PCR based mutation screening of the laminin α2 chain gene (LAMA2): application to prenatal diagnosis and search for founder effects in congenital muscular dystrophy

Pascale Guicheney, Nicolas Vignier, Xu Zhang, Yi He, Corinne Cruaud, Véronique Frey, Anne Helbling-Leclerc, Pascale Richard, Brigitte Estournet, Luciano Merlini, Haluk Topaloglu, Marina Mora, Jean-Paul Harpey, Charles-Antoine Haenggeli, Annie Barois, Bernard Hainque, Ketty Schwarz, Fernando M S Tomé, Michel Fardeau, Karl Tryggvason

Abstract
Classical congenital muscular dystrophy with merosin deficiency is caused by mutations in the laminin α2 chain gene (LAMA2). Extended sequencing of the introns flanking the 64 LAMA2 exons was carried out and, based on these sequences, oligonucleotide primers were designed to amplify the coding region of each exon separately. By PCR-SSCP analysis, we identified eight new mutations in nine families originating from various countries. All induced a premature truncation of the protein, either in the short arm or in the globular C-terminal domain. A 2 bp deletion in exon 13, 2098delAG, was found in three French non-consanguineous families and a nonsense mutation of exon 20, Cys514stop, in two other non-consanguineous families originating from Italy. Determination of rare intragenic polymorphisms permitted us to show evidence of founder effects for these two mutations suggesting a remote degree of consanguinity between the families. Other, more frequent polymorphisms, G to A 1905 (exon 12), A to G 2848 (exon 19), A to G 5551 (exon 37), and G to A 6286 (exon 42), were used as intragenic markers for prenatal diagnosis. This study provides valuable methods for determining the molecular defects in LAMA2 causing merosin deficient congenital muscular dystrophy.

Keywords: congenital muscular dystrophy; merosin; laminin-2

Merosin deficient congenital muscular dystrophy (MD CMD) is an autosomal recessive muscle disorder with homogeneous clinical features represented by early onset of muscle hypotonia, weakness, severe contractures, and white matter changes in the central nervous system. The children generally have normal intelligence and in a number of cases epileptic seizures. Muscle biopsies show marked variation in fibre size, necrotic and regenerating processes, at early stages, and interstitial fibrosis. Immunohistochemical analyses have shown absence or reduction of the laminin α2 chain around the skeletal muscle fibres. Disruption of the basement membrane of muscle fibres based on electron microscopy examination has been reported in a few cases. Brain magnetic resonance imaging (MRI) has indicated diffuse white matter changes clearly associated not only with complete deficiency but also with partial deficiency of the laminin α2 chain. This typical pattern is usually detectable after 6 months of age. Abnormal somatosensory and visual evoked potentials, as well as motor nerve conduction, have been described. Extensive brain abnormalities, such as cortical dysplasia, have been reported in some cases.

Several mutations causing either a partial or a complete deficiency of the laminin α2 chain have been identified in the laminin α2 chain gene. The laminins are a family of large trimeric basement membrane glycoproteins composed of α, β, and γ chains. The three subunit chains associate to form a2:12:1y, a unique molecule with specificity for basement membranes surrounding skeletal muscle fibres. The high tissue specificity of these isoforms is provided only by the α2 chain, as the other component chains of the laminin isoforms, the β1 or β2 and γ1 chains, are ubiquitously expressed. The α2 polypeptide chain contains 3110 residues. Detailed characterisation of the gene encoding this chain, LAMA2, showed that it exceeds 260 000 base pairs in size and contains 64 exons.

In the present study, we extended the sequencing of introns flanking all exons in order to enable PCR amplification of large enough segments for thorough mutation analysis of all the 64 exon regions. Oligonucleotide primers were designed to amplify each exon, which was then studied by PCR-SSCP analysis and direct sequencing. New mutations and several polymorphisms were identified and applications for prenatal diagnosis and search for founder effects were shown.
Patients, material, and methods

PATIENTS
In the present study, mutation analysis or prenatal diagnosis was carried out on 14 families (nine consanguineous and five non-consanguineous) with at least one affected child with a clinicopathological diagnosis of CMD, according to the criteria of the International Consortium on Congenital Muscular Dystrophy. These families originated from various countries: France (490, 1272, 1672, 2656, BE), Italy (4835, 4836, SI, LI), Turkey (1663, 1830), Israel (IF), Belgium (BU), and Morocco (2324). Some of them have been referred to us for prenatal diagnosis. All families gave informed consent before inclusion in this study. A muscle biopsy was performed on one affected child from each family and studied by immunohistochemistry, using an antibody against the C terminal part of the globular G domain of the human laminin α2 chain (Chemicon), as previously reported. Complete deficiency of the laminin α2 chain was observed in patients in 13 families, whereas partial deficiency was shown in one family (1830).

MICROSATELLITE TYPING
D6S407 and D6S1620 microsatellite markers were provided by Génethon human genetic linkage maps. PCR amplifications were performed on 40 ng genomic DNA, as previously described.

SEQUENCING OF INTRONS AND PCR-SSCP ANALYSIS
Sequencing of all 64 exons and their immediate intronic boundaries (30 bp) has previously been reported. Further sequencing of the introns was carried out directly on our previously purified λ clones or subcloned restriction fragments using exon specific oligonucleotide primers as well as the AmpliCycle kit (Perkin-Elmer). Oligonucleotide primers were designed to amplify each LAMA2 exon, based on the intronic sequences using the OLIGO 4.0 program. The primers were chosen so that at least 30 to 50 bp of flanking intron sequences were readable. Amplifications from genomic DNA were performed using the PCR “touchdown” method. The primer pairs and the initial and final annealing temperatures are given in table 1, with decreasing of the annealing temperature by 1°C after each two cycles and with 30 cycles at lower temperature. At each cycle, denaturation was performed at 94°C for 40 seconds and extension at 72°C for one minute. Final extension was at 72°C for 2.5 minutes. For SSCP analysis, PCR products were denatured for two minutes at 95°C after dilution (1:4) in 10% saccharose buffer, according to Maruya et al with minor modifications, kept in ice, loaded onto 10% polyacrylamide (37.5:1) minigels, then run at 8 mA (per gel) at 7°C and 20°C in a Hoeffer apparatus. The bands were visualised after silver staining of the gels (Biorad). All abnormal conformers were sequenced. When polymorphisms were determined, allele frequencies were estimated by the analysis of 200 normal chromosomes from unrelated subjects.

DIRECT SEQUENCING OF THE PCR PRODUCTS
PCR fragments were centrifuged through a Centricon 100 membrane (Amicon) and sequenced with a Taq cycle sequencing kit (Applied Biosystems) using fluorescent dideoxynucleotides and one of the PCR primers. Reaction products were run on an automated DNA sequencer (Applied Biosystems). All sequences were determined on both strands.

Results

DETERMINATION OF EXON FLANKING INTRON SEQUENCES AND DESIGN OF PRIMER PAIRS FOR MUTATION DETECTION
Subcloned restriction fragments of genomic λ phage clones containing the 64 exons were sequenced using cycle sequencing. At least 50 bp of intron sequences flanking each exon were determined to enable PCR amplification and sequencing of intron sequences likely to be essential for the splicing of the primary transcript. These sequences might be altered in some cases of CMD.

The intron sequences were used to design oligonucleotide primers (table 1) for PCR amplification of the exon regions of the LAMA2 gene. This gene contains exons with sizes varying between 6 and 270 bp. Sixty-three primer pairs allowed a direct PCR amplification of an exon with the exon/intron boundaries. For exon 5, two overlapping primer pairs were designed. The sizes of the PCR products ranged between 129 and 345 bp, allowing SSCP analysis and direct sequencing.

IDENTIFICATION OF MUTATIONS IN THE LAMA2 GENE
Eight new mutations were identified in LAMA2 associated with MD CMD in nine families (table 2). M1 is a 2 bp deletion at position 1539 in exon 10, 1539delGT in a French non-consanguineous family (1272). This deletion resulted in the formation of a stop codon (T[GT]AG→TAG) in the domain V that should produce a truncated protein of 496 residues. M2 is also a 2 bp deletion at position 2098 in exon 13, 2098delAG, resulting in a frameshift and a premature termination within 20 amino acid residues in domain IVb. This deletion was identified in three French non-consanguineous families (490, 1272, 1672) (figs 1 and 2). M3 is a C to A transversion at position 2950 in exon 20 changing a cysteine codon to a stop codon (TG to TGA) in domain IIIb. This nonsense mutation, Cys967stop, was identified in two non-consanguineous families originating from Italy (LI, 4835) (figs 1 and 2). M4 is another nonsense mutation in exon 20 at position 3011, a C to T transition changing a glutamine codon to a stop codon (CAA to TAA). This mutation, Gin988stop, was identified in a consanguineous Italian family (4836). M5 is a 1 bp deletion at position 3171 in exon 21, 3171delG, resulting in a frameshift and a premature stop codon within 33 amino acid

Department of Neuromuscular Diseases, Istituto Nazionale Neurologico "Carlo Besta", 20133 Milano, Italy
M Mora
Clinique de Pédiatrie et de Génétique Médicale, Groupe Hospitalier Pitie-Salpétrière, 47 Boulevard de l'Hôpital, 75651 Paris Cedex 13, France
J-P Harpey
Hôpital des Enfants, Genève, Switzerland
C-A Haengeli
Correspondence to: Dr Guicheney.
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residues in domain IIIb. This deletion was identified in a consanguineous Turkish family (1963). M6 is a C to A transversion at position 4687 in exon 31, changing a cysteine residue to a stop codon (TGC to TGA). This nonsense mutation in domain IIIa, Cys1546stop, was identified in a consanguineous Italian family (SI). M7 is a G to A transition at position 6916+1 of the consensus donor splice site of exon 47 in the DNA of the affected child of family 1672. It is the second mutation transmitted by the father. The mutation occurred in the coreboxy terminal G globular domain and its exact effect at the protein level is not known. M8 is a 1 bp deletion at position 8314 in exon 58, 3814delA, in a French consanguineous family (256), resulting in a frameshift and a premature stop codon within five conserved acids in the carboxy terminal G globular domain. The six first mutations should result in truncated proteins lacking domains I and II of the long arm of the laminin α2 chain which normally forms a coil with the carboxy termini of the α1 and α2 chains. The M7 and M8 mutations occurred in the carboxy terminal G globular domain. M8 should result in the truncation of the two last G repeat domains again, which the commercial antibody is raised to. The different mutations detected by SCCP, as well as the modifications of restriction site they induce, are given in table 2.

Table 1: Oligonucleotide primers and conditions for PCR amplification of LAMA2 exons

<table>
<thead>
<tr>
<th>Exon</th>
<th>Position on mRNA (nt)</th>
<th>Exon size (bp)</th>
<th>Location upstream from exon</th>
<th>Forward primer sequence (5’-3’)</th>
<th>Reverse primer sequence (5’-3’)</th>
<th>Location downstream from exon</th>
<th>PCR product size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
</table>
IDENTIFICATION OF LAMA2 POLYMORPHISMS

We detected 13 polymorphisms in nine exons (table 3). Eight were silent polymorphisms and five induced amino acid changes in exons 12, 37, 55, and 56. Several polymorphisms induce a loss or creation of a restriction site and all can be detected by SSCP analysis at 7°C or 20°C, or at both temperatures, as mentioned in table 3. The frequencies of the less frequent alleles were estimated for most of the polymorphisms (table 3). The A→G polymorphism at position 5551 in exon 37 is the most informative with a frequency of 38% for allele G and 62% for allele A. Several rare variants were identified in 2 to 5% of the chromosomes which were analysed. In exon 55, the presence of at least three polymorphisms accounts for the various SSCP patterns observed, each corresponding to a different allelic combination. The respective frequencies of these polymorphisms have not been determined. We identified three additional polymorphisms in introns 4, 30, and 52 in the PCR products corresponding to exons 5, 30, and 52, respectively.

APPLICATION TO RESEARCH OF FOUNDER EFFECTS

SSCP analysis of exon 13 and exon 20 PCR products showed the same deletion, 2098delAG, in three French families and the same nonsense mutation, Cys967stop, in two families originating from Italy (fig 1). These five families were non-consanguineous, suggesting that two different mutations cause the disease. We identified the two mutations in only two families, family 1272 (2098delAG and 1539delGT) and family 1672 (2098delAG and 6916+1 G→A). Analysis of the microsatellite markers D6S407 and D6S1620, which are the closest so far known to the LAMA2 locus, did not show whether the mutations 2098delAG and Cys967stop were independent mutational events or related ones (fig 2).

By the study of intragenic polymorphisms, we were able to show that the three French families shared the same haplotype associated with the 2098delAG mutation, suggesting that these families probably have a remote degree of consanguinity (fig 3). The G at position 2848, which is the less common allele, is associated with the 2098delAG deletion in these families.

In the Italian families a rare variant of exon 37, A at position 5579, was found associated with the Cys967stop mutation. The two families shared the same haplotype, which also suggests a founder effect (fig 2).

APPLICATION TO PREGNATAL DIAGNOSIS

Six families were referred to us for prenatal diagnosis (fig 3). In addition to the two microsatellites bordering the LAMA2 locus, D6S407 and D6S1620, we analysed at least exons 12, 19, 37, and 42 in each family for polymorphism detection. As shown in fig 3, by haplotyping the microsatellites and polymorphisms, we could determine the two at risk haplotypes in each family and their presence in the DNA samples from trophoblastic biopsies (fig 3). The polymorphisms of exons 12, 19, and 42, 1905 G→A, 2848A→G, and 6286 G→A, were informative in two, two, and one of the six families, respectively, whereas the polymorphism of exon 37, 5551 A→G, was informative in all the six families. For the cases described here, all the fetuses have received only one or no disease haplotype, without any evidence of recombination. In two cases, study of the mutations already identified (family 1272, see above, and family 2324, 2418delC in exon 16) have strengthened the conclusions of the haplotype analysis.

Discussion

The laminin α2 chain is a large protein of the extracellular matrix. The gene, LAMA2, contains 64 exons, 62 with sizes varying between 87 and 270 bp and two unusually small ones, exon 43 and exon 52, with 6 and 12 base pairs, respectively. Further sequencing of exon flanking intron regions allowed PCR amplification of sufficiently long intron sequences to ensure sequencing and mutation detection of

Table 2  New LAMA2 mutations causing laminin α2 chain deficiency

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Exons</th>
<th>Domains</th>
<th>Sequence variation</th>
<th>Size of the polymorphism</th>
<th>SSCP</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 Cys497stop</td>
<td>10</td>
<td>V</td>
<td>1539delGT</td>
<td>496</td>
<td>7°C</td>
<td>New SpeI and Mdi</td>
</tr>
<tr>
<td>M2 2098delAG</td>
<td>13</td>
<td>IVb</td>
<td>2098delAG</td>
<td>702</td>
<td>7 and 20°C</td>
<td>No change</td>
</tr>
<tr>
<td>M3 Cys967stop</td>
<td>20</td>
<td>IIIb</td>
<td>2950 C→A</td>
<td>906</td>
<td>7 and 20°C</td>
<td>No change</td>
</tr>
<tr>
<td>M4 Gln988stop</td>
<td>20</td>
<td>IIIb</td>
<td>3011 C→T</td>
<td>987</td>
<td>7°C</td>
<td>Loss NalV and BanI</td>
</tr>
<tr>
<td>M5 3171delG</td>
<td>21</td>
<td>IIIb</td>
<td>3171delG</td>
<td>1073</td>
<td>20°C</td>
<td>No change</td>
</tr>
<tr>
<td>M6 Cys1546stop</td>
<td>31</td>
<td>IIia</td>
<td>4687 C→A</td>
<td>1545</td>
<td>20°C</td>
<td>Loss FluI and BstAI</td>
</tr>
<tr>
<td>M7 6916+1 G→A</td>
<td>37</td>
<td>G</td>
<td>6916+1 G→A</td>
<td>Unknown</td>
<td>7°C</td>
<td>No change</td>
</tr>
<tr>
<td>M8 8314delA</td>
<td>58</td>
<td>G</td>
<td>8314delA</td>
<td>2759</td>
<td>7 and 20°C</td>
<td>New Taq</td>
</tr>
</tbody>
</table>

Figure 1  SSCP analysis of the three French families with a 2098delAG mutation (A) and the two Italian families with a Cys967stop mutation (B). Abnormal conformers were detected for the probands compared to the control (C). In each family, the proband and one of his parents was heterozygous for the mutation in agreement with the haplotypes shown in fig 2.
the splice consensus sequences. Optimal PCR conditions were determined for each exon region of the LAMA2 gene. By PCR-SSCP and direct sequencing, we identified 14 different mutations, including the eight new ones of this report, in CMD patients with complete merosin deficiency. All these mutations were 1 or 2 bp deletions, nonsense, or splice mutations, leading to truncated proteins and absence of immunoreactivity directed against the C-terminal part of the protein. Three additional mutations have been reported by other groups, including a 5 bp deletion and a large deletion. Systematic screening, exon by exon, of such a large gene is expensive and time consuming. Nevertheless, such an approach cannot be avoided to detect missense mutations, such as the Cys996Arg, causing CMD with partial merosin deficiency. To detect mutations causing CMD with a complete deficiency, the protein truncation test (PTT) would be a more appropriate approach, especially since we have only identified 60 to 70% of the mutations by PCR-SSCP mutation screening.

By analysing the various LAMA2 exons, we identified 16 polymorphisms, three in introns and 13 in exons. Most of them are silent polymorphisms, but five induce an amino acid change without any known physiological consequence. Four of these polymorphisms, 1905G→A, 2848 A→G, 5551 A→G, and 6286 G→A, in exons 12, 19, 37, and 42, respectively, have an allele frequency above 16% and can be useful as polymorphic markers, especially for prenatal diagnosis. The A to G polymorphism

Table 3 LAMA2 polymorphisms

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>Frequency</th>
<th>Restriction site</th>
<th>SSCP conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1540 T→C</td>
<td>497 TGT(Cys)→TGC(Cys)</td>
<td>2%</td>
<td>New PstI</td>
<td>7°C, 20°C</td>
</tr>
<tr>
<td>12</td>
<td>1847 G→A</td>
<td>600 GGA (Gly)→AGA(Arg)</td>
<td>2%</td>
<td>Loss MstI</td>
<td>7°C</td>
</tr>
<tr>
<td>12</td>
<td>1905 G→A</td>
<td>619 CGT(Arg)→CAT(His)</td>
<td>18%</td>
<td>Loss BstII</td>
<td>7°C</td>
</tr>
<tr>
<td>19</td>
<td>2848 A→G</td>
<td>933 CAA(Glu)→CAG(Gln)</td>
<td>24%</td>
<td>No change</td>
<td>7°C, 20°C</td>
</tr>
<tr>
<td>33</td>
<td>5005 C→G</td>
<td>1663 ACC(Thr)→AGC(Thr)</td>
<td>3%</td>
<td>Loss EcoRI</td>
<td>7°C</td>
</tr>
<tr>
<td>37</td>
<td>5551 A→G</td>
<td>1834 GAA(Glu)→GAG(Glu)</td>
<td>5%</td>
<td>New MspI</td>
<td>7°C</td>
</tr>
<tr>
<td>37</td>
<td>5579 C→A</td>
<td>1844 GTG(Arg)→AGT(Ser)</td>
<td>2%</td>
<td>No change</td>
<td>7°C</td>
</tr>
<tr>
<td>42</td>
<td>6286 G→A</td>
<td>2079 ACG(Thr)→ACA(Thr)</td>
<td>16%</td>
<td>Loss BsmI</td>
<td>20°C</td>
</tr>
<tr>
<td>54</td>
<td>7669 C→G</td>
<td>2540 TCC(Ser)→TGC(Ser)</td>
<td>17%</td>
<td>No change</td>
<td>7°C</td>
</tr>
<tr>
<td>55</td>
<td>7809 T→C</td>
<td>2587 GTA(Val)→GCA(Val)</td>
<td>ND</td>
<td>No change</td>
<td>20°C</td>
</tr>
<tr>
<td>55</td>
<td>7879 C→G</td>
<td>2610 GTC(Val)→GTC(Val)</td>
<td>ND</td>
<td>New BstI</td>
<td>20°C</td>
</tr>
<tr>
<td>55</td>
<td>7894 G→A</td>
<td>2615 CGA(Arg)→CCA(Pro)</td>
<td>ND</td>
<td>Loss Hinfl</td>
<td>20°C</td>
</tr>
<tr>
<td>56</td>
<td>7955 A→G</td>
<td>2636 ACA(Thr)→ACA(Ala)</td>
<td>6%</td>
<td>No change</td>
<td>7°C</td>
</tr>
</tbody>
</table>

ND=not determined.
at position 5551 is the most informative one and induces the creation of a new MspI restriction site. It was informative in our experience for the 11 prenatal diagnoses we performed (data not shown). These polymorphisms can be analysed to obtain more information in case of recombination between the two flanking microsatellite markers. Some of them do not induce the creation or the loss of a restriction site but they can all be easily detected by SSCP. The pattern associated with each polymorphism can be established using a reference panel of DNA samples which have been sequenced.

We present here two new mutations in the LAMA2 gene in non-consanguineous families. A 2 bp deletion at position 2098 in exon 13, 2098delAG, which results in the truncation of the laminin a2 chain in domain IVb, was found in three French families. Analysis of intragenic polymorphisms suggests that these three families are related. The nonsense mutation, Cys967stop, in exon 20, which results in the truncation of the laminin a2 chain in domain IIIb, was identified in two families of Italian origin. These two families also probably have a remote degree of consanguinity since a rare polymorphism, adenine at position 1847, was found linked to the mutation. The two families originate from the south of Italy on the Adriatic coast. Analysis of the various polymorphisms of the gene could be used to identify founder effects in the populations. We previously reported that the rare polymorphism in exon 10, cytosine at position 1540, was linked to the Gln1241stop nonsense mutation identified in a family originating from Tunisia.21

This study provides valuable methods for determining the molecular defects in LAMA2 causing merosin deficient congenital muscular dystrophy.

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