FKRP (826C>A) frequently causes limb-girdle muscular dystrophy in German patients


Limb-girdle muscular dystrophies (LGMD) and congenital muscular dystrophies (MDC) represent two heterogeneous groups of genetic diseases differing in clinical severity and age of onset. Mutations in the LAMA2 gene can result in either MDC or, more rarely, LGMD. Laminin α2 forms a link between α-dystroglycan and the basal lamina. α-Dystroglycan is a heavily glycosylated peripheral membrane component of the dystrophin-associated-glycoprotein complex (DAG), whilst β-dystroglycan, derived from the same gene, is a transmembrane protein that links to dystrophin intracellularly. Dystroglycan therefore plays a pivotal role in linking the actin-associated cytoskeleton to components of the extracellular matrix, and disruption of this axis is associated with several forms of muscular dystrophy. Recently, abnormalities of α-dystroglycan glycosylation have been reported for several forms of MDC and for LGMD2I. A novel gene encoding a putative glycosyltransferase, fukutin-related protein (FKRP), was found to be responsible for both a novel form of MDC (MDC1C) and for LGMD2I. Interestingly, the single homozygous point mutation (826C>A) leading to an amino acid exchange (Leu276Ile) is associated with a relatively benign clinical phenotype, whereas patients compound heterozygous for the 826C>A mutation may show a more severe clinical phenotype. We investigated the frequency of the FKRP mutation (826C>A) in 124 LGMD patients and correlated these findings with the clinical phenotype.

METHODS

Patients

All patients described in this study were examined by one of the coauthors. Most patients described are of German descent, but lives in Hungary. One patient is Croatian (patient 2 in the table), and one patient (patient 19 in the table) is of German descent, but lives in Hungary.

Consanguinity was not reported. Pedigrees were compatible with autosomal recessive traits. Electromyography of affected muscles in all patients was performed with chronic myopathy. The clinical findings of all patients are summarised in the table. Diagnostic muscle biopsies were performed in 19 out of 20 patients and showed various degrees of chronic necrotic myopathy without inflammatory or specific structural changes on standard histological examinations. In all samples, the number of necrotic fibers was below 10% of the total. Muscle tissue was available for immunohistochemistry and immunoblotting in all but three patients. Venous blood samples were used for DNA extraction and FKRP mutation analysis. Morphological and genetic data are shown in the table, and clinical phenotypes are presented in figure 1. In all patients available for testing brain MRI, pulmonary function and cardiac ultrasound were performed.

Key points

- A novel gene (FKRP) was found to be responsible for both a form of congenital muscular dystrophy (MDC1C) and a form of limb-girdle muscular dystrophy (LGMD2I). Our work aimed at investigating the frequency of FKRP (826C>A) in a large cohort of limb-girdle muscular dystrophy (LGMD) patients and correlating these findings with the clinical phenotype.
- We present clinical and genetic data of 20 LGMD2I patients from 19 unrelated families. Neurological examinations consisting of diagnostic muscle biopsies including immunohistochemistry and sequence analysis of FKRP were performed in this cohort.
- Interestingly, one single point mutation (826C>A) leading to an amino acid exchange (Leu276Ile) was associated with a relatively benign clinical phenotype. We identified the previously described FKRP mutation (826C>A) in 20 patients. Thirteen patients were homozygous for the mutation and seven were compound heterozygous. In all patients heterozygous for the 826C>A mutation, a second heteroallelic mutation was detected; two mutations have been described before, four mutations are novel. Interestingly, muscle pain and myoglobinuria were the earliest presenting symptoms in most patients.
- In summary, LGMD2I is frequently caused by a single missense mutation of the FKRP gene, namely 826C>A (Leu276Ile). This knowledge may simplify the diagnostic workup for LGMD patients of European origin.

Immunohistochemistry

Serial 10 μm cross-sections were obtained in a cryostat, placed on gelatinised slides and fixed with acetone for 1 min. Commercially available monoclonal antibodies to α-dystroglycan (Clone VIA4-1, Upstate Biotechnology, Lake Placid, NY, USA) and merosin (MAB 1922, Chemicon, Temecula, Canada) were used at conditions recommended by the suppliers. This was followed by incubation with secondary biotin-conjugated anti-mouse IgG (Amersham, Pharmacia Biotech, Uppsala, Sweden) and streptavidin-coupled Cy3 (Dianova, Hamburg, Germany).

Abbreviations: DAG, dystrophin-associated-glycoprotein complex; FKRP, fukutin-related protein; LGMD, limb-girdle muscular dystrophy; MDC, congenital muscular dystrophy.
## Clinical and genetic findings of 20 LGMD2I patients from 19 independent families

<table>
<thead>
<tr>
<th>No</th>
<th>Sex</th>
<th>Age at onset</th>
<th>Disease duration</th>
<th>Age at exam</th>
<th>Distribution of weakness (MRC)</th>
<th>Average CK levels (normal value = 80 U/l)</th>
<th>Biopsy muscle</th>
<th>Mer</th>
<th>αDG</th>
<th>Protein analysis IHC</th>
<th>Mode of inheritance</th>
<th>FKRP analysis</th>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>5</td>
<td>35</td>
<td>40</td>
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<td>Biopsy brochii</td>
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<td>n.d.</td>
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<td>826C–A</td>
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<tr>
<td>2</td>
<td>M</td>
<td>8</td>
<td>4</td>
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<td>Rectus femoris</td>
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<tr>
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<td>12</td>
<td>19</td>
<td>31</td>
<td>4/5 proximal arm muscles, 3/5 proximal leg muscles, 4/5 foot extensors</td>
<td>2000</td>
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<td>Compound heterozygous</td>
<td>826C–A, 899T&gt;C</td>
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</table>

*Patients 1 and patient 3 are siblings.

αDG, α-dystroglycan; CK, creatine kinase; IHC, immunohistochemistry; Mer, merosin; MRC, Medical Research Council; +, normal; ( ), mildly reduced; - , reduced; - -, severely reduced or missing; n.d., not done.
Clinical phenotype of independent LGMD2I patients according to the table (A is patient 5, B is patient 17, C is patient 12). Patients are homozygous (A, C) or compound heterozygous (B) for the FKRP Leu276ile mutation. Calf hypertrophy is seen in some (A, B) but not all patients (C).

Analysis of two polymorphisms at the FKRP gene locus reveals a common core founder haplotype of the 826C>A allele. (A) Exons 1–4 are indicated by boxes and the coding region is represented by a filled black box. The newly identified polymorphism -326C/G is located upstream of the transcriptional start site in the promoter region. The intragenic polymorphism 135C/T has been described previously. (B) Fisher’s exact two-tailed test reveals a common haplotype carrying -326G, 135T, and 826A.

<table>
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<tr>
<td>Number of alleles (normal controls)</td>
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p = 0.036

<table>
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<th>135C</th>
<th>Total</th>
</tr>
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<td>Number of alleles (826C&gt;A homozygous)</td>
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<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Number of alleles (normal controls)</td>
<td>7</td>
<td>27</td>
<td>34</td>
</tr>
</tbody>
</table>

p = 0.0016
Mutation analysis of the FKRP gene

Venous blood samples were obtained from the patients as well as from their affected and unaffected family members, if available. Genomic DNA was isolated using a blood and tissue culture DNA extraction kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). A total of 124 patients (81 patients from the University of Munich, 33 patients from the University of Bonn, and 10 patients from other centers) with so far unclassified LGMD were screened for the FKRP (826C>A) mutation. Muscle biopsies were taken in all but seven patients, and screened for deficiencies in dystrophin, sarcoglycans, dysferlin, caveolin-3, and calpain-3 by standard immunohistochemical and immunoblotting methods. No abnormalities were found except for a mild or moderate reduction of calpain-3 on immuno blot of 29 patients. However, mutations in CAPN3 were excluded in these patients by direct sequencing of all exons, and calpain-3 deficiency was therefore considered to be secondary.

A PCR fragment encompassing the common 826C>A mutation was amplified using primers 3F[5’-ACCAGGCTTCTTCTCGAGACC-3’] and 4R[5’-CCTTCTCCCATACGAGGC-3’]. Standard PCR conditions were applied: initial melting at 95°C for 5 min; followed by 32 cycles at 52°C for 90 s, 72°C for 3 min, and 94°C for 1 min; and a final extension at 72°C for 7 min. The resulting 532 bp fragment was purified using the NucleoSpin Extract kit (Macherey-Nagel, Düren, Germany), digested with the restriction endonuclease BfaI (New England Biolabs, Frankfurt, Germany), and the resulting fragments separated on 2% agarose gels by electrophoresis. The mutant fragment (826C>A) remains uncut, whereas wild-type DNA is digested into two fragments of 198 and 270 bp length. A PCR fragment encompassing 899T>C was amplified using primers 3F and 4R. The resulting 532 bp fragment was purified and then digested with the restriction endonuclease BclI (Promega, Mannheim, Germany). The mutant fragment (362T>A) remains uncut, whereas wild-type DNA is digested into two fragments of 198 and 270 bp length. A PCR fragment encompassing 899T>C was amplified using primers 3F and 4R. The resulting 532 bp fragment was purified and then digested with the restriction endonuclease SacII (MBI Fermentas, St. Leon-Rot, Germany). Mutant DNA (899T>C) is digested into three fragments of 68, 174, and 290 bp whereas wild-type DNA is digested into two fragments of 68 and 464 bp length. A PCR fragment encompassing 1054C>G was amplified using primers 3F and 4R. The resulting 532 bp fragment was reamplified using primer 352s (TACCCTCTACGAGGCAGCCGAGC) and mismatch primer 352as (TCCCATGAGATGATGCCCCGAGC; mismatch indicated by bold letter). The resulting 161 bp fragment was purified and digested with the restriction endonuclease Eco52I (MBI Fermentas, St. Leon-Rot, Germany). Mutant DNA (1054C>G) is digested into two fragments of 135 and 26 bp length whereas wild-type DNA remains uncut.

Furthermore, we determined the frequency of two intragenic polymorphisms, -326C/G (promoter) and 135C/T (exon 4) in mutant (826C>A) and normal alleles by direct sequencing (fig 2). Alleles of patient 3, the brother of patient 1, were not used for the calculation. For statistical analysis Fisher's two-tailed exact test was employed to ascertain whether individual polymorphisms were linked to 826C>A.

Sequence comparison

To compare the amino acid sequences of human FKRP with FKRP homologues from other species, sequences were
accessed through public domains (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and aligned with FASTA software. The accession numbers are: Homo sapiens (NP_077277), Mus musculus (NP_775606), Rattus norvegicus (XP_218425), Drosophila melanogaster (NP_611531), and Anopheles gambiae (XP_314789).

RESULTS
Clinical data
We identified FKRP (826C>A) either homozygously or compound heterozygously in 20 patients from 19 unrelated families. A summary of the clinical, morphological, and genetic findings for all patients is shown in the table and in figure 1.

Onset of symptoms occurred from 3 to 32 years of age, with a mean age of 15 ± 7 years. Mean disease duration was 19 ± 7 years and mean age at examination was 34 ± 10 years. In 15 out of 20 (75%) patients, myalgia and rhahdomyolysis were the first signs of the disease. Patients frequently complained about muscle cramps, predominantly in calf muscles, and exercise-induced muscle pain. In one patient (patient 14), a malignant hyperthermia-like reaction along with myoglobinuria (CK levels up to 23 000 U/l, normal value <80 U/l) during adenotomy (general anaesthesia, application of succinylcholine) represented the first sign of muscle disease at the age of 3 years. This reaction was not investigated further, and there is no report of malignant hyperthermia in the relatives of patient 14. The phenotype is variable: some patients (patients 4, 5, 6, and 11) do not suffer from severe paralyses even decades after the onset of symptoms, while others are more severely affected. All patients are still able to walk without support. However, some patients (patients 7, 9, 12, 15, 16, 17, and 20) show difficulties in walking and climbing stairs because of marked proximal leg weakness. Distribution of weakness was mainly proximal, affecting the hip girdle more than the shoulder girdle. In most patients, foot extensors became affected with ongoing disease. Calf hypertrophy was noted in eight out of 20 patients. Lumbar lordosis, scoliosis, and scapular winging were not commonly found, and none of the patients showed facial weakness, tongue hypertrophy, or contractures.

CK levels were examined on several occasions and were found to be elevated 4- to 60-fold in our patients (CK mean 1800 ± 1600 U/l). The highest CK levels were found during episodes of myoglobinuria, but levels never returned back to normal values. Most of the patients with a disease duration of more than 20 years showed less dramatic CK elevations as compared to patients with a shorter disease duration. Brain MRI scans were performed in 12 out of 20 patients and showed no abnormalities. Pulmonary function and cardiac ultrasound were carried out in all patients. Two patients (patients 3 and 9) showed mildly to moderately impaired cardiac function (ejection fraction; patient 3: 55%; patient 9: 38%), while in one patient vital capacity was mildly reduced to 80% (patient 1).

Immunohistochemistry
The histological changes were characteristic of a muscular dystrophy in all patients. Immunohistochemistry revealed a variable reduction of α-dystroglycan in most patients (table). However, the degree of α-dystroglycan reduction did not clearly correlate with the severity of clinical symptoms nor with the age of onset. Immunohistochemistry for merosin showed a reduction in seven out of 15 examined patients.

DISCUSSION
In this study, we present clinical and genetic data of 20 LGMD2I patients from 19 unrelated families. The severity of muscular dystrophies due to FKRP mutations is described to vary remarkably, ranging from severe MDC to mild LGMD of adulthood. In this study, the average clinical phenotype is relatively mild and includes patients presenting with muscle pain, myoglobinuria, and elevated CK levels only. This is partly due to a sampling bias since more affected patients (patient 2), who presented with a more severe phenotype, moved to pediatric, not neurologic departments, and only patients carrying the frequent mutation FKRP (826C>A) were included. Similarly, previous studies have reported a more benign LGMD phenotype to be associated with this polymorphism (336G>C) and a polymorphism in the promoter region (-326G>C) were analysed. Both polymorphisms were identified homozygously in all patients homozygously harboring the FKRP (826C>A) mutation. A Fisher’s exact test indicated strong linkage disequilibrium for both polymorphisms when FKRP (826C>A) alleles were compared to controls (fig 2). Moreover, FKRP (826C>A) was identified heterozygously in one out of 300 healthy controls (600 control chromosomes).

Immunohistochemistry
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Genetic analysis
In a cohort of 124 LGMD patients (81 patients from Munich neurological department, 33 patients from Bonn neurological department, and 10 from other departments) the previously described FKRP mutation 826C>A (Leu276Ile) was identified in 20 patients, of which 13 were homozygous for the mutation and seven were compound heterozygotes (table). None of the homozygous individuals was the offspring of consanguineous marriages. In all patients heterozygous for the 826C>A mutation we were able to detect a second heteroallelic mutation by sequence analysis (table). Two of these mutations were previously described (919T>G resulting in Tyr307Asn11; 1486T>G resulting in Stop496Arg11). Interestingly, in two of our patients the latter mutation was associated with Ala114Gly (341C>G) which was also seen in patients from Tunisia.11 Four mutations are novel: 1475delC is predicted to result in a frameshift close to the termination codon which may cause an elongation of the amino acid chain. None of the three novel missense mutations Val121Glu (362T>A), Val300Ala (896T>C), and Arg352Gly (1054C>G) were found in 100 normal alleles each. Val300 and Tyr307 are highly conserved across species, while Val121 and Arg352 are only conserved among mammals, but not in insects (fig 3).

To evaluate the hypothesis that the FKRP mutation 826C>A is due to a founder effect, a previously described polymorphism in exon 4 (1335C>T10) and a polymorphism in the promoter region (-326G>C) were analysed. Both polymorphisms were identified homozygously in all patients homozygously harboring the FKRP (826C>A) mutation. A Fisher’s exact test indicated strong linkage disequilibrium for both polymorphisms when FKRP (826C>A) alleles were compared to controls (fig 2). Moreover, FKRP (826C>A) was identified heterozygously in one out of 300 healthy controls (600 control chromosomes).
normal German control (data not presented). Therefore, Ala114Gly should be considered as a rare polymorphism, while Stop496Arg is likely to represent the pathogenic mutation in our and the Tunisian patients. Similarly to Stop496Arg, the novel mutation 1475delC close to the termination codon may cause an elongation of the amino acid chain by several missense codons. We hypothesise that the three novel missense mutations (Val121Glu, Val300Ala, and Arg352Gly) constitute pathogenic changes, since (i) they were not detected in 100 normal alleles, (ii) they affect highly conserved amino acid residues (Val300Ala) or alter the charge of the residue (Val121Glu, Arg352Gly), (iii) they are heteroallelic to Leu276Ile, and (iv) they lead to the typical clinical and biochemical alterations. However, functional studies of mutant FKRP would need to be undertaken to firmly prove the pathogenic nature of these mutations.

The frequent mutation Leu276Ile (826C>A) was found to be associated with two intragenic polymorphisms in all cases and may therefore constitute an old founder allele. This is partly at variance with a previous study that found evidence for several founders through haplotyping using two extragenic, polymorphic repeats spanning 3 cM of the FKRP region. However, two rare alleles of D19S606 that appeared to be in linkage disequilibrium with the mutation were observed, suggesting it may have arisen on a limited number of occasions. Therefore, further studies on a larger number of mutant alleles including both intra- and extragenic, polymorphic markers need to be undertaken to determine whether Leu276Ile (826C>A) has arisen several times, or whether it appeared only once. In the latter case, this event may have happened a long time ago to allow for the observed heterogeneity of close, extragenic markers through recombination events and to explain the Europe-wide distribution of the mutation. Interestingly, Leu276Ile (826C>A) has also been observed in the Hutterites, an endogamous population that emigrated from Europe to North America several hundreds of years ago. Haplotyping of the Hutterite Leu276Ile (826C>A) allele may help to better understand the history of the mutation.

Overall, LGMD2I appears to be one of the most common muscular dystrophies in adult patients, within the range of LGMD2A (calpainopathies), LGMD2B (dysferlinopathies), and LGMD2C-F (sarcoglycanopathies). Although we did not undertake epidemiological studies and a sampling bias needs to be considered, LGMD2I due to Leu276Ile (826C>A) allele is responsible for 19% (15 out of 81) and 18% (six out of 33) of so far unclassified LGMD in two German cohorts (from the neurology departments of Munich and Bonn, respectively). This is in keeping with a recent study that identified LGMD2I in 11% of all LGMD2 patients. A previous study from the UK identified one Leu276Ile (826C>A) mutant allele in 200 normal control chromosomes, whereas identified one mutant allele in 600 control chromosomes in Germany. Therefore, Leu276Ile (826C>A) was detected at a similar frequency in the general populations of both Germany and the UK. Accordingly, a prevalence for LGMD2I could be estimated that is within the range of other, more frequent muscular dystrophies. However, epidemiological studies are warranted to prove this hypothesis.

So far, accurate diagnosis of LGMD was based on a muscle biopsy and subsequent immunohistochemical and immunoblotting examinations of the tissue in most cases. This was partly caused by the large phenotypic variability of most forms of LGMD and the large clinical overlap among different forms. Furthermore, few patients carry recurrent mutations of LGMD genes, while many carry rare or novel mutations. The findings presented in our and other studies suggest that LGMD2I is frequent in adult patients and usually, at least in Europe, caused by a single missense mutation of the FKRP gene, namely Leu276Ile (826C>A). This may simplify the diagnostic workup at least for some patients. In male patients, the high prevalence of dystrophinopathies (Becker muscular dystrophy) and the overlap of clinical findings with LGMD2I may still make a muscle biopsy, early in the diagnostic process, necessary. In contrast, we propose to test directly for FKRP (Leu276Ile) in female LGMD patients using a blood sample prior to undertaking a muscle biopsy.

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