LAMA2 Gene Analysis in Congenital Muscular Dystrophy

New Mutations, Prenatal Diagnosis, and Founder Effect

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Objective: To determine if laminin-α2 deficiency is due to mutations in the LAMA2 gene or secondary to mutations in other congenital muscular dystrophy genes.

Methods: We performed molecular analysis of LAMA2, by single-strand conformation polymorphism and sequencing, in 15 patients with undetectable or greatly reduced laminin-α2 expression. We also performed 4 prenatal diagnoses and investigated a founder effect.

Results: We found 1 known and 9 previously undescribed LAMA2 mutations spanning all protein domains. These were nonsense or frameshifts causing laminin-α2 absence or, in 1 case, a homozygous missense mutation producing partial protein expression and milder phenotype. LAMA2 mutations were undetected in 5 patients, in 2 of whom FKRP mutations explained the phenotype. In 3 prenatal cases, the fetus was heterozygous for the mutation of interest and pregnancy continued; in 1 case, the fetus was affected and aborted. In 2 patients, the Cys967Stop mutation and identical haplotypes flanking the LAMA2 gene indicated a founder effect.

Conclusions: The clinical phenotype was severe in most patients with LAMA2 mutations and associated with undetectable protein expression. One case with no protein and another with partial expression had milder phenotypes. Typical white matter alterations on magnetic resonance imaging were found in all patients with LAMA2 mutations, supporting the utility of magnetic resonance imaging in differential diagnosis. The founder mutation (Cys967Stop) probably originated in Albania. Genetic characterization of affected families is mainly of use for prenatal diagnosis.

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The congenital muscular dystrophies (CMDs) are early-onset, recessively inherited, and clinically and genetically heterogeneous disorders. At least 11 genes and 1 locus have been associated with the 12 clinically recognized CMD forms1 that are variably characterized by muscle involvement, mental retardation, and brain structure alterations. Patients with CMD may have normal or reduced expression of the laminin-α2 chain, a protein present in the heterotrimERIC complexes laminin-2 and laminin-42 expressed in the basal lamina of skeletal muscle fibers. Laminin-α2, also expressed in Schwann cells, epidermis, and fetal trophoblastic tissue,3 is encoded by the LAMA2 gene.4 Defects in this gene are responsible for merosin-deficient CMD, autosomal recessive (MDC1A),5 the most frequent western form of CMD.1

All patients with primary laminin-α2 deficiency have abnormalities of the central white matter on magnetic resonance imaging (MRI) that, however, are usually asymptomatic.1 While clinically severe phenotypes are always associated with total lack of the protein,1 milder, though heterogeneous, phenotypes are usually associated with partial protein deficiency.6,7 Secondary reduction of laminin-α2 occurs in several CMD forms associated with reduced α-dystroglycan glycosylation1 and also in other variants with atypical phenotypes not linked to the LAMA2 locus.8,9 It is therefore important to determine the primary defect in the LAMA2 gene, particularly for prenatal diagnosis.

In this study, we sought LAMA2 mutations in patients with partial or complete laminin-α2 deficiency and related protein defect to clinical phenotype. We detected several previously undescribed mutations, performed prenatal diagnoses, and investigated the founder effect of a specific mutation.
Fifteen patients with clinical and pathological findings typical of CMD were included. All except patient 15 (with symptom onset at 9 months) had generalized hypotonia and severe weakness from birth. Multiple contractures were present at birth or became evident early in all patients except patients 10 and 13. In all muscle biopsy specimens, laminin-α2/H9251 expression was undetected or greatly reduced. Clinical features are summarized in Table 1.

### IMMUNOHISTOCHEMISTRY

Muscle or skin biopsy specimens, obtained after parental informed consent, were frozen and stored in isopentane cooled in liquid nitrogen. Laminin-α2 was analyzed on 6-µm thick cryosections using 2 commercially available monoclonal antibodies.6

### MOLECULAR ANALYSIS

Genomic DNA was extracted from blood and analyzed by the polymerase chain reaction (PCR) touchdown method using oligonucleotide primers flanking the intron-exon junctions of each of the 65 LAMA2 exons. Single-strand conformation polymorphism (SSCP) analysis was performed as described elsewhere10; both strands of all aberrant conformers were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Exon numbering according to the Leiden muscular dystrophy database11 differs from that reported by Zhang et al,12 whose exon 5 includes 2 exons (now exons 5 and 6).

New primers were synthesized to amplify exons 5 and 6:

- 5F, GCTCGCTATATTCGCCTGAG
- 5R, GTGGGATATCATTTGTAGGCTCT
- 6F, CTCTGGATTGCTTTTTGCAG
- 6R, CAGGGCTTCATTTTGCCTAA

(M) direction.

Each new missense mutation was verified in more than 100 normal, unrelated controls by SSCP. To determine the individual mutations in heterozygous cases, purified PCR

### METHODS

#### PATIENTS

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### Table 1. Clinical Findings in 15 Patients With Congenital Muscular Dystrophy

<table>
<thead>
<tr>
<th>Patient/ Sex</th>
<th>Laminin-α2 Chain Expression</th>
<th>Age Last Seen</th>
<th>Maximum Motor Ability</th>
<th>Highest Creatine Kinase Level, U/L</th>
<th>Brain MRI Results</th>
<th>Neurupathy (Age at Examination)</th>
<th>Neuropathy/ Mental Retardation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/F</td>
<td>Absent</td>
<td>24 mo</td>
<td>Sitting</td>
<td>&gt;1000</td>
<td>Not done</td>
<td>Not checked</td>
<td>No</td>
</tr>
<tr>
<td>2/M</td>
<td>Absent</td>
<td>22 mo</td>
<td>Sitting</td>
<td>&gt;1000</td>
<td>Abnormal-classic</td>
<td>Not checked</td>
<td>No</td>
</tr>
<tr>
<td>3/F</td>
<td>Absent</td>
<td>4.6 y*</td>
<td>Sitting</td>
<td>&gt;1000</td>
<td>Abnormal-classic</td>
<td>No, pathological EEG results</td>
<td>No</td>
</tr>
<tr>
<td>4/M</td>
<td>Absent; died at 12 y</td>
<td>9 y; died</td>
<td>Sitting</td>
<td>&lt;1000</td>
<td>Abnormal-classic</td>
<td>No</td>
<td>Absent (12 mo); Language delay</td>
</tr>
<tr>
<td>5/M</td>
<td>Absent</td>
<td>30 mo</td>
<td>Sitting</td>
<td>&gt;1000</td>
<td>Abnormal-classic</td>
<td>No</td>
<td>Absent (3 mo)</td>
</tr>
<tr>
<td>6/M</td>
<td>Absent</td>
<td>18 mo; died</td>
<td>Poor head control</td>
<td>&lt;1000</td>
<td>Normal at 4 mo;</td>
<td>Not checked</td>
<td>Absent (8 mo)</td>
</tr>
<tr>
<td>7/F</td>
<td>Absent</td>
<td>7 y</td>
<td>Walking</td>
<td>&lt;1000</td>
<td>Abnormal-classic</td>
<td>No</td>
<td>Absent (16 mo and 4 y); motor nerve demyelination (8 y)</td>
</tr>
<tr>
<td>8/F</td>
<td>Absent</td>
<td>9 y*</td>
<td>Sitting</td>
<td>&lt;1000</td>
<td>Abnormal-classic</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>9/M</td>
<td>Absent</td>
<td>5 y*</td>
<td>Sitting</td>
<td>&lt;1000</td>
<td>Abnormal-classic and cortical dysplasia</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>10/M</td>
<td>Reduced</td>
<td>10 y</td>
<td>Walking</td>
<td>&gt;1000</td>
<td>Abnormal-classic</td>
<td>Yes</td>
<td>Absent (3 y); Moderate</td>
</tr>
<tr>
<td>11/F</td>
<td>Absent</td>
<td>24 mo</td>
<td>Sitting</td>
<td>&gt;1000</td>
<td>Abnormal-classic</td>
<td>No</td>
<td>Absent (12 mo); Moderate</td>
</tr>
<tr>
<td>12/M</td>
<td>Absent</td>
<td>3 mo</td>
<td>Sitting</td>
<td>&gt;1000</td>
<td>Abnormal-classic</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>13/M</td>
<td>Reduced</td>
<td>6 y</td>
<td>Walking</td>
<td>&gt;1000</td>
<td>Normal</td>
<td>1 seizure</td>
<td>Absent (3 y); Moderate</td>
</tr>
<tr>
<td>14/M</td>
<td>Absent</td>
<td>6 y</td>
<td>Sitting</td>
<td>&lt;1000</td>
<td>Abnormal-classic</td>
<td>No</td>
<td>Milder reduced motor nerve conduction velocity (5 y)</td>
</tr>
<tr>
<td>15/F</td>
<td>Reduced</td>
<td>9 y</td>
<td>Standing with support</td>
<td>&gt;1000</td>
<td>Normal</td>
<td>No</td>
<td>Absent (18 mo)</td>
</tr>
</tbody>
</table>

Abbreviations: Abnormal-classic, white matter changes; EEG, electroencephalography; MRI, magnetic resonance imaging.

*Age at time of publication.
products were subcloned into a pMOSBlue vector (Amersham Pharmacia Biotech, Rainham, Essex, England) and the DNA sequenced using the T7 promoter and U19 primers.

RNA ISOLATION AND COMPLEMENTARY DNA AMPLIFICATION

To detect alternative transcripts in cases with splice-site mutations, total RNA was prepared from muscle or myoblasts using RNAWIZ (Ambion, Austin, Tex) and reverse transcribed using the First-Strand cDNA (complementary DNA) Synthesis kit (Amersham Pharmacia Biotech). The resulting complementary DNA was amplified by reverse transcriptase PCR and sequenced.

GENOTYPING

The region including the LAMA2 gene was analyzed for the microsatellite markers D6S407, D6S1620, and D6S1705 (Généthon genetic linkage maps). The genomic DNA PCR products were generated with fluorescent primer sets, pooled, and separated electrophoretically along with PCR products from a reference individual (CEPH 1347 02). Fractionation and data collection used an ABI Prism 377 (Applied Biosystems). The markers were typed with GeneScan 3.1.2 software (Applied Biosystems).

PRENATAL DIAGNOSIS

DNA from chorionic villus or amniocytes of at-risk pregnancies was tested for placental contamination by analysis of polymorphic regions. In 1 fetus, we first analyzed the 3 microsatellite markers bordering the LAMA2 locus and 5 intragenic polymorphisms; subsequent confirmation was by direct mutation detection, following our identification of the LAMA2 mutation in the family. In the 3 other fetuses, the previously identified mutation was investigated directly.

RESULTS

By immunohistochemistry, laminin-α2 expression was undetectable or greatly reduced in all 15 patients (Figure) (Table 1). The 2 antibodies provided closely similar results. LAMA2 mutations were found in 10 patients from 9 families (Table 2). The mutations in patients 1, 2, and 3, from different consanguineous families, were homozygous nonsense mutations in exons 2, 12, and 21, respectively. Those in patients 1 and 2 were novel; affected domains VI and IVb of the protein, respectively; and resulted in undetectable protein expression. The mutation in patients 3 and 4 (Cys967Stop) has been described previously in 2 Italian families originating from the southern Adriatic coast. Analyzed members of the families of patients 3 and 4 had the same Cys967Stop-associated haplotype. Investigation of the microsatellite markers flanking the LAMA2 gene in these 2 families indicated that both had an identical mutation-associated haplotype between the more widely spaced markers D6S407 and D6S1620, suggesting remote consanguinity and a founder effect. Cys967Stop truncates laminin-α2 in domain IIIb and the protein expression is undetectable.
The second mutation in patient 4 was a deletion in exon 6 (825delC) affecting domain V, causing a frameshift and subsequent stop codon.

We detected novel homozygous deletions in exons 34 (4861delC) and 57 (8007delT) in patients 5 and 6, respectively (Table 2). Both resulted in a frameshift, subsequent stop codon, and undetectable protein expression (Figure).

A novel heterozygous deletion in exon 30 (4375delG) was found in patient 7; the mutation on the second allele was not found. The protein expression was undetectable; however, at 7 years of age, she retained the ability to walk unaided.

Two novel mutations were found in patients 8 and 9 (siblings) from a nonconsanguineous family; a deletion in exon 25 (3630delT) and a duplication in exon 32 (4695-4698dupTGCA), resulting in undetectable protein expression.

A homozygous substitution (500A>C) in exon 4 was found in patient 10 from a consanguineous family, resulting in the substitution Gln167Pro. The SSCP indicated this change was absent in more than 100 normal, unrelated subjects. Sequence homology analysis has shown the glutamine-167 (domain VI) to proline. The whole N-terminal domain VI is in fact highly conserved in numerous human and nonhuman laminin isoforms and participates in calcium ion–dependent intermolecular interactions and integrin binding. In the spontaneous mutant dy2J/dy2J mouse, the LAMA2 mutation results in deletion of a section of domain VI concerned with laminin trimer polymerization but nearly normal expression of laminin-α2; the phenotype is severe but less so than in other laminin-α2-deficient models.

We have identified 1 known and 9 new LAMA2 mutations, all resulting in primary laminin-α2 deficiency and responsible for the MCD1A form of CMD. The Leiden muscular dystrophy database shows that mutations occur in most of the 65 exons and affect all protein domains. We identified nonsense, missense, and frameshift mutations affecting all domains. Since the second mutation in patient 4 was present in one of the 2 exons previously included in exon 5,12 we recommend analyzing both these exons separately or reanalyzing them in patients in whom a mutation has not been found.

Most children with LAMA2 mutation only manage unsupported sitting but may achieve standing with support or, more rarely, walking with support; however, these abilities are lost as scoliosis progresses.1 The phenotype was similarly severe in most of our patients with LAMA2 mutation. However, patient 7 was less affected; she achieved independent walking and was still walking at 7 years of age. She is similar to another case with a homozygous out-of-frame deletion and almost undetectable protein expression.13 Patient 10, with partial protein expression, also has a somewhat milder phenotype, as reported by others.6,7 He achieved independent walking and was still walking at 12 years of age. He has a homozygous missense mutation changing the conserved glutamine-167 (domain VI) to proline. The whole N-terminal domain VI is in fact highly conserved in numerous human and nonhuman laminin isoforms and participates in calcium ion–dependent intermolecular interactions and integrin binding. In the spontaneous mutant dy2J/dy2J mouse, the LAMA2 mutation results in deletion of a section of domain VI concerned with laminin trimer polymerization but nearly normal expression of laminin-α2; the phenotype is severe but less so than in other laminin-α2-deficient models.

Our data confirm the importance of MRI in patients with laminin-α2 deficiency. The classic white matter abnormalities associated with the clinical picture and laminin-α2 deficiency should direct attention to the LAMA2 gene. By contrast, normal brain MRI findings should direct attention to the clinically similar MDC1C, due to mutations in the FKRP gene, with secondary laminin-α2 deficiency.3 However, patients with MDC1C with mental
retardation and cerebellar cysts or atrophy, with or without white matter alterations, have been described recently. Furthermore, structural changes (including occipital polymicrogyria/agryria and hypoplastic pons or cerebellum) may be present in some patients with complete or mutation-proven partial laminin-α2 deficiency.1

Motor and sensory nerve conduction velocities were mostly normal in our patients (Table 1). This contrasts with altered motor nerve conduction reported elsewhere and is probably due to the young age at examination.

Among patients 11 through 15, with no detected LAMA2 mutation (Table 2), FKRP mutations were found in patients 13 and 15, both with normal MRI findings and reduced α-dystroglycan glycosylation. In patient 12, MRI findings were complex, with features similar to lissencephaly, as often observed in other forms of CMD; α-dystroglycan was absent suggesting a glycosylation defect. Patients 11 and 14 had undetectable laminin-α2 expression and white matter changes suggesting MDC1A. In patient 11, α-dystroglycan glycosylation was normal; in patient 14, muscle was no longer available.

Our results support a founder effect for the Cys967Stop mutation in the families of patients 3 and 4, as previously proposed for other families.10 Our families originated from the southern Adriatic, but patient 4’s family is from Albania. Since there are several enclaves of Albanian migrants in southern Italy but migration in the other direction practically nonexistent, the mutation probably originated in Albania.

MDC1A can be diagnosed prenatally by linkage analysis,10,17 protein testing of fetal tissue,18 or direct mutation analysis.17 Laminin-α2 is expressed in normal trophoblasts from 9 weeks,18 allowing immunohistochemical detection in chorionic villus. However, in cases of partial laminin-α2 deficiency or small sample size, protein detection is not reliable; furthermore, placental contamination may occur. Linkage analysis can also be problematic because it is based on the proximity and informativeness of genetic markers. Both methods together are usually necessary (provided the family is genetically informative) for reliable prenatal diagnosis. However, direct mutation detection is more reliable and is present in the only practical application of molecular diagnosis in the absence of effective treatment.

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