, were found to be hypoventilating at night and have been ventilated, by use of nocturnal ventilation via

Table 1

Summary of Clinical Features

	Severity of Clinical Features of Patients in[a]					
	Family 1				Family 2	
Clinical Feature	II.1	II.2	II.3	II.4	II.1	II.2
Sex	F	F	F	M	F	F
Age:						
In 1999 (years)	D	D	12	9	8	3
At presentation (mo)	20	24	28	0	3	18
At sitting (mo)	8	8	8	10	12	7
At walking (mo)	20	24	28	18	30	18
Muscle weakness:						
Facial muscles	+	+	+	+	+	+
Neck muscles	+++	+++	+++	+++	++	++
Shoulder girdle	++	++	++	++	++	++
Limb girdle	++	++	++	++	++	++
Distal muscles	+	+	+	+	+	+
Muscle hypertrophy	++	++	++	++	++	++
Contractures:						
Spine	NA	NA	+	+	−	−
Achilles tendon	+	+	+	+	+	+
Elbows	−	−	−	−	−	−
Respiratory involvement	+++	+++	+++	+++	NA	NA
CK elevation	+++	+++	+++	+++	+++	+++
Muscle biopsy:						
Dystrophic changes	+++	+++	+++	+++	+++	NA
Reduction of laminin-α2	++	++	++	+	++	NA

[a] D = deceased; + = mild; ++ = moderate; +++ = severe; − = absent; NA = not available.

a biphasic positive airway pressure (BiPAP) machine, with excellent results. Intellect was normal. Investigations revealed grossly elevated creatine kinase (CK) levels in all the children (range 2,270–7,650 IU/liter; normal values <190 IU/liter). Muscle needle-biopsy samples showed a dystrophic picture in all four children (fig. 2*a*), with normal immunolabeling for dystrophin and for α, β, and γ sarcoglycans (not shown). Antibodies to laminin α2 showed reduced labeling on several fibers (fig. 2*b*). Expression of integrins α7 and β1D was also reduced on the same fibers (not shown). Laminin α5 was overexpressed in the youngest girl but not in the biopsy samples from the other children.

Family 2.—Family 2 is a German nonconsanguineous family with two affected sisters and a healthy older boy (fig. 1*B* and table 1). The older sister, now age 11 years, presented with generalized hypotonia and delayed motor milestones during the 1st year of life. She sat at age 12 mo and walked at age 30 mo. At age 18 mo, she had an episode of spontaneous rhabdomyolysis. She is currently able to walk a distance of 200–300 m. At a recent examination, she showed symmetric weakness that was more pronounced proximally than distally. She had facial weakness and hypertrophy of the gastrocnemius and quadriceps. She could rise from the floor, by use of a Gower maneuver, in 5 s. Her younger sister, age 5 years, was similarly affected. She sat unsupported at age 7 mo and walked at age 18 mo. At age 15 mo she had a rhabdomyolysis develop during an intercurrent infection. On examination at age 5 years, she had generalized muscle weakness and rose from the floor by use of a Gower maneuver. She had mild bilateral hypertrophy of the gastrocnemius and quadriceps, and there was a mild facial weakness. Cognitive development was normal. Findings from cardiological examination and from echocardiography were normal in the younger sister; in the older sister, the left-ventricular fractional shortening was in the lower range of normal. Serum CK levels were grossly elevated in both children (range 1,729–4,000 IU/liter).

The muscle-biopsy sample showed a dystrophic picture with normal expression of dystrophin and of proteins of the sarcoglycan complex. Immunostaining for the laminin-α2 chain of merosin showed marked variation of expression from fiber to fiber (not shown). Laminins α5 and α4 were overexpressed.

Genotypic Analysis

After informed consent was obtained, DNA extraction from whole-blood samples was performed by use of proteinase K/SDS digestion, followed by phenol/chloroform

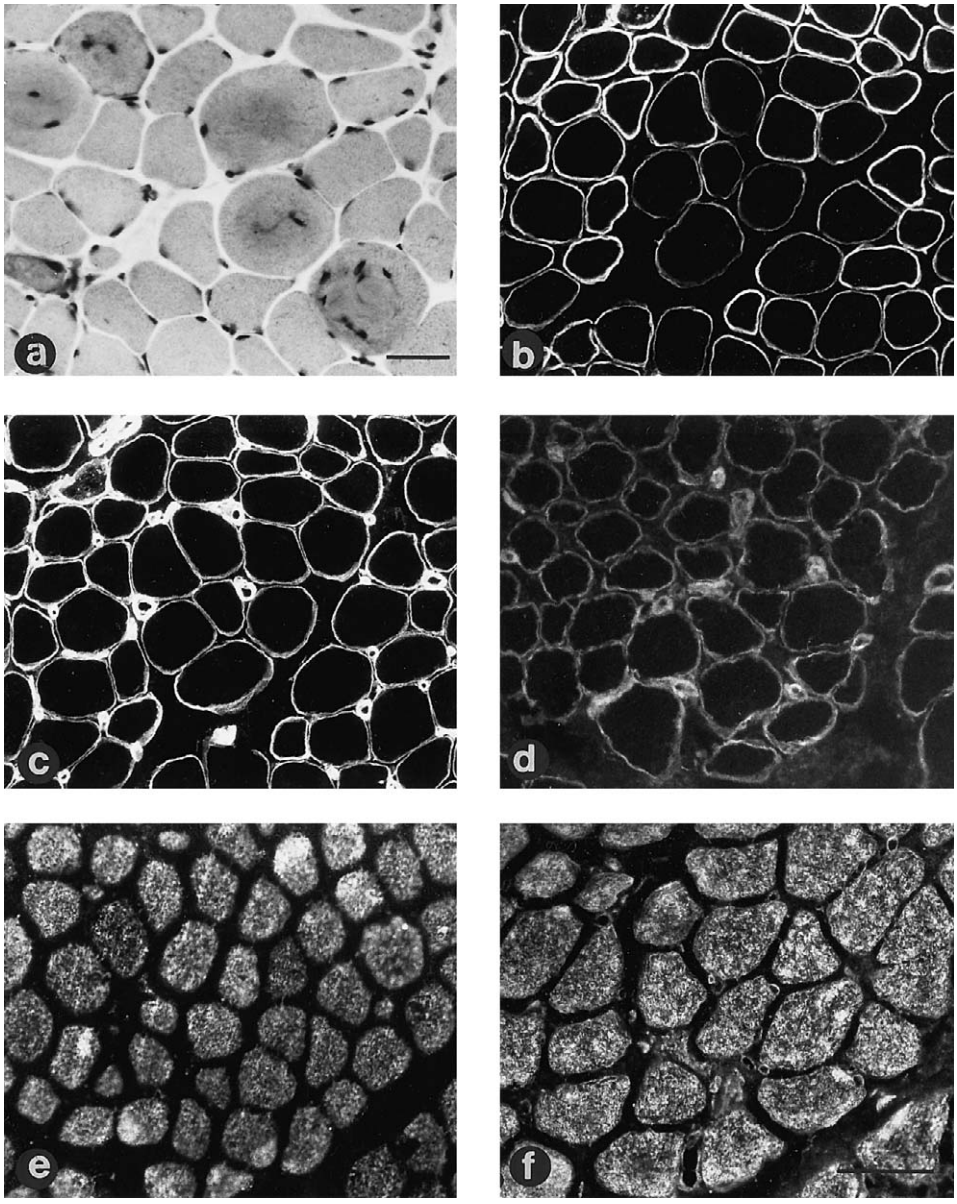

Figure 2 Frozen sections of the muscle-biopsy samples from patients II.4 (*a–c, e,* and *f*) and II.3 (*d*) from family 1; sections were stained with hematoxylin and eosin (*a*) and were immunolabeled for laminin-α2 (*b*), heparan sulfate proteoglycan (*c*), nidogen (*d*), α-actinin 2 (*e*), and actin (*f*). Note the dystrophic morphology; the reduced laminin-α2 on some fibers but normal expression of heparan sulfate proteoglycan; and the presence of nidogen, α-actinin 2, and actin. Bars = 50 μm (bar in *a* also applies to panels *b–d,* and *e*).

extraction, according to standard procedures. Each member of family 1 was typed with the use of the ABI linkage mapping set I. This consisted of 358 fluorescent, highly informative $(CA)_n$-repeat microsatellite markers located at average intervals of 10 cM.

The PCR reactions were performed with the use of 12.5 ng genomic DNA, 1 × GeneAmp PCR buffer II, 250 μM each dNTP, 2.5 mM $MgCl_2$, 1.25 pmol of both forward and reverse primers, and 0.25 U Ampli*Taq* Gold DNA polymerase (PE Biosystems) in a final volume of 5 μl. PCR amplification was done by means of a 9600 thermal cycler (PE Biosystems), with use of the parameters recommended by the manufacturer.

Amplified products were separated by electrophoresis, with use of a denaturing 5% polyacrylamide gel (Gene Page Plus; Amresco), on an ABI PRISM 377 DNA sequencer (PE Biosystems). Markers were typed by use of GENESCAN, version 2.02, and GENOTYPER, version 2.01, software (PE Biosystems). For the refinement of the candidate area on chromosome 1q42, markers

D1S2860, D1S2894, D1S2871, D1S2833, D1S2709, and D1S459, selected from the Généthon collection (Dib et al. 1996), were studied in addition to markers D1S213 and D1S2800 from the ABI panel. Primers were purchased from PE Biosystems. In all cases, the forward primer was modified, at the 5′ end, with a FAM, TET, or HEX fluorescent label. The order and distance between markers used in the linkage analysis are depicted in figure 3.

Linkage Analysis

Linkage analysis was performed with the use of the LINKAGE 5.2 package, according to the assumptions of autosomal recessive inheritance, an equal recombination frequency for males and females, and a disease-gene frequency of .0001. Two-point LOD scores were calculated by use of the MLINK program. Allele frequencies were calculated either with reference to the Généthon database, where possible, or on the basis of our own data from a total of 92 individuals from 18 consanguineous families primarily of Turkish and Asian origin.

Muscle Biopsy and Immunohistochemistry

Muscle needle-biopsy samples were available from all affected children in family 1 and from the oldest sister in family 2. All samples were frozen and were stained with a panel of histological and histochemical stains, according to standard procedures (Dubowitz 1985). Unfixed cryostat sections were immunolabeled with antibodies against the following candidate proteins: nidogen (rabbit polyclonal; kindly provided by Dr. R. Timpl), skeletal-muscle α-actin (mouse monoclonal; Dako), α-actinin 2 (rabbit polyclonal; kindly provided by Dr. A. Beggs), and heparan sulfate proteoglycan (perlecan)(rat monoclonal; Chemicon). This was followed by use of an appropriate biotinylated secondary antibody and Texas red conjugated to streptavidin, as described elsewhere (Sewry et al. 1995).

Results

Exclusion of the Known CMD Loci

Linkage to the following loci responsible for CMD was excluded: LAMA2 (Hillaire et al. 1994; Helbling-Leclerc et al. 1995*a*, 1995*b*; Naom et al. 1997), RSMD1 (MIM 602771) (Moghadaszadeh et al. 1998), MEB (Cormand et al. 1999), FCMD (Toda et al. 1993), and the locus for integrin α7 with markers (Hayashi et al. 1998).

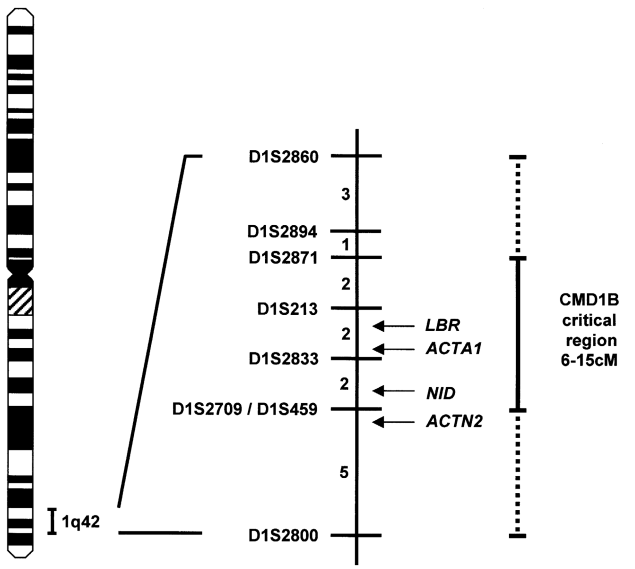

Figure 3 Ideogram of chromosome 1 and markers within the CMD1B critical region. The unbroken-and-dashed line represents the minimal and maximal critical regions. These regions are defined by the recombination events in family 1 (in patient II.2, between D1S459 and D1S2800; in patient II.4, between D1S2860 and D1S2871). Distances between adjacent markers are in centimorgans, on the basis of the data from the Généthon database. The map positions of four functional candidate genes, on the basis of the GeneMap'98 (International Radiation Hybrid Mapping Consortium) GB4 map, are indicated as follows: *LBR, ACTA1, NID,* and *ACTN2*.

Assignment of the Novel Locus in Family 1

The genomewide search identified five markers for which all four affected children were homozygous. These markers were located on chromosomes 1p, 1q, 11q, 18p, and 19q. We subsequently selected additional markers located immediately adjacent to and closely flanking each of these five markers, by use of the Généthon database. These markers allowed us to exclude all but one of these regions, since the neighboring markers did not identify a stretch of homozygosity (at least one affected individual was heterozygous). Fine mapping around D1S213 on chromosome 1q confirmed this marker to be part of an original area of homozygosity by descent, an area that included markers D1S2871, D1S213, D1S2833, D1S2709, and D1S459.

Patient II.2 in family 1 had a recombination of the maternal chromosome, between D1S2860 and D1S2871. Her younger brother (patient II.4) had a recombination of the paternal chromosome, between markers D1S459 and D1S2800. These two recombination events define the critical region as being 6 < 15 cM, depending on the exact site of recombination.

Since the clinical and histopathological features of family 2 were identical to those of family 1, we tested

the hypothesis that this family was also linked to the chromosome 1q42 locus. As can be seen in figure 1*B*, the two affected sibs shared the same genotype for the chromosome 1q markers, whereas the healthy sib inherited only one of the two at-risk haplotypes.

Two markers gave significant positive cumulative LOD scores (>3), with the highest score being 3.57 (θ = .00), for D1S213 (table 2). Similar LOD scores were obtained by use of allele frequencies calculated on the basis of CEPH families and our own data.

We also studied 17 additional consanguineous families with CMD, for linkage to this locus. We found no evidence of linkage to this locus in any of these families (data not shown). This was not surprising, since none of these 17 families had either clinical features similar to those of our two linked families or a reduction of the laminin-α2 chain of merosin in the muscle-biopsy samples.

Muscle Biopsy: Exclusion of Possible Candidate Proteins

As previously reported, the biopsy samples showed dystrophic changes and a reduction of laminin α2 on some fibers in the affected members in family 1 (fig. 2*b*) and in the propositus in family 2 (not shown). Expression of integrins α7 and β1D was also reduced on the same fibers (data not shown), whereas heparan sulfate proteoglycan was normal on all fibers (fig. 2*c*), including those fibers with reduced laminin α2 and integrins.

Several proteins expressed in skeletal muscle have been mapped to the interval D1S2860–D1S2800. This region includes the genes for nidogen (*NID*), skeletal-muscle α-actin (*ACTA1*), α-actinin 2 (*ACTN2*), and the lamin B receptor (*LBR*). We observed normal immunocytochemical expression of nidogen, α-actin, and α-actinin 2 in the skeletal muscle–biopsy samples from two affected individuals in family 1 (figs. 2*d–f*) and from the propositus in family 2.

Table 2

Two-Point LOD Scores for Linkage between the CMD1B Locus and Chromosome 1q42 Markers

Locus and Family	LOD Score at θ = .00	.01	.05	.10	.20	.30	.40
D1S2860:							
1	−3.70	.26	.78	.87	.74	.48	.20
2	.72	.71	.62	.50	.30	.14	.03
Total	−2.98	.97	1.40	1.37	1.04	.62	.23
D1S2894:							
1	.24	.23	.19	.15	.08	.03	.01
2	.43	.40	.32	.23	.08	.02	.01
Total	.67	.63	.51	.38	.16	.05	.02
D1S2871:							
1	2.29	2.24	2.04	1.79	1.28	.74	.23
2	.72	.70	.61	.50	.30	.13	.03
Total	3.01	2.94	2.65	2.29	1.58	.87	.26
D1S213:							
1	2.85	2.80	2.59	2.32	1.75	1.16	.56
2	.72	.70	.61	.50	.30	.13	.03
Total	3.57	3.50	3.20	2.82	2.05	1.29	.59
D1S2833:							
1	1.52	1.49	1.36	1.19	.86	.54	.23
2	.42	.41	.37	.31	.20	.10	.02
Total	1.94	1.90	1.73	1.50	1.06	.64	.25
D1S2709:							
1	1.43	1.31	1.19	1.05	.75	.46	.19
2	.43	.42	.37	.32	.20	.10	.02
Total	1.86	1.73	1.56	1.37	.95	.56	.21
D1S459:							
1	1.38	1.35	1.23	1.08	.78	.48	.19
2	.42	.41	.37	.31	.20	.10	.02
Total	1.80	1.76	1.60	1.39	.98	.58	.21
D1S2800:							
1	−3.20	.74	1.25	1.31	1.10	.76	.37
2	.73	.70	.61	.51	.31	.14	.03
Total	−2.47	1.44	1.86	1.82	1.41	.90	.40

Discussion

The two families reported in the present study have similar clinical and histopathological features, which suggests that they are affected by the same disease. Some features of their presentation and clinical symptoms are shared between CMD and LGMD. The muscle hypertrophy, in particular, is common to several forms of LGMD; however, the selective muscle weakness affecting the neck muscles, the sternomastoids, the facial muscles, and the diaphragm is unusual in the early stages of LGMD. These children all presented in infancy, and long-term follow-up showed that the muscle weakness is static, with the exception of the progressive respiratory failure, as is observed in several forms of CMD. Intellect is normal, and there is no evidence of either CNS or peripheral-nervous-system involvement. Levels of serum CK are grossly elevated, and the muscle-biopsy samples show similar dystrophic changes in all cases, with reduced expression of the laminin-α2 chain of merosin. The normal expression of perlecan indicates that there is no generalized abnormality of the basal lamina. However the reduced expression of the integrins associated with the laminin-α2 chain suggests that the primary defect may be in the interaction between the basal lamina and the sarcolemma.

The various known loci responsible for CMD have been excluded by linkage analysis, indicating that the merosin deficiency is a secondary phenomenon. The normal expression of proteins of the sarcoglycan complex excludes the corresponding forms of LGMD (Campbell et al. 1995). Whole-genome linkage data and recombinant analysis allowed us to exclude >95% of the genome and to establish the locus for this form of CMD, on

chromosome 1q42 (fig. 3). In these families, we were able to refine the mapping to 1q to an interval of 6–15 cM. Fine mapping of the recombination breakpoints will allow us to further define this interval (fig. 3). Families 1 and 2 potentially share a haplotype at markers D1S2709 and D1S459, suggesting that the CMD1B disease locus could be very close to them (the markers are <1 cM apart; fig. 3). Allele 2 at D1S2709 is the most-common allele at this locus (frequency .32), and allele 1 at D1S459 is the second-most-common allele (frequency .23). The frequency of these two alleles occurring together in the population studied, on the basis of 48 chromosomes, is .125. Taken together with the different ethnic origins of the two families, these data reduce the likelihood of a disease-associated haplotype at these two markers, although it cannot be discounted.

Four functional candidates have been mapped to this region: nidogen, an extracellular-matrix protein; α-actinin 2; α-actin; and lamin B receptor. For three of these proteins (nidogen, α-actinin 2, and α-actin), we have been able to rule out a "complete knockout." In light of the reduced expression of the laminin-α2 chain and the integrins, the normal immunocytochemistry for nidogen makes it an unlikely candidate. Mutations in the α-actin gene have been reported in children who have a form of congenital myopathy with actin accumulation but with no reduction in extracellular-matrix proteins (Goebel et al. 1997; Nowak et al. 1999). It is unlikely that the gene for α-actinin 2 is responsible for this form of CMD, since we observed normal immunocytochemical expression and since we are not aware of any direct links between this protein and the extracellular matrix. We have not been able to study the lamin B receptor with the use of a suitable antibody; however, other studies have shown that this protein is predominantly expressed in interstitial cells in skeletal and cardiac muscle (Manilal et al. 1999).

In summary, we have mapped a locus for a clinically and pathologically distinct form of CMD to chromosome 1q42. We suggest calling this disorder "CMD1B" (CMD1A representing CMD with primary merosin deficiency). These patients have early-onset weakness, generalized muscle hypertrophy, selective muscle weakness, ankle contractures, and a rigid spine. Follow-up data show that there is little functional skeletal-muscle deterioration over many years. These patients have a secondary, partial deficiency of the laminin-α2 chain, suggesting that the primary defect may be in the assembly of the basal lamina.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

CEPH, http://www.cephb.fr/
GeneMap'98, http://www.ncbi.nim.nih.gov/genemap98/
Généthon, http://www.genethon.fr/ (for markers used for the refinement of the candidate area on chromosome 1q42)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for Duchenne and Becker muscular dystrophies [MIM 310200], Bethlem myopathy [MIM 158810], FCMD [MIM 253800], MEB [MIM 253280], and RSMD1 [MIM 602771])

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