Clinical and Molecular Characterization of Patients With Limb-Girdle Muscular Dystrophy Type 2I

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**Background**: Limb-girdle muscular dystrophy type 2I is caused by mutations in the fukutin-related protein gene (FKRP). FKRP encodes a putative glycosyltransferase protein that is involved in α-dystroglycan glycosylation.

**Objectives**: To identify patients with limb-girdle muscular dystrophy type 2I and to derive genotype-phenotype correlations.

**Design**: Two hundred fourteen patients who showed muscle histopathologic features consistent with muscular dystrophy or myopathy of unknown etiology were studied. The entire 1.5-kilobase FKRP coding sequence from patient DNA was analyzed using denaturing high-performance liquid chromatography of overlapping polymerase chain reaction products, followed by direct sequencing of heteroduplexes.

**Results**: Thirteen patients with limb-girdle muscular dystrophy type 2I (6% of all patients tested) were identified by FKRP mutation analysis, and 7 additional patients were identified by family screening. Six missense mutations (1 novel) were identified. The 826C>A nucleotide change was a common mutation, present in 35% of the mutated chromosomes. Clinical presentations included asymptomatic hyperCKemia, severe early-onset muscular dystrophy, and mild late-onset muscular dystrophy. Dilated cardiomyopathy and ventilatory impairment were frequent features. Significant intrafamilial and interfamilial clinical variability was observed.

**Conclusions**: FKRP mutations are a frequent cause of limb-girdle muscular dystrophies. The degree of respiratory and cardiac insufficiency in patients did not correlate with the severity of muscle involvement. The finding of 2 asymptomatic patients with FKRP mutations suggests that modulating factors may ameliorate the clinical phenotype.

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PATIENTS

About 6000 consecutive muscle biopsy specimens from the tissue bank of the Neuromuscular Center, University of Padova, Padova, Italy, were screened for patients meeting the following clinical and laboratory criteria: (1) muscle histopathologic features consistent with muscular dystrophy or myopathy and (2) normal findings for dystrophin, α-sarcoglycan, calpain, and dysferlin on immunohistochemistry or immunoblotting. Two hundred fourteen muscle biopsy specimens from unrelated patients were selected and analyzed for FKRP mutation studies.

α-DYSTROGlycan IMMUNOBLOTTING ANALYSIS

α-Dystroglycan immunoblotting analysis was performed using a mouse monoclonal antibody directed against a glycosylated epitope of α-dystroglycan (IIH6).12

FKRP MUTATION STUDIES

DNA was extracted from peripheral blood or, if not available, from muscle biopsy specimens, using standard procedures. The entire 1.5-kb FKRP coding region was amplified using sets of overlapping primers in all 214 patients selected for the study (primer sequences and polymerase chain reaction conditions are available from the author). Denaturing high-performance liquid chromatography analysis was performed using a 3500HT WAVE DNA fragment analysis system (Transgenomic, Omaha, Neb).

CONFIRMATION ANALYSIS

The FKRP variants identified were confirmed using the appropriate restriction endonuclease digestion or single-strand conformational polymorphism analysis. Each novel mutation was assessed on 150 control chromosomes.

RESULTS

FKRP mutations were identified in 13 (6%) of 214 patients with muscular dystrophy or myopathy. Seven additional patients with the mutation were identified through family screening.

CLINICAL FEATURES

Twenty patients (12 male and 8 female) from 13 families were studied. The age at onset of LGMD2I ranged from 2 to 50 years (mean±SD age, 19±17 years). Ten of 20 patients had onset of symptoms before the age of 15 years, and 3 patients had onset in the fourth and fifth decades of life. Symptoms and signs at clinical presentation included proximal muscle weakness in the lower limbs (7 patients), weakness in both the upper and the lower limbs (2 patients), myalgia (2 patients), and myalgia associated with muscle weakness (1 patient); 4 patients ambulated slower than their peers. In one patient (patient 11) (Table 1), muscle stiffness during effort was the initial symptom at age 37 years, and in another patient (patient 18), dyspnea with muscle weakness was the initial symptom. Two patients (patients 10 and 19) were asymptomatic and were identified through family screening.

Two patients died, one at age 16 years (patient 1) (Table 1) and another at age 53 years (patient 13). At birth, patient 1 had dyspnea, polypnea, and cyanosis. Transposition of the great arteries was diagnosed and surgically corrected at age 3½ months with the atrial reduction procedure (Senning operation). His motor skills developed normally, but he had increasing difficulties in climbing stairs and rising from the floor at age 2 years. Muscle weakness progressed, and he lost the ability to ambulate at age 13 years. Subsequently, the patient experienced several episodes of right ventricular failure and died at age 16 years. Several echocardiographic studies revealed right ventricle dilatation, a minimal interventricular septal defect, and normal left ventricle function.

Patient 13 (Table 1) was not clinically evaluated, and all information about him was derived from medical records. The patient died at 53 years of age of dilated cardiomyopathy. He was reported to have had a waddling gait and difficulties in climbing stairs and rising from a chair since he was 20 years old.

Fifteen of 20 patients were still ambulatory at ages ranging from 19 to 65 years. Five patients lost the ability to ambulate when they were aged between 13 and 69 years. Distribution of weakness was predominantly proximal and symmetrical in the pelvic girdles in all patients. Shoulder girdle weakness developed subsequent to lower limb weakness but remained minor in all patients. Distal hand muscles were affected only in patient 20 (Table 1), but distal leg muscle weakness was present in 4 patients (patients 5, 14, 16, and 20) with progression of the disease. One patient (patient 5) had mild weakness of eye closure. Winging of the scalp was seen in 3 patients. Seven patients had calf hypertrophy, and 3 had macroglossia. Patient 11 had ptosis of the right eye; neuroimaging results demonstrated no abnormal findings in this patient. Patient 2 had minimal proximal weakness and slight hypertrophy of the right calf. Patient 16 had severe muscular dystrophy with hyperlordosis, a marked waddling gait with difficulties in climbing stairs, and the inability to rise from the floor. She also had severe weakness of the neck extensor muscles, with head dropping. Patient 20 was born at term, with the delivery complicated by a left brachial plexus lesion from fetal macrosomia due to maternal diabetes mellitus. At age 40 months, the patient had increasing difficulties in climbing stairs, walking long distances, and lifting the upper arms, and she had a tingling sensation in the distal portion of the lower extremities. Neurological examination at age 69 years showed diffuse muscle wasting in the upper and lower limbs and interosseous muscle atrophy, bilateral pes cavus, and severe proximal and distal muscle weakness (grade 3 or 3.5 on the Medical Research Council scale). The patient was able to stand with bilateral support and to take only a few steps. Electromyography at age 69 years showed a neurogenic pattern in all muscles examined.

In all patients, creatine kinase levels were markedly increased in the early stages of LGMD2I but declined with advancing age. All patients had normal cognitive function.
RESPIRATORY AND CARDIAC STUDY

Twelve patients underwent conventional spirometry. Three patients (patients 2, 19, and 20) (Table 1) had normal pulmonary function. In 9 patients, forced vital capacity (mean±SD, 1.9±1 L; 46±19 of the predicted percentage) and forced expiratory volume (1.8±0.9 L; 50±16 of the predicted percentage) were markedly reduced; however, the severity of the restrictive lung impairment did not clearly correlate with disease duration. Following onset of ventilatory failure with severe carbon dioxide retention, 3 patients (patients 6, 11, and 16) required long-term noninvasive mechanical ventilation at the ages of 34, 47, and 56 years, respectively, 29, 10, and 21 years, respectively, after the onset of LGMD2I.

A complete cardiac evaluation was obtained in 13 patients, including history, physical examination, electrocardiography, and echocardiography. Two patients (patients 13 and 18 [Table 1]) had symptoms of heart failure. Patient 13 died at age 53 years of severe dilated cardiomyopathy after several episodes of left ventricular failure. Patient 18 had severe dilated cardiomyopathy since age 46 years, and a biventricular pacemaker for synchronization therapy was placed at age 51 years. At age 63 years, the patient is on a waiting list for heart transplantation. Although most of the 13 patients appeared to have symptoms of cardiac failure, 5 of 13 had neither signs of left ventricular dilatation nor left ventricular ejection fraction reduction. One patient had left ventricular wall motion ab-

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**Table 1. Clinical Data Among 20 Patients With Limb-Girdle Muscular Dystrophy Type 2I**

<table>
<thead>
<tr>
<th>Patient No./Sex/Age at Onset, y</th>
<th>Family History</th>
<th>Onset Symptoms</th>
<th>Age at Last Evaluation, y</th>
<th>Muscle Hypertrophy</th>
<th>Muscle Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/2</td>
<td>No</td>
<td>Muscle weakness in the LL</td>
<td>16†</td>
<td>No</td>
<td>LL &gt;&gt; UL</td>
</tr>
<tr>
<td>2/M/3</td>
<td>No</td>
<td>Myalgia</td>
<td>19</td>
<td>Slight right calf</td>
<td>LL = UL</td>
</tr>
<tr>
<td>3/M/13</td>
<td>Adopted</td>
<td>Myalgia and muscle weakness in the LLs and UL</td>
<td>42</td>
<td>No</td>
<td>LL &gt;&gt; UL, winging of the scapula</td>
</tr>
<tr>
<td>4/F/14</td>
<td>No</td>
<td>Muscle weakness in the LL</td>
<td>26</td>
<td>Calves</td>
<td>LL &gt;&gt; UL</td>
</tr>
<tr>
<td>5/M/7</td>
<td>No</td>
<td>Slower than peers</td>
<td>30</td>
<td>No</td>
<td>LL = UL, proximal and distal, weakness of eye closure</td>
</tr>
<tr>
<td>6/M/5</td>
<td>No</td>
<td>Slower than peers</td>
<td>35</td>
<td>No</td>
<td>LL &gt;&gt; UL</td>
</tr>
<tr>
<td>7/M/22</td>
<td>No</td>
<td>HyperCKemia</td>
<td>42</td>
<td>Calves</td>
<td>LL &gt;&gt; UL</td>
</tr>
<tr>
<td>8/M/7</td>
<td>Yes</td>
<td>Muscle weakness in the LL</td>
<td>42</td>
<td>Calves</td>
<td>LL &gt;&gt; UL</td>
</tr>
<tr>
<td>9/M/10</td>
<td>Yes</td>
<td>Slower than peers</td>
<td>48</td>
<td>Calves</td>
<td>LL = UL</td>
</tr>
<tr>
<td>10/M/asymptomatic</td>
<td>Yes</td>
<td>Asymptomatic</td>
<td>52</td>
<td>Calves</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>11/M/37</td>
<td>No</td>
<td>Muscle stiffness</td>
<td>53</td>
<td>Tongue</td>
<td>LL &gt;&gt; UL, winging of the scapula, ptosis of the right eye</td>
</tr>
<tr>
<td>12/F/25</td>
<td>Yes</td>
<td>Muscle weakness in the LL</td>
<td>53</td>
<td>No</td>
<td>LL &gt;&gt; UL</td>
</tr>
<tr>
<td>13/M/20</td>
<td>Yes</td>
<td>Muscle weakness in the LL</td>
<td>53†</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>14/F/10</td>
<td>Yes</td>
<td>Muscle weakness in the LL</td>
<td>54</td>
<td>No</td>
<td>LL &gt;&gt; UL, proximal and distal</td>
</tr>
<tr>
<td>15/F/50</td>
<td>No</td>
<td>Myalgia</td>
<td>58</td>
<td>No</td>
<td>LL &gt;&gt; UL</td>
</tr>
<tr>
<td>16/F/35</td>
<td>Yes</td>
<td>Muscle weakness in the LL</td>
<td>61</td>
<td>Tongue</td>
<td>LL &gt;&gt; UL, proximal and distal</td>
</tr>
<tr>
<td>17/M/12</td>
<td>Yes</td>
<td>Slower than peers</td>
<td>61</td>
<td>Calves</td>
<td>LL &gt;&gt; UL, winging of the scapula</td>
</tr>
<tr>
<td>18/F/49</td>
<td>Yes</td>
<td>Muscle weakness in LL and dyspnea</td>
<td>63</td>
<td>Tongue</td>
<td>LL = UL, scoliosis</td>
</tr>
<tr>
<td>19/F/asymptomatic</td>
<td>Yes</td>
<td>Asymptomatic</td>
<td>65</td>
<td>No</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>20/F/40</td>
<td>No</td>
<td>Muscle weakness in the LL and UL</td>
<td>69</td>
<td>No</td>
<td>LL = UL, proximal and distal</td>
</tr>
</tbody>
</table>

Abbreviations: LL, lower limbs; NA, not available; UL, upper limbs; >>, much greater than.

*Patients 8, 9, 10, 12, 13, 14, and 17 are from the same family. Patients 16 and 19 are sisters. The following 5 patients lost the ability to ambulate: patient 1 at age 13 years, patient 3 at age 42 years, patient 4 at age 24 years, patient 6 at age 25 years, and patient 20 at age 69 years.

†Deceased.

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**Figure 1. α-Dystroglycan expression in muscle biopsy specimens from patients (Table 1) and a healthy control subject. A, α-Dystroglycan immunoblotting analysis of the patient specimen using the IIH6 antibody directed against a glycosylated epitope of the protein. B, Corresponding posttransfer gel stained with Coomassie blue to visualize the myosin heavy-chain protein.**
normalities, 5 patients had mild dilated cardiomyopathy, and 2 patients had severe dilated cardiomyopathy.

**α-DYSTROGLYCAN IMMUNOBLOTTING ANALYSIS**

α-Dystroglycan immunoblotting analysis was performed in 10 patients. A broad band of approximately 156 kDa was seen in normal muscles. Five patients (patients 1, 3, 4, 7, and 16 [Table 1]) had only traces of glycosylated α-dystroglycan in their muscle biopsy specimens, and 4 patients (patients 2, 9, 10, and 15) had decreased intensity and narrowing of the band, with a shift toward lower molecular weight of α-dystroglycan. Patient 5 had only minor alterations in the molecular weight of α-dystroglycan (**Figure 1**).

**FKRP MUTATION STUDIES**

Among 214 patients analyzed, 13 elution profiles differed from the wild-type DNA sample profile (**Figure 2**). We identified 6 disease-causing mutations (1 novel) in 13 patients, and 7 polymorphisms (4 novel) in 104 patients. In 9 patients, both FKRP mutated alleles were identified, and in 4 patients, only one was identified.
All FKRP mutations were missense mutations, and all coded for amino acids located in the luminal domain of the protein (Table 2). The mutations were distributed through the entire FKRP coding sequence, without significant clustering; however, the 826C>A nucleotide change was present in 9 (35%) of 26 mutated chromosomes. The 731G>A nucleotide change, coding for Arg244His, is a novel mutation.

Of the 7 FKRP polymorphisms identified, 5 were silent nucleotide changes (135C>T [Ala45Ala], 201A>G [Val67Val], 1164T>C [Asp388Asp], 1242C>T [Hys414Hys], and 1405C>T [Leu469Leu]), and 1 was in the 5’ untranslated region (−34C). A 341C>G nucleotide change resulted in an alanine to a glycine amino acid change (Ala114Gly). The 341C>G nucleotide change was present in 1 of 100 chromosomes studied.

We describe a large series of Italian patients affected by LGMD2I. FKRP mutation studies were conducted using denaturing high-performance liquid chromatography in 214 patients affected by muscular dystrophy or myopathy of unknown etiology. Thirteen patients with FKRP mutations were identified, and 7 additional family members proved to have the same mutations as those of the probands.

We identified both mutant alleles in 9 (70%) of 13 patients. Only one mutation was detected in 4 heterozygous patients (30%). The 4 patients in whom only one mutated allele was identified carried the same 427C>A nucleotide change, resulting in an arginine-to-serine amino acid substitution (Arg143Ser). We do not have compelling evidence that these patients are indeed affected by LGMD2I, but several findings suggest that they are. First, the 427C>A mutation has previously been reported in LGMD2I; second, in our cohort an individual was compound heterozygous for this mutation and another previously reported missense FKRP mutation (1073C>T)*; third, the clinical presentation in these 4 patients was consistent with LGMD2I; and fourth, the 427C>A mutation meets the commonly accepted criteria for validation of the mutation (it is absent in >200 control chromosomes, it involves an evolutionary conserved residue, and the resulting mutant protein is predicted to have lost a positively charged amino acid, with a dramatic effect on protein function).

Clinically, the age at onset, rate of disease progression, and severity varied greatly at the interfamily and intrafamily levels in our cohort of patients. One patient carrying a homozygous FKRP mutation and another patient carrying a compound heterozygous FKRP mutation, ascertained through family screening, were asymptomatic, had mildly elevated creatine kinase levels, and were unaware of any clinical problems, while their affected siblings displayed a severe LGMD2I phenotype. The findings in these 2 patients suggest that FKRP mutations are not the only determinant in phenotype severity and that polygenic modifiers may affect age at onset and disease progression. Despite only sporadic reports of asymptomatic mutation carriers in limb-girdle muscular dystrophies, asymptomatic carriers seem to be common among patients with LGMD2I. Four other asymptomatic carriers have previously been described,* suggesting that modulation of phenotype expression may be not only at the transcriptional level but also at the posttransductional level, based on protein glycosylation states. The theory that enhanced glycosylation may be beneficial in muscular dystrophy has already been explored, and the findings among the patients described herein may strengthen the hypothesis that bypass rather than correction of genetic defects could effectively treat muscular dystrophies.

The premise that protein glycosylation is a dynamic process amenable to multiple modulations is also suggested by the occurrence of unusual clinical presenta-
tions in LGMD2I. For example, patient 1 (Table 1) had a severe phenotype similar to Duchenne muscular dystrophy and was born with transposition of the great arteries. Although these 2 findings may be unrelated, a high incidence of malformation of the cardiac outflow tract was observed in perlecan-deficient mice embryos.14 Perlecan, a heparin sulfate proteoglycan, binds to the cell surface in a dystroglycan-dependent manner.15 Abnormal α-dystroglycan glycosylation, which is a hallmark of LGMD2I, may interfere with this binding and eventually result in cardiac malformation.14 Patient 20 in our study, who had a clinical phenotype with combined myopathic and neurogenic features, also demonstrates the importance of proper glycosylation for normal binding of α-dystroglycan to ligands (such as perlecan or laminin) in the extracellular matrix. Peripheral nerve involvement has not previously been reported in LGMD2I, to our knowledge, although it is common in laminin α2 deficiency in which LAMA2 mutations affect muscles and nerves.16 The reasons for this discrepancy are unknown, but it is surprising, considering that secondary loss of laminin α2 or improper processing of the protein is common in LGMD2I.1,3

Respiratory and cardiac impairment was frequent among our LGMD2I cohort and has been previously reported.10 In our series, no clear correlations between disease duration or disease severity with the onset of respiratory or cardiac involvement were detected. Respiratory failure was not a late complication in the natural history of the disease, as it preceded the onset of profound muscle weakness in several patients. On the other hand, the severity of cardiac involvement also did not parallel the degree of respiratory failure in any patient. The selective vulnerability of respiratory muscles to glycosylation is not well investigated, but it is possible that the degree of glycosylation of dystroglycan or other target proteins (through the resulting ability to cross-link and aggregate with various extracellular matrix proteins) affects the viscoelastic properties and compliance of the diaphragm earlier than in other muscles.

In conclusion, understanding the molecular basis of clinical heterogeneity in LGMD2I is challenging. The questions raised herein may inspire new treatment approaches among patients with limb-girdle muscular dystrophies.

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