Clinical and molecular analysis of a large family with three distinct phenotypes of progressive muscular dystrophy

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Summary

We describe a unique six-generation, highly consanguineous family originating from an isolated mountainous village in the Russian province of Daghestan. Three separate clinical phenotypes of progressive muscular dystrophy were identified in this large family. Seven patients developed a classical limb-girdle variant of muscular dystrophy (LGMD), with disease onset at 15–30 years and loss of ambulation within a 25-year course. The second group included three patients with a slowly progressive distal myopathy first manifested in the late teens and confined to the tibial and calf muscles. Each of these two phenotypes segregated independently as an autosomal recessive trait, and muscle biopsies showed non-specific myopathic changes. Lastly, two male siblings exhibited an atypical variant of Duchenne muscular dystrophy confirmed by detection of a deletion in the dystrophin gene.

To clarify the molecular basis of the polymorphic autosomal recessive form of muscular dystrophy in this kindred, we performed molecular genetic studies on 67 family members and obtained significant evidence for linkage to chromosome 2p. A maximum pairwise lod (logarithm of odds) score of 5.64 was achieved at the zero recombination fraction (i.e. at $\theta = 0.00$) for locus D2S29; multipoint linkage analysis confirmed the most likely location of a mutant gene near D2S291. The patients with LGMD and those with the distal muscular dystrophy phenotype share a common affected homozygous haplotype associated with the same founder chromosome; key recombinants defined D2S286 and D2S292 to be the closest loci flanking the mutant gene. Remarkably, two clinically distinct forms of autosomal recessive muscular dystrophy, LGMD type 2B (LGMD2B) and Miyoshi myopathy, were recently mapped to the same locus. We suggest that all three chromosome 2p-linked conditions may represent allelic disorders, i.e. different phenotypic expressions of a single gene.

Keywords: autosomal recessive muscular dystrophy; clinical phenotypes; molecular genetics; linkage analysis; chromosome 2p

Abbreviations: CK = creatine phosphokinase; LGMD = limb-girdle muscular dystrophy; PCR = polymerase chain reaction

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Introduction

Autosomal muscular dystrophies represent a heterogeneous group of rare genetic disorders with an estimated frequency of >50 per million (Emery, 1991). Until recently the definition of these conditions and even their nosological existence were in dispute because of substantial overlap with different well-delineated phenocopies, such as Duchenne/Becker muscular dystrophy, metabolic myopathies, mitochondrial myopathies and spinal muscular atrophies (Bradley, 1979). Significant progress in this field was marked in the past few years by the development of the concept of the dystrophin–glycoprotein complex (Ervasti et al., 1990; Yoshida and Ozawa, 1990; Tinsley et al., 1994), followed by cloning or mapping of the causative genes and discoveries of the specific structural-protein deficiencies in a number of autosomal dominant and autosomal recessive forms of muscular dystrophy (Beckmann et al., 1991; Ben Othmane et al., 1992; Speer et al., 1992; Toda et al., 1993; Bashir et al., 1994; Hillair et al., 1994; Roberds et al., 1994; Bejaoui et al., 1995; Bönnemann et al., 1995; Helbling-Leclerc et al., 1995; Lim et al., 1995; Noguchi et al., 1995; Richard et al., 1995).

With advances in molecular genetics it was clearly demonstrated that neurological manifestations of particular genetic forms of muscular dystrophy may vary significantly from family to family, thus making precise distinction between the forms, based solely on clinical grounds, virtually impossible (van der Kooi et al., 1994). This polymorphism may be explained, at least in part, by the occurrence of different mutations resulting in variable effects on an abnormal protein function (Bönnemann et al., 1995; Lim et al., 1995; Piccolo et al., 1995). However, the pathogenesis of muscular dystrophies remains poorly characterized, and contributions of other endo- or exogenic factors to the phenotypic expression of mutant genes have yet to be explored.

In this connection the most challenging questions arose from the few observations of large inbred families in which separate, obviously different forms of muscular dystrophy cosegregated within the same genealogy (Udd et al., 1991, 1992; Mahjneh et al., 1992). Various hypotheses were proposed to explain these unusual findings, but no molecular studies on such families have been reported to date. Undoubtedly, such studies are invaluable for elucidating the basic mechanisms of the genotype–phenotype interaction and the causes of the clinical diversity within a complex group of muscular dystrophies, as well as for elaborating a comprehensive system of classification of these disorders.

Here we describe a unique multigeneration highly consanguineous family in which Duchenne muscular dystrophy, LGMD and distal myopathy manifested in separate affected members; the transmission pattern of LGMD and the distal myopathy is consistent with typical autosomal recessive inheritance. Results of the molecular genetic analyses suggest that the LGMD and distal muscular dystrophy phenotypes in this kindred are likely to be caused by the same mutant gene located on chromosome 2p.

Subjects and methods

Family examination

Information was obtained on a total of 258 members belonging to six generations of the family. The pedigree is shown in Fig. 1. Fourteen individuals from three generations were found to be affected by muscular dystrophy (eight males and six females), and 12 of them were personally examined by the authors. Five patients and their parents were examined at the Institute of Neurology, Russian Academy of Medical Sciences, Moscow. Seven affected members, their living parents and available non-affected siblings were evaluated as outpatients by at least one of the authors (I.A.I.-S., S.N.I. or V.V.P.) during several trips to the area where the family members resided. In addition, for three patients, medical records were obtained from other hospitals.

Muscle biopsies

Biopsy specimens were taken from the clinically affected muscles in cases IV-7, IV-30, IV-31, IV-65 and V-31. Cryostat or paraffin sections were stained with haematoxylin and eosin, modified Gomori trichrome, van Gieson and various histochemical methods including PAS, Sudan black, von Kossa, succinic dehydrogenase and adenosine triphosphatase stains. Specimens were processed for electron microscopy by standard procedures.

DNA studies

Blood samples were collected with informed consent from 67 members of the family, including 12 affected persons. High-molecular-weight DNA was extracted as described elsewhere (Miller et al., 1988). Polymorphic microsatellite markers were amplified by polymerase chain reaction (PCR) in a total volume of 25 µl containing 125 ng of sample DNA, 2.5 pmol of both CA strand primer and fluorescently labelled GT strand primer, 50 mM KCl, 10 mM Tris–HCl (pH 8.4), 1.5 mM MgCl₂, 100 mM deoxyribonucleoside triphosphates and 0.5 U AmpliTag DNA polymerase (Perkin-Elmer Cetus). The mixture was denatured at 94°C for 4 min followed by 25 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 3 min); the final extension was at 72°C for 5 min. The PCR products were electrophoresed through a 5% denaturing polyacrylamide gel using an automated A.L.F. DNA Sequencer II (Pharmacia LKB, Sweden), and the data were processed with the Fragment Manager software (Pharmacia). A mixture of fluorescently labelled DNA fragments (50–500 bp) and PCR products of known lengths from a CEPH (Centre d’Études du Polymorphism Humain, Paris, France) family reference member (Gyapay et al., 1994) were used as size markers for an accurate assessment of the allele sizes.

Amplification of dystrophin gene exons was performed using the Chamberlain original primers (Chamberlain et al., 1992). Various hypotheses were proposed to explain these unusual findings, but no molecular studies on such families have been reported to date. Undoubtedly, such studies are invaluable for elucidating the basic mechanisms of the genotype–phenotype interaction and the causes of the clinical diversity within a complex group of muscular dystrophies, as well as for elaborating a comprehensive system of classification of these disorders.

Here we describe a unique multigeneration highly consanguineous family in which Duchenne muscular dystrophy, LGMD and distal myopathy manifested in separate affected members; the transmission pattern of LGMD and the distal myopathy is consistent with typical autosomal recessive inheritance. Results of the molecular genetic analyses suggest that the LGMD and distal muscular dystrophy phenotypes in this kindred are likely to be caused by the same mutant gene located on chromosome 2p.

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Fig. 1 Family pedigree. Squares indicate males and circles indicate females. The numbers inside the diamonds represent the numbers of siblings within sibships. Open symbols represent unaffected individuals and solid symbols affected individuals; asterisks indicate individuals selected for detailed molecular genetic study; slashed symbols, deceased individuals; arrow, the proband; double lines, consanguineous marriages.
Fig. 2 Clinical phenotypes of muscular dystrophy in the present family. (A) Patient with proximal LGMD (case IV-56). Generalized muscle wasting with predominant involvement of the limb-girdle musculature is evident. (B) Patient with distal myopathy (case V-31). Note the thin lower legs with marked wasting confined to the anterior tibial and calf muscles. (C) Patient with severe early onset muscular dystrophy (case IV-30). Generalized muscle wasting is evident; note mild equinovarus contractures of the ankle joints and lack of calf enlargement.

1988) and an additional primer pair for exon 50 (Beggs et al., 1990). The PCR conditions were as described by those workers. Exons amplified with the Chamberlain primer set and PCR products from exon 50 were analysed on separate 1.5% agarose gels at 5 V cm⁻¹.

Linkage analysis
Two-point lod scores were calculated using the MLINK program of the LINKAGE package, version 5.1 (Lathrop and Lalouel, 1984) under a model of autosomal recessive inheritance of the trait. In the calculations a mutant gene frequency of 0.001% and no sex differences in recombination rates were assumed. We took into account age-dependent penetrance and introduced four liability classes which were established through analysis of the cumulative age at onset curve in the affected persons (Ott, 1991). Marker-allele frequencies were derived from 17 ethnically matched control subjects (34 normal control chromosomes). Multipoint linkage analysis was performed between the disease locus and six loci on chromosome 2p using the LINKMAP program (Lathrop and Lalouel, 1984), assuming published relative genetic map distances among the loci (Gyapay et al., 1994).

Because of computer constraints, the number of alleles used in the multipoint linkage analysis was reduced at each locus, and the consanguineous loops containing genotyped parents were broken.

Results

Pedigree analysis
All family members are ethnic Avars originating from a relatively isolated mountainous village which is located in the northern Caucasus (Russian province of Daghestan). Since no sources of long-term genealogical records (e.g., church archives) were available in this village, the family history could not be traced back more than a few generations. According to the available ethnographic data, the community inhabiting this place was established ~300 years ago. Because of the geographic isolation only limited migration occurred between the village and the neighbouring regions; as a result, the population comprising the community became highly consanguineous.

One pair of parents of the affected members were first cousins, and two couples were known to be more distant relatives, although their exact kinship could not be determined with certainty. Parents of the other patients were born in the same village. As one may see in Fig. 1, two other consanguineous loops were also identified in this kindred.

Results of genealogical analysis of the pedigree clearly indicate an autosomal recessive mode of inheritance; this conclusion is based on the presence of consanguinity in the pedigree, the estimated proportion of affected siblings of slightly over 0.25 (with both sexes being affected equally) and the lack of cases of vertical disease transmission from a parent to offspring (Fig. 1). However, a visual inspection of
the pedigree chart does not allow differentiation between autosomal recessive and X-linked recessive inheritance in the two affected male siblings IV-30 and IV-31.

Case histories
Below we describe in detail the cases of three patients representing the entire spectrum of clinical variants of the muscle disorder in this family.

Case IV-56 (Fig. 2A)
This patient was normal at birth, and his medical history during childhood and his teens was unremarkable. At the age of 16 years he complained of awkwardness in moving his knees and hips, followed by difficulty in climbing stairs and raising his knees when running. Four years later he experienced fatigue and weakness in the proximal arm muscles. At this stage the distal portions of the lower and upper extremities were spared. Over the years, however, the limb weakness and wasting worsened and spread steadily so that he had to stop working on his farm; he gradually lost ambulation and became wheelchair-bound by the age of 38 years. On examination at age 43 his general health was good. Neurological examination revealed intact muscles innervated by the cranial nerves, except the sternocleidomastoids which were moderately atrophic. The patient exhibited severe and generalized limb-girdle muscle weakness and wasting. The lower extremities were diffusely atrophic and hypotonic, and only minimal strength and movement could be detected in the legs distally. There was prominent diffuse involvement of the scapular, shoulder girdle and humeral musculature, in particular the biceps, triceps, deltoid and supraspinatus, and, to a lesser degree, infraspinatus, trapezius, rhomboidei and pectoralis major muscles; distal muscles of the upper extremities were also paretic but remained stronger than proximal ones, and the patient was able to perform limited exercise and other activities with his hands and forearms. The long muscles of the back were moderately atrophic, but he could maintain an erect trunk position when sitting unassisted. Tendon reflexes were totally lost. He had prominent contractures in the ankle joints. There were no symptoms of sensory or sphincter abnormalities, and no signs of intellectual decline.

Case IV-30 (Fig. 2C)
This patient was delivered after an uncomplicated pregnancy and had slightly delayed early developmental milestones. He was described by his parents as a ‘passive’ child who could not keep up with his peers in active games. The disease became clear when he was 5 years old; he exhibited a waddling gait with frequent falls and premature fatigue when walking. Marked generalized progressive muscle weakness and wasting became evident by the age of 7 years, and the ability to walk was lost by the age of 10 years. He first came to our observation at the age of 11 years, at which time he looked somewhat underdeveloped for his age, but otherwise the results of a general physical examination were unremarkable. There were no apparent signs of cardiac involvement. Neurological examination revealed normal masticatory/facial muscle functions and moderate wasting and paresis of the sternocleidomastoid and trapezius muscles. He exhibited severe diffuse atrophy and weakness of the limb and torso musculature, prominent winged scapulae and a lordotic posture. The boy could not elevate his arms or maintain them against gravity, he was unable to stand up without assistance and could take only a few steps, even with support. No muscle hypertrophy, fasciculations or myotonia were observed. Tendon reflexes were absent except the brachioradial ones. There were mild equinovarus contractures of the ankle joints. Sensation and sphincter functions were normal. The patient showed no apparent signs of mental retardation. Examination 2 years later revealed evidence of further progression of the muscle disorder; he was totally incapacitated and wheelchair-bound, and he had prominent contractures of the ankles, knees, elbows and wrists.

Case V-31 (Fig. 2B)
This patient was born after a full-term uneventful pregnancy and an uncomplicated delivery. Early development was normal. The patient had always been in good health as a child and a youth; he was active in athletic games and had no difficulties with his ordinary schooling. At 15 years of age the patient first noticed some awkwardness in moving his feet while playing soccer. Thereafter, slowly progressive weakness and wasting of the distal leg muscles, clumsy gait, stumbling and an inability to walk on the heels and toes were observed. He was admitted to our clinic at 23 years of age and showed no abnormalities on general physical examination. Neurological examination revealed marked symmetrical muscle weakness and diffuse wasting confined to the distal lower extremities. Both anterior and posterior muscle compartments were clearly affected, but the involvement of the anterior tibial and the long toe extensor muscles was somewhat greater than that of the gastrocnemius. The patient was barely able to stand on his toes and could not stand or walk on his heels. He had bilateral footdrop, could not hop on one leg and exhibited a typical steppage gait with slight waddling. Intrinsic foot musculature was apparently spared, and the range of toe movement was preserved. Slight weakness against resistance in the hamstrings could be detected bilaterally, but other muscles of the thigh and the pelvic girdle were normal. No abnormalities in the trunk or upper extremity musculature were observed, the neck and facial muscles were also normal. The patient exhibited loss of Achilles reflexes; other tendon reflexes were preserved. The remainder of the neurological examination was unremarkable. On examination 2 years later he exhibited no significant change in the disability level.

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**Summary of clinical findings and laboratory data**

**Neurological features**
The affected individuals exhibited three different and separate clinical phenotypes of progressive muscular dystrophy which could be clearly identified by routine neurological examination. Neurological features are summarized in Table 1.

Seven patients developed a proximal-type muscular dystrophy with an insidious onset of symptoms around the age of 20 years (range 15–30 years), predominant involvement of the pelvifemoral and, several years later, involvement of the scapulohumeral muscles and a relentlessly progressive course. Symmetrical muscle atrophy generally paralleled paresis. Over the years weakness and wasting gradually spread to the distal portions of the extremities, but the distal limb muscles remained stronger than the proximal ones throughout the disease course. At the late stage, mild to moderate involvement of the neck muscles, total areflexia and contractures of the ankle, knee and wrist joints were observed. Faciobulbar and respiratory musculature was invariably spared. The patients usually became confined to a wheelchair by their early fifties; the duration of the disease in the deceased patients varied from 20 to 51 years. In patients IV-55 and IV-58, who were not examined by us personally, the clinical picture was similar to that described above, as concluded from the available medical records and descriptions by their relatives.

Three patients (IV-65, V-30 and V-31) exhibited a completely different phenotype of distal myopathy. The presenting symptoms at the ages of 15–21 years were fatigue and weakness of the distal leg muscles in physically demanding situations, followed by overt paresis and wasting in the distal portions of the legs. Weakness and atrophy tended to involve the anterior and posterior muscular compartments equally, though minor patient-to-patient variations were observed. The proximal leg and pelvic girdle muscles were intact, except for the hamstrings which were slightly paretic in two patients. Neither patient exhibited appreciable weakness or wasting of the arm, trunk, neck or facial muscles. Achilles reflexes were absent, but other deep tendon reflexes were brisk in all the patients. The condition showed a rather slow progression and to date, with a disease duration of up to 10 years, all the patients have remained ambulant and are able to perform their home and farm duties.

The third group of patients included two brothers (IV-30 and IV-31) who exhibited a severe early-onset muscular dystrophy phenotype with the debut of symptoms at 5 years of age; the disorder developed on a background of slightly delayed early motor milestones. This clinical variant was characterized by generalized symmetrical weakness and atrophy in the limb, trunk and neck muscles (starting from the proximal portions of the legs and the pelvic girdle), total areflexia, contractures and a 'malignant' course, with severe disability occurring by the end of the first decade.

On general physical examination none of the patients in this family exhibited cardiomyopathy, hepatosplenomegaly or any other symptoms of internal organ disease.

**Laboratory studies**
The following investigations were each performed in two (at least) or more of the patients and yielded normal results: routine haematology, liver and kidney function tests, evaluation of serum glucose, protein, lipids, vitamin E, electrolytes, lactate, pyruvate and lactate/pyruvate ratio (both at rest and after an exercise), leucocytar α-glucosidase activity, routine urine analysis, cranial and chest X-rays, abdominal ultrasound, electro- and echocardiography, and cerebral CT.

Serum creatine phosphokinase (CK) activity levels were determined in cases IV-7, IV-30, IV-31, IV-65, V-30 and V-31. In all examined patients the CK values were found to be increased >10-fold, with the highest increase (up to 56 times the upper normal level) in patients with the distal myopathy phenotype. Six patients underwent neurophysiological examination. The patients with proximal muscular dystrophy (case IV-7) and severe early onset muscular dystrophy (cases IV-30 and IV-31) exhibited marked generalized myopathic changes. In the distal myopathy group (cases IV-65, V-30 and V-31) the EMG studies showed polyphasic short-duration motor unit potentials of moderately decreased amplitude in the distal leg muscles, and minor myopathic changes in the clinically unaffected scapulohumeral muscles. None of the patients showed fibrillation potentials, sharp positive waves or pseudomyotonic discharges. The results of nerve conduction studies were normal in all examined persons.

**Histopathological investigations**
Morphological studies of the muscle biopsies obtained from one patient with proximal muscular dystrophy and two patients with severe early onset muscular dystrophy revealed similar abnormalities consistent with an advanced myopathic process. Marked variation in fibre size with numerous atrophic and hypertrophic fibres, fibre splitting, few necrotic and regenerating fibres and moderate phagocytosis were observed. A few isolated hyalinated muscle fibres were occasionally seen. Other abnormalities included extensive proliferation of intramuscular connective tissue, partial replacement of the muscle with fibrous and adipose tissue and an increased number of internalized nuclei. There were no obvious changes in the distribution of the fibre types. Electron microscopic examination revealed prominent disorganization and disruption of myofibrils, alterations of the sarcoplasmic reticulum, numerous autophagic vacuoles (mostly subsarcolemmal), scattered lipid droplets and degenerating mitochondria; there were only scanty signs of muscle fibre regeneration.

In two patients with distal myopathy, studies of the
Table 1  Characteristics of clinical phenotypes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age* at disease onset</th>
<th>Age* at examination</th>
<th>Initial symptoms</th>
<th>Muscle weakness</th>
<th>Muscle atrophy</th>
<th>Contractures</th>
<th>Loss of tendon reflexes</th>
<th>Functional milestones in the disease course and age* at death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PF</td>
<td>T</td>
<td>G</td>
<td>SH</td>
<td>DA</td>
</tr>
<tr>
<td>III-4</td>
<td>30</td>
<td>70</td>
<td>Leg muscle tiring</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>III-6</td>
<td>18</td>
<td>60</td>
<td>Proximal leg muscle weakness</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>III-20</td>
<td>16</td>
<td>61</td>
<td>Stumbling in walking</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>III-26</td>
<td>15</td>
<td>64</td>
<td>Proximal leg muscle weakness</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>IV-6</td>
<td>19</td>
<td>39</td>
<td>Proximal leg muscle weakness</td>
<td>++++</td>
<td>++</td>
<td>++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>IV-7</td>
<td>18</td>
<td>31</td>
<td>Proximal leg muscle weakness</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>IV-56</td>
<td>16</td>
<td>43</td>
<td>Knee and hip awkwardness</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Distal muscular dystrophy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1V-65</td>
<td>21</td>
<td>23</td>
<td>Frequent stumbling in running</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V-30</td>
<td>18</td>
<td>24</td>
<td>Distal leg muscle tiring</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V-31</td>
<td>15</td>
<td>23</td>
<td>Stumbling, foot awkwardness</td>
<td>+</td>
<td>++++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Severe early onset muscular dystrophy</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1V-30</td>
<td>5</td>
<td>11</td>
<td>Motor passoveness, leg muscle tiring</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>1V-31</td>
<td>5</td>
<td>7</td>
<td>Motor passoveness, leg muscle tiring</td>
<td>++++</td>
<td>++</td>
<td>++</td>
<td>++++</td>
<td>++</td>
</tr>
</tbody>
</table>

PF = pelvifemoral muscles; T = tibial muscles; G = gastrocnemius; SH = scapulohumeral muscles; DA = distal arm muscles; N/F = neck/facial muscles; Pr = present; Abs = absent; ++++ = total weakness; +++ = severe weakness; ++ = moderate weakness; + = minimal weakness; - = no detectable weakness *In years.
specimens taken from the clinically affected muscles (anterior tibial and gastrocnemius) revealed clear myopathic changes which were more extensive in the case with longer disease duration (9 versus 2 years). Variation in fibre size, numerous atrophic, hyalinated and lobulated fibres, fibre necroses with marked phagocytosis, focal lymphocytic infiltrates and an increased number of internalized nuclei were the main pathological features observed in both these cases. Signs of endomysial and perimysial fibrosis were moderate in the less disabled patient and prominent in the more severe case; in the latter, partial replacement of the muscle with adipose tissue was observed. There was a slight predominance of type 1 fibres, but without the fibre type grouping which would have suggested a neurogenic process. One patient had a few isolated ragged-red fibres. Ultrastructural abnormalities were unspecific and included disruption of the Z line, fragmentation of myofibrils, degenerative changes of mitochondria and numerous scattered autophagic vacuoles.

The studied biopsy specimens showed no signs of abnormal accumulation of glycogen, lipids or calcific deposits.

**Dystrophin gene analysis**

To test the possibility of Duchenne muscular dystrophy in two male siblings with the severe early onset muscular dystrophy phenotype (cases IV-30 and IV-31), we analysed several microsatellite polymorphisms located within the dystrophin gene (Beggs and Kunkel, 1990; Clemens et al., 1991; Fee et al., 1991). We also performed conventional deletion screening via multiplex PCR and amplification of the deletion-prone exons (Chamberlain et al., 1988; Beggs et al., 1990). In both patients we observed failure of DNA amplification at locus STR-49 from intron 49 of the gene (not shown), and lack of PCR products from the adjacent exon 50 (Fig. 3A). These findings clearly indicate presence of a deletion in this dystrophin gene region, implying the diagnosis of Duchenne muscular dystrophy in the affected boys. Both patients retain all the exons amplified with the Chamberlain primer set, including exon 48 (Fig. 3B), and have no allele loss at intronic locus STR-50 (not shown). These data enabled us to assess the extent of the deletion and to show that it was located between introns 48 and 50.

Patients with the proximal and the distal muscular dystrophy phenotypes do not carry the above-mentioned deletion in the dystrophin gene, since we observed neither lack of PCR products from exon 50 in males (Fig. 3A) nor loss of heterozygosity at locus STR-49 in females (not shown).

**Linkage studies**

We performed linkage analyses of the autosomal recessive form of progressive muscular dystrophy in this family. The boys with Duchenne muscular dystrophy were included in the whole cohort for linkage calculations as unaffected individuals. We initially studied genetic linkage with several known gene loci responsible for autosomal recessive forms of LGMD and congenital muscular dystrophy in order to investigate the possible involvement of these genes in the present family. The tested polymorphic microsatellite marker loci included an intragenic locus within the LGMD type 2D gene adhalin on chromosome 17q12-q21.33 (Roberds et al., 1994) and the following dinucleotide marker loci: D15S779, linked to LGMD2A on chromosome 15q15.1-q21.1 (Allamand et al., 1995); D2S291, linked to LGMD2B on chromosome 2p13-p16 (Passos-Bueno et al., 1995); D13S232, linked to LGMD2C on chromosome 13q12-q13 (Ben Othmane et al., 1995); D4S518, linked to LGMD2E on chromosome 4q12 (Lim et al., 1995); D6S270, linked to merosin-deficient congenital muscular dystrophy on chromosome 6q2 (Hillaire et al., 1994); and D9S58, linked to Fukuyama-type congenital muscular dystrophy on chromosome 9q31-q33 (Toda et al., 1993).
Pairwise linkage analyses definitively excluded linkage (i.e. yielded lod scores of \(-2\)) with the loci on chromosomes 4, 6, 9, 13, 15 and 17 (data not shown). Lod scores were positive for the LGMD2B locus (chromosome 2). After obtaining preliminary data that indicated linkage to this locus we tested 7 additional markers around the critical region of chromosome 2p (Gyapay et al., 1994). Combined lod scores from pairwise linkage analysis with the eight microsatellite marker loci on chromosome 2p are summarized in Table 2. Significant linkage was observed for locus D2S291, with a maximum lod score (Z) of 5.64 at \(\theta = 0.00\). High positive lod scores suggestive of linkage (though not reaching a statistically significant level) were also obtained for D2S292, D2S358 and D2S285. The marker locus D2S169 was not fully informative.

Extended haplotypes of the key family members, constructed assuming a minimal number of recombinations, are shown in Fig. 4. Sibships both with proximal and with distal muscular dystrophy the affected pairs of siblings (III-4 and III-6, IV-6 and IV-7, and V-30 and V-31) clearly displayed a region of homozygosity with at least four markers studied and inherited identical pairs of chromosomes from their parents. Various portions of the same homozygous segment were also evident in 'single' cases (patients without affected siblings) of either the proximal or the distal muscular dystrophy phenotype (III-20, III-26, IV-56 and IV-65). Analysis of the recombination events in the cases of patients III-8 and III-20 and individual III-27 (who transmitted her recombinant chromosome to her children IV-6 and IV-7) revealed that all patients with either of these two phenotypes share the chromosomal region lying between D2S286 and D2S292. Haplotype analysis in the cases of the healthy individual V-36, who is homozygous for the entire region studied, is presently 5 years old, i.e. much younger than his two affected siblings were at the disease onset (18 and 15 years); therefore, he is likely to be a presymptomatic homozygous carrier of the mutation.

Multipoint linkage analyses were performed using the following six marker loci: D2S286, D2S291, D2S292, D2S358, D2S285 and D2S380. These calculations confirmed that the most likely location of the mutant gene in the present family was between D2S286 and D2S292, with a maximum multipoint lod score of 4.82 achieved at the position of marker locus D2S291 (Fig. 5).

### Discussion

**Clinical features and differential diagnosis**

In the modern classification of non-sex-linked progressive muscular dystrophy three major and genetically heterogeneous clinical groups comprised of cases with predominant limb-girdle, distal or facioscapulohumeral muscle involvement are distinguished (Gardner-Medwin and Walton, 1994). Amongst these the autosomal recessive forms, mostly occurring as sporadic cases, have always been a matter of great controversy. The recent molecular achievements in recessive muscular dystrophy, which resulted in subdivision of the limb-girdle form into several genetic subtypes (Bushby and Beckmann, 1995) and localization of a gene for one distal form (Bejaoui et al., 1995), seemed to validate the common clinical approach of categorizing these syndromes primarily by the distribution of muscle weakness. Our findings, however, raise new questions concerning the relationship between the various entities of recessive muscular dystrophy and the appropriateness of their generally accepted nomenclature.

The present family provided the unique opportunity to investigate the simultaneous occurrence of the three clinically different variants of progressive muscular dystrophy. The largest group comprised seven patients whose clinical picture satisfied all the diagnostic criteria for LGMD (Bushby, 1995). The disorder always started in the second or third decade, primarily affected the pelvic girdle and proximal leg muscles,
Fig. 4 Haplotypes of the key family members. Black symbols indicate patients with the proximal LGMD phenotype, grey symbols, patients with the distal myopathy phenotype; checkered symbols, patients with Duchenne muscular dystrophy. Clinically unaffected individuals are indicated by open symbols. Reconstructed haplotypes are boxed, and the haplotype segregating with the disease locus is shaded. Marker loci are listed on the left-hand side of the diagram; *tel* = telomere; *cen* = centromere. The individuals' numbers correspond to those in Fig. 1.
Chromosome 2p-linked muscular dystrophy

Fig. 5 Multipoint analysis with six loci on chromosome 2p. Recombination fractions between loci were transformed into genetic distances using the Kosambi mapping function (Ott, 1991). The hatched line defines the 95% confidence interval.

spread from the lower to the upper extremities (with marked predilection for the shoulder girdle and proximal arm muscles) and, in general, showed no specific clinical, electrophysiological or morphological features compared with other described forms of autosomal recessive LGMD (Bradley, 1979; Shields, 1994; van der Kooi et al., 1994).

The clinical presentations in the cases of three other patients were totally different from those described above and fitted the criteria of distal myopathy (Griggs and Markesbery, 1994). The specific combination of clinical features in our cases (age at onset of around 20 years, equal involvement of the anterior tibial and calf muscles, sparing of the upper extremities, notably elevated serum CK activity level and lack of rimmed vacuoles) makes it difficult to assign this particular phenotype to any of the main diagnostic categories of distal myopathies (Welander, 1951; Markesbery et al., 1974; Nonaka et al., 1981; Miyoshi et al., 1986; Udd et al., 1993). The disorder tended to progress more slowly than in the cases of the LGMD phenotype.

Both the LGMD and the distal muscular dystrophy phenotypes are apparently transmitted in an autosomal recessive manner, which is not unexpected in this highly inbred family.

Two brothers belonging to one sibship are affected by a severe form of the muscle disorder with remarkably early onset at around 5 years of age and rapid deterioration. The mode of inheritance in their small nuclear family could not be determined with certainty. We showed that both boys are hemizygous carriers of the dystrophin gene deletion, which is consistent with the diagnosis of Duchenne muscular dystrophy. It is noteworthy, however, that the clinical picture in both patients is rather atypical, since they lack calf enlargement and cardiomyopathy, the hallmarks of Duchenne muscular dystrophy (Emery, 1993). Our observation illustrates clinical polymorphism of the dystrophinopathies and highlights the significance of the dystrophin analyses in the differential diagnosis of muscular dystrophies.

Reports on only two other families with co-occurrence of different muscular dystrophy phenotypes have been published. One is an inbred Palestinian family, in which congenital muscular dystrophy and LGMD (both variants exhibiting autosomal recessive inheritance) occurred in two groups of patients belonging to closely related branches of the family (Mahjneh et al., 1992). The clinical features in this family, especially the tendency of stabilization over the years, differ significantly from those in our observation. The authors speculated that the two phenotypes described might be caused either by the same gene or by different, independent pathological genes. Interestingly, the branch of this family with LGMD was one of the families in which chromosome 2p linkage was demonstrated later by Passos-Bueno et al. (1995); however, the authors did not provide any molecular genetic data concerning the family branch with congenital muscular dystrophy. Another example was presented in a series of papers by Udd and associates, who described muscular dystrophy with two separate clinical phenotypes in a large consanguineous Finnish family (Udd et al., 1991, 1992; Udd, 1992). One clinical variant with proximal muscle weakness, observed in eight patients and transmitted as an autosomal recessive trait, was almost identical to the LGMD phenotype in our family. The second group described by Udd and colleagues comprised 14 patients exhibiting late-onset non-vacuolar distal myopathy with anterior tibial involvement, a benign course and a mildly elevated serum CK level. The histopathological differences between the two phenotypes were quantitative, not qualitative (Udd et al., 1992). On the basis of segregation analysis it was suggested that the patients with distal myopathy might have a dominant muscle disorder, and that the limb-girdle variant might
represent homozygous expression of the same mutant gene (Udd, 1992). In our opinion, however, the pedigree described by Udd and associates allows alternative explanations. For instance, pseudodominant inheritance of a recessive trait in this markedly inbred kindred could explain both the occurrence of several cases of vertical disease transmission and the high proportion of affected siblings. It is not clear whether the differences between the phenotypes in the Finnish and the present family (such as age at onset or serum CK activity level) are essential or just reflect an interfamilial polymorphism commonly observed in hereditary disorders. Therefore, at this point the possibility that the two conditions are identical cannot be excluded.

**Molecular genetic analysis**

As the first step in an attempt to clarify the molecular basis for unusual autosomal recessive muscular dystrophy in the present family we performed systematic genomic screening of the candidate loci and eventually found evidence of linkage to the locus on chromosome 2p, where a gene responsible for one form of autosomal recessive LGMD (LGMD2B) had initially been mapped (Bashir et al., 1994; Passos-Bueno et al., 1995). The extended linkage analyses yielded a highly significant lod score for the locus D2S291 \((Z = 5.64 \text{ at } \theta = 0.00)\) and also positive but non-significant lod scores for D2S292, D2S358 and D2S285.

Reconstruction of the entire haplotypes for the genotyped markers in this family revealed a unique disease-associated haplotype which is present in the homozygous state in all patients with either the limb-girdle or the distal variant of the muscle disorder. These findings clearly indicate that affected individuals are homozygous by descent at the chromosomal region surrounding the locus of the mutant gene. The obtained results suggest a founder effect in this pedigree; such an effect is commonly observed in highly consanguineous populations. Identification of a common homozygous haplotype perfectly cosegregating with LGMD and distal muscular dystrophy suggests that both autosomal recessive conditions are associated with an abnormal gene located on the same founder chromosome, and thus may serve as direct evidence for the genetic unity of these distinct clinical phenotypes in the present family.

Study of the critical crossing-over events in our pedigree allowed location of the interval containing a mutant gene to a narrow 6 cM region lying between D2S286 and D2S292. Results of multipoint linkage analysis supported this conclusion and suggested that the most likely location for the mutant gene is at D2S291.

Strikingly, two clinically different entities of autosomal recessive muscular dystrophy, LGMD2B and Miyoshi myopathy, have been shown to be associated with the same chromosomal region. The gene for LGMD2B was assigned to a locus flanked by D2S286 and D2S292 (Passos-Bueno et al., 1995). Clinically, the LGMD2B variant is very similar to the LGMD phenotype in our family, including age at onset, course and significant elevation of serum CK level (Bashir et al., 1994). The gene for Miyoshi myopathy was mapped to essentially the same region, with the strongest linkage at a zero recombination fraction to the marker locus D2S291 (Bejaoui et al., 1995). Although our patients with the distal phenotype do not exhibit the selective involvement of the calf muscles typical of Miyoshi myopathy (Miyoshi et al., 1986), they bear some similarities to the Miyoshi variant, such as age at onset in the late teens and a very high serum CK level.

Taken together, these findings raise the intriguing possibility that all three conditions (LGMD2B, Miyoshi myopathy and the present LGMD/distal polymorphic syndrome) represent allelic variants of the same abnormal gene located at 2p12–14. Alternatively, there might be a cluster of functionally related genes in the defined region, each being responsible for a particular muscle disorder; this, however, seems very unlikely. In the case of a single gene, the underlying causes of the considerably wide range of clinical presentations may include differences in the nature and biological consequences of the particular mutations in different families. Indeed, the severity of other muscular dystrophies, such as dystrophinopathies (Hoffman and Kunkel, 1989), LGMD2D (Piccolo et al., 1995) and LGMD2E (Bönnemann et al., 1995; Lim et al., 1995) was clearly found to vary with the type of mutation. Remarkable qualitative interfamilial polymorphism resulting from different mutations within the same gene has also been detected in a number of allelic neurohereditary disorders, for instance, Pelizaeus–Merzbacher disease and X-linked spinal paraplegia (Saugier-Veber et al., 1994), Charcot–Marie–Tooth disease type 1A and hereditary neuropathy with pressure palsies (Chance and Fischbeck, 1994). Future research will help to elucidate the molecular biology of chromosome 2p-associated myopathies and resolve the question of whether they have a common genetic basis. In view of our results, it would be of great interest to conduct linkage studies with chromosome 2p markers in cases of other informative families with recessive muscular dystrophy exhibiting similar unusual clinical features.

While phenotypic variability between families is likely to reflect different properties of the causative mutations, the remarkable interfamilial polymorphism is yet another puzzle that needs to be explained. From this viewpoint, the important finding in our pedigree is that all affected members belonging to the same sibship always developed the same phenotype, either proximal or distal (Fig. 4). This implies that expression of the mutation is influenced by parental genetic factors which are common within nuclear families, for instance, by specific alleles at, as yet unidentified, regulatory genetic elements surrounding the mutant gene. An alternative possibility, proposed by Richard et al. (1995) for LGMD2A, assumes an important role for a specific mitochondrial genetic, or energetic, background for expression of an abnormal gene in muscular dystrophy (considering that mitochondrial...
compartments are identical within sibships and influence the fate of energy supply-dependent muscle cells).

Identification of a distinct molecular basis for the muscle disorder in the two brothers with severe early onset muscular dystrophy, namely the dystrophin gene deletion, is highly unexpected. These boys are close relatives of other affected individuals in the present kindred, and they were originally thought to suffer from the same disease. The deletion identified in these cases is relatively small in length, encompasses at most two exons (exon 50 and, possibly, exon 49) and is located in the typical deletion-prone region of the dystrophin gene (Den Dunnen et al., 1989; Clemens et al., 1991). This particular type of deletion (exon 50 with or without exon 49) was detected in 15 out of 416 patients with Duchenne/Becker muscular dystrophies in a recent large multicentre study (Multicenter Study Group, 1992). The present work represents, to our knowledge, the first molecularly proven report of genetically different forms of progressive muscular dystrophy within the same kindred.

Conclusions
A rapidly growing number of developments in autosomal muscular dystrophies has brought new insights into the molecular biology of these conditions, and at the same time highlighted a striking clinical diversity even within genetically homogeneous groups. Therefore, detailed analysis of families with the established linkage is a matter of high priority, as was stressed at the 30th and 31st International Workshops on LGMDs (Bushby and Beckmann, 1995).

We examined a large consanguineous kindred with co-occurrence of highly polymorphic autosomal recessive muscular dystrophy and an atypical variant of Duchenne muscular dystrophy. Molecular genetic studies in this family demonstrated linkage of the autosomal recessive disorder to chromosome 2p12–14, exactly to the locus containing causative genes for two other recessive forms of muscular dystrophy, LGMD2B and Miyoshi myopathy. Haplotype reconstruction showed that all patients with either proximal or distal muscular dystrophy are homozygous by descent at the critical chromosomal region; these findings provide evidence for the existence of a common genetic basis of these two clinical phenotypes in the present kindred. We suggest that all chromosome 2p-linked muscular dystrophies may represent allelic disorders, and yet they are another example of extreme phenotypic polymorphism probably resulting from complex interactions between a particular mutation and non-allelic modifying or regulatory genes.

The nomenclature of muscular dystrophies undergoes rapid changes reflecting new achievements in this hot spot of neurogenetics. For instance, in view of the results of recent studies on phenotype–genotype correlations the term 'severe childhood autosomal recessive muscular dystrophy' is considered to be inappropriate (Bushby and Beckmann, 1995). A definitive classification system may be developed only on the basis of precise information regarding underlying structural and biochemical defects. The results of our work illustrate this issue and show that descriptive terms such as 'distal' or 'limb-girdle' muscular dystrophy' (in other words, the primary nosological significance of muscle weakness distribution) should be used with caution, at least until the molecular nature of all known conditions belonging to this group is clarified. Cloning of a gene (or genes) and identification of causative mutations in the chromosome 2p-associated disorders is an exciting challenge and important step which will greatly enhance our understanding of the gene expression and clinical spectrum of autosomal recessive muscular dystrophies.

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