Refinement of the laminin $\alpha 2$ chain locus to human chromosome 6q2 in severe and mild merosin deficient congenital muscular dystrophy

Isam S Naom, Mariella D'Alessandro, Haluk Topaloglu, Caroline Sewry, Alessandra Ferlini, Anne Helbling-Leclerc, Pascale Guicheney, Jean Weissenbach, Ketty Schwartz, Kate Bushby, Jo Philpot, Victor Dubowitz, Francesco Muntoni

Abstract

About half of the children with classical congenital muscular dystrophy (CMD) show an absence in their skeletal muscle of laminin $\alpha 2$ chain, one of the components of the extracellular matrix protein, merosin. Linkage analysis implicated the laminin $\alpha 2$ chain gene (LAMA2) on chromosome 6q2, now confirmed by the discovery of mutations in the laminin $\alpha 2$ chain gene. We have further investigated the location of the LAMA2 locus on chromosome 6q2, using both linkage analysis in nine informative families and homozygosity mapping in 13 consanguineous families. Four of these families only had mild or moderate down regulation of laminin $\alpha 2$ chain expression and a milder phenotype; the rest had no protein or only a trace. Haplotype analysis in all the informative families, including those with partial laminin $\alpha 2$ expression, was compatible with linkage to chromosome 6q2. This observation expands the spectrum of the phenotype secondary to laminin $\alpha 2$ chain deficiency. Our results suggest that the LAMA2 locus is more centromeric than previously proposed. Recombinant events place the locus between markers D6S470 and D6S1620 in an interval of less than 3 cM.

(*J Med Genet* 1997;34:99–104)

Keywords: congenital muscular dystrophy; merosin; linkage.

Congenital muscular dystrophies (CMD) are autosomal recessive muscular disorders presenting with muscle weakness and hypotonia at birth or within the first 6 months of life.¹ Joint contractures of variable degree are also frequently present at birth or appear early during the course of the disease. The central nervous system (CNS) can also be affected, and brain magnetic resonance imaging (MRI) shows structural abnormalities (characteristically present in Fukuyama, Walker-Warburg, and muscle-eye-brain diseases) or white matter changes, resembling a leucodystrophy, in a proportion of children with the "classical" form of CMD.^{2–4} Histological changes seen in muscle biopsies include variability in fibre size, marked increase in endomysial collagen and

adipose tissue, and the presence of regenerating and necrotic fibres.¹

A classification of these various subtypes was suggested at two recent CMD workshops.^{5,6} A deficiency of the laminin $\alpha 2$ chain of merosin was subsequently reported in a proportion of patients presenting with the classical form of CMD.^{7–9} Merosin is a basement membrane specific glycoprotein composed of $\alpha 2$, $\beta 1$, and $\gamma 1$ subunit chains that are assembled in a cross shaped heterotrimer.¹⁰ We have shown that these merosin deficient patients have a more severe disease course and lower motor functional achievements compared to children with normal merosin expression.⁴ They invariably have increased signal in the white matter on T2 weighted magnetic resonance imaging (MRI) in the brain,¹¹ minor neurological and perceptuomotor deficits,¹² a motor demyelinating peripheral neuropathy,¹³ and may also have a cardiomyopathy.¹⁴

The laminin $\alpha 2$ chain locus (LAMA2) has been mapped to chromosome 6q2¹⁵ and analysis of informative families with merosin deficient CMD has confirmed linkage to the LAMA2 locus, suggesting that the deficiency of this protein is the primary defect.⁸ Further refinement of the LAMA2 localisation on chromosome 6q2 has been recently reported¹⁶ and mutations in the LAMA2 gene have now been discovered in affected children, confirming the primary role of this gene in families with this subtype of CMD.^{17,18}

The aim of our present study was to refine the localisation of the LAMA2 locus using our informative or consanguineous merosin deficient CMD families, or both. We have also looked for linkage at the LAMA2 locus in families with mild or moderate reduction in laminin $\alpha 2$ chain expression in the muscle and a milder disease course, to verify the hypothesis that these patients have a condition allelic to the typical, severe CMD phenotype.

Patients, material, and methods

PATIENTS

Blood samples and muscle or skin biopsies were obtained from 114 members from 28 families. Twenty-one families resided in the United Kingdom (13 white and eight of Asian descent) and the other seven in Turkey. Thirteen of the 28 families were consanguineous and nine more were informative (with at least one affected and one healthy sib), while

Neuromuscular Unit,
Department of
Paediatrics and
Neonatal Medicine,
Royal Postgraduate
Medical School,
Hammersmith
Hospital, Du Cane
Road, London
W12 0NN, UK
I S Naom
M D'Alessandro
C Sewry
A Ferlini
J Philpot
V Dubowitz
F Muntoni

Department of
Paediatric Neurology,
Hacettepe Children's
Hospital, Ankara,
Turkey
H Topaloglu

MRC Muscle Cell
Biology Group,
Hammersmith
Hospital, London, UK
C Sewry

INSERM U153,
Hôpital
Pitié-Salpêtrière,
Paris, France
A Helbling-Leclerc
P Guicheney
K Schwartz

Généthon-CNRS URA
1922, Paris, France
J Weissenbach

Department of Human
Genetics, Newcastle
upon Tyne, UK
K Bushby

Correspondence to:
Dr Muntoni.

Received 24 June 1996
Revised version accepted for
publication
4 September 1996

Figure 1 Homozygosity mapping in 13 consanguineous CMD families with total or almost complete absence of laminin $\alpha 2$ expression. The microsatellite markers studied and their genetic distance is shown on the left. Each vertical bar represents the result of the typing in the proband of one family. The dark bars represent regions of homozygosity, while the hatched bars are regions of heterozygosity for the microsatellite markers. The vertical open bar represents the minimal homozygosity region that the various families had in common, between the D6S407 and D6S1620 markers, where the LAMA2 locus lies.

the remaining six were uninformative (with one single affected child in the family).

Patients resident in the UK were assessed clinically at the Hammersmith Hospital Neuromuscular Unit (13 families) or at the Department of Human Genetics, Newcastle

upon Tyne (eight families), while Turkish patients were assessed at the Department of Paediatric Neurology in Ankara (seven families).

Patients were subdivided into two groups on the basis of the laminin $\alpha 2$ chain expression as judged by the immunocytochemical results (see below): (1) total absence or only traces of the protein, and (2) mild to moderate reduction.

MUSCLE AND SKIN BIOPSY

Needle muscle biopsies or skin biopsies or both were obtained from all subjects included in the study and processed as previously reported.⁹⁻¹⁹ Age at biopsy ranged from 8 days to 14 years. Unfixed 5 μ m cryostat sections were immunolabelled with a mouse monoclonal antibody to the C-terminus of the laminin $\alpha 2$ chain of merosin (Chemicon, diluted 1:1000) and visualised using a biotin-streptavidin-Texas Red method, as previously described.⁹ Serial sections were also labelled with antibodies to the $\alpha 1$, $\beta 1$, and $\gamma 1$ laminin subunits and to β -spectrin, to ensure that fibres were positively labelled, indicating that both the basement membrane and the plasmalemma were well preserved.

MICROSATELLITE GENOTYPING

Genomic DNA was extracted from whole blood or tissue samples by the salt-chloroform method.²⁰ Thirteen highly informative CA

Figure 2 Immunocytochemical labelling of laminin $\alpha 2$ in muscle biopsies from a control (A) and three cases of CMD with a deficiency of laminin $\alpha 2$ (B, C, D). (A) Normal expression in a control; (B) total absence; (C) traces of protein on some fibres; (D) reduced expression on most fibres.

repeat polymorphisms spanning an area of 12 cM around the LAMA2 locus on chromosome 6q2 were selected (fig 1). The primer sequence of markers D6S292, D6S270, D6S472, D6S413, D6S457, D6S262, and D6S407 have been published in Genethon human genetic linkage map,²¹ while that of markers D6S1626, D6S957, D6S1656, D6S1572, D6S1705, D6S1620 were obtained from Genethon and have been reported recently.²² The microsatellite markers were typed in all available family members using the radionuclide end labelling method described previously.²³ The forward primer was 5' end labelled with polynucleotide kinase and [γ 32p]ATP for two to three hours before amplifying the DNA. PCR amplification was performed in a total volume of 12.5 μ l containing 75 ng genomic DNA, 2.5 pmol of reverse primer, 2.5 mmol/l dNTP, 1.5 mmol/l MgCl₂, and 0.75 units of *Taq* DNA polymerase (Promega). The DNA was denatured for five minutes in a DNA thermocycler (Perkin Elmer-Cetus) and amplified for 23 cycles (45 seconds at 94°C, 45 seconds at 61°C, one minute at 72°C, followed by five minutes elongation at 72°C). Amplified products were then electrophoresed on a Model S2 sequencing apparatus (BRL), dried, and processed for autoradiography.

Two point lod scores were calculated using the program MLINK of the Linkage package

(version 5.2) assuming a recessive disease gene frequency of 0.001 with complete penetrance.

Results

PATIENTS

In 23 index cases from the 28 families, we observed a total or almost complete absence of laminin $\alpha 2$ chain expression in the muscle (fig 2B and C, respectively), while in four there was only a mild to moderate reduction (fig 2D).

All patients with total or almost complete absence of laminin $\alpha 2$ chain were severely affected, as also noted in our earlier review of CMD.⁴ No patient in this group achieved independent walking and the maximal motor ability was walking with support in two cases and standing with support in nine. One patient in this group was just able to stand unsupported. All had the typical white matter changes on MRI found in merosin deficient cases¹¹ and evidence of peripheral nerve involvement on neurophysiological study.¹³

The four patients belonging to the group with moderate expression of the laminin $\alpha 2$ chain varied in clinical severity: one (family 2) had a severe phenotype indistinguishable from that of patients with no expression of laminin $\alpha 2$ chain, while the other three (families F-19, F-4, and F-33) were mildly affected. The 5 year old girl in family F-19 had a moderate delay in motor milestones (sat at 8 months and walked

Figure 3 Result of the haplotype analysis in three consanguineous families with total or almost complete absence of laminin $\alpha 2$ expression. The open and dark symbols represent unaffected and affected subjects, respectively. The open bars are the regions where homozygosity for the various markers was found. A centromeric recombinant was found in family F-28, while telomeric recombinant events are shown in families F-26 and F-36.

Figure 4 Result of the haplotype analysis in four families with mild reduction of laminin $\alpha 2$ expression. The open and dark symbols represent unaffected and affected subjects, respectively. At risk haplotypes are represented by open bars. Only the affected subjects in each family inherited the at risk haplotypes.

at 28 months) but is now able to walk for relatively long distances without assistance and only has mild ankle contractures. Her CK is only moderately raised (800 IU/l). She has the typical changes on T2 weighted MRI imaging of the brain. The two affected children in family F-36 presented with delayed motor milestones (walked at 22 and 24 months, respectively) and frequent falls. Serum CK levels were considerably raised in both children (2500 and 4000 IU/l, respectively). Their disease course has shown a slow but definite improvement in clinical function and both children remain fully mobile at the ages of 6 and 8 years, respectively. The muscle immunocytochemistry in these cases showed only a slight reduction in laminin $\alpha 2$ chain immunostaining (muscle biopsy of F-19 is shown in fig 2D).

The four patients in family F-33 were even more mildly affected, and had onset of symptoms in the second decade. They all had white matter changes on brain MRI. A detailed description of the phenotype of this family is part of a separate clinical report.²⁴ The laminin $\alpha 2$ chain expression in the muscle biopsy of the

proband was only mildly reduced compared to controls using the commercially available C-terminus antibody.

No secondary reduction in immunostaining was observed using the same antibodies in a variety of disease control patients, including children affected by Duchenne and Becker muscular dystrophies, adhalin deficient (sarcoglycan) limb-girdle muscular dystrophy, Emery-Dreifuss muscular dystrophy, facio-scapulohumeral muscular dystrophy, and juvenile dermatomyositis.

HOMOZYGOSITY MAPPING²⁵

Thirteen microsatellite markers spanning a region of 12 cM on chromosome 6q2 were genotyped in 13 consanguineous families. In seven of these families the parents were first cousins; in the remaining six the interrelationship was more distant or inferred by the sharing of haplotypes between parents originating from the same geographical areas. In particular, five families of this latter group (families F-4, F-15, F-27, F-28, and F-29) came from the same village in West Pakistan, had the same surname,

and carried the same haplotype, while the sixth family was a gypsy family of Irish origin.

Various telomeric recombinations (see families F-4, F-15, F-16, F-27, F-25, F-26, and F-35, fig 1) suggested that the locus is more centromeric than D6S1620. We also found a centromeric recombination (family F-28) suggesting that the locus is telomeric to D6S407 (fig 3). The minimal homozygosity region that these families had in common was therefore contained between the microsatellite markers D6S407 and D6S1620.

HAPLOTYPE ANALYSIS IN NON-CONSANGUINEOUS FAMILIES WITH ABSENCE OF LAMININ $\alpha 2$
Of the remaining 11 families with absent or only traces of laminin $\alpha 2$ chain expression, five were informative. The genotyping in these families was suggestive of linkage to the LAMA2 locus. In particular, there was no sharing of the at risk haplotypes between affected and healthy children, and when two affected children were available in the same family they always shared the at risk haplotypes.

HAPLOTYPE ANALYSIS IN PATIENTS WITH MILD REDUCTION IN MEROSIN EXPRESSION
Haplotype analysis of the four families in this group was also compatible with a primary role for the LAMA2 gene (fig 4). Family F-33 was of particular interest as the four affected members all shared the identical haplotypes. A recombinant event was found in the maternally derived haplotype of member II.3 between markers D6S407 and D6S1620. This is in keeping with the observation that the LAMA2 locus is located between these two markers.

LINKAGE ANALYSIS
Studying nine informative non-consanguineous and 13 consanguineous families, a maximum lod score of 11.69 was achieved at $\theta=0.01$ with the D6S407 marker, which was shown to flank the LAMA2 locus centromerically (table 1). The second highest lod score was obtained with marker D6S1620 (lod score of 9.08 at $\theta=0.01$), which flanks the LAMA2 locus telomerically (table 1). The results of the lod scores obtained with the remaining markers are shown in table 1.

Discussion
Mapping to LAMA2 locus on chromosome 6q2 has been previously reported in CMD families with a deficiency of the laminin $\alpha 2$ chain of merosin.⁸ Readjustment of the localisation of this locus on chromosome 6q2 suggested that the gene is more centromerically located.¹⁶ In the present study we have used the combined approach of homozygosity mapping in 13 consanguineous families and linkage analysis in nine informative families with a deficiency of laminin $\alpha 2$ chain on muscle or skin biopsies. Our results in the consanguineous families suggest that the LAMA2 locus is between markers D6S407 and D6S1620. This is in keeping with a previous report suggesting that the LAMA2 locus is located between markers D6S407 and D6S1705¹⁶ and further refines the localisation of the locus between markers D6S407 and D6S1620, in an interval of less than 3 cM.

We have also studied four families with partial deficiency of laminin $\alpha 2$ chain expression in the muscle. Interestingly, the phenotype in these four families was quite variable and ranged from children as severely affected as those with no protein expression, to patients who developed their first symptom in the second decade.²⁴ Intermediate phenotypes between these two extremes were also seen. In all these families the haplotype analysis was suggestive of linkage to the LAMA2 locus. In one Turkish family in particular (F-33), the four affected subjects all shared the same haplotypes. These observations suggest that the spectrum of the conditions secondary to laminin $\alpha 2$ chain deficiency encompasses milder disease courses than those observed in children with the typical neonatal onset CMD. It is likely that allelic mutations at the same locus might be responsible for this variable phenotypic expression.

Two patients with a significant amount of laminin $\alpha 2$ chain expression had a severe phenotype, while the remaining two had a milder disease course. The amount of staining obtained with the commercially available C-terminus antibody does therefore not correlate with the severity of the phenotype in these patients. A severe phenotype in patients with partial laminin $\alpha 2$ chain expression and a

Table 1 Cumulative lod scores obtained in 22 CMD families (13 consanguineous and nine informative non-consanguineous families). The highest lod score was achieved at $\theta = 0.01$ with the D6S407 marker, while second highest lod score was obtained with the D6S1620 marker. These two markers flank the LAMA2 locus centromerically and telomerically respectively

Marker	Recombination fraction					
	0.001	0.01	0.05	0.1	0.2	0.3
D6S407	11.21	11.69	10.66	8.92	5.50	2.80
D6S1620	8.72	9.08	8.69	7.32	4.38	2.86
D6S1705	6.68	7.40	7.10	5.98	3.62	1.76
D6S1572	4.66	6.24	7.82	6.33	3.83	1.87
D6S262	2.42	4.09	4.09	3.58	2.15	1.04
D6S457	-2.03	0.95	3.11	3.08	2.14	1.40
D6S1656	-1.08	2.16	4.05	3.54	2.20	1.08
D6S413	-1.05	-0.27	-0.06	-0.01	0.03	0.07
D6S472	-2.71	0.52	3.08	3.06	2.05	1.07
D6S975	1.10	2.51	3.06	2.57	1.45	0.63
D6S270	-4.10	0.09	1.21	2.81	1.81	1.20
D6S1626	-7.52	-2.45	1.33	1.91	1.47	0.77
D6S292	0.23	2.63	4.08	3.65	2.27	1.11

mutation in the short arm of the molecule (N-terminus) has been recently reported by others.¹³ It is likely that the mutations in our patients are also located in the N-terminus and that the resulting phenotype varies depending on the disturbance of the structure or function of the merosin trimer. The haplotype analysis obtained in all the informative families analysed so far shows that all cases, including four cases of partial laminin $\alpha 2$ chain deficiency, are linked to chromosome 6q2.

Mutations in the human LAMA2 gene have been reported in two CMD cases. One had a nonsense mutation, the other a splice site mutation.¹⁷ The direct role of LAMA2 in CMD with partial laminin $\alpha 2$ chain deficiency was evidenced by the recent identification of the first missense mutation in the coding sequence of a severely affected child.¹⁸ The task of identifying mutations in this gene has proved difficult, hampered by its large size and lack of definition of exon-intron boundaries. As a result of these limitations, it is likely that in future the molecular genetic studies of this form of CMD will be mainly conducted using linkage analysis. In this respect we show here that the LAMA2 locus is located between the D6S407 and D6S1620 markers. This information can be used for prenatal diagnosis together with the direct visualisation of laminin $\alpha 2$ chain expression in the trophoblast.²⁶ Our study also suggests that patients with a milder disease course, white matter change on brain MRI, and mild or moderate impairment of laminin $\alpha 2$ chain expression in the muscle biopsy are also linked to the LAMA2 gene and probably represent allelic variants at the same locus.

The authors wish to thank all the families that participated in the study and Karen Davison for the photographs. We are indebted to Deborah Ford and Richard Houlston, Institute of Cancer Research, Sutton, Surrey for the support given for the analysis of linkage. This work was supported by the Muscular Dystrophy Group (MDG) of Great Britain and Northern Ireland (IN and AF). MD'A is an Italian TELETHON PhD student. AH-L is supported by the Association Française contre les Myopathies (AFM). We also wish to thank the European Neuromuscular Centre (ENMC), who hosted the workshops of the CMD consortium, The Medical Research Council (MRC) of Great Britain, and the Institut National de la Santé et de la Recherche Médicale (INSERM), and the Associazione Malattie Rare Baschirotto.

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