Disease severity in dominant Emery Dreifuss is increased by mutations in both emerin and desmin proteins


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Individuals with the same genetic disorder often show remarkable differences in clinical severity, a finding generally attributed to the genetic background. We identified two patients with genetically proven Emery-Dreifuss muscular dystrophy (EDMD) who followed an unusual course and had uncommon clinicopathological findings. We hypothesized digenic inheritance and looked for additional molecular explanations. Mutations in additional separate genes were identified in both patients. The first patient was a member of a family with molecularly proven X-linked EDMD. However, the clinical features were unusually severe for this condition in the propositus: he presented at 2.5 years with severe proximal weakness and markedly elevated serum creatine kinase. Muscle weakness rapidly progressed, leading to loss of independent ambulation by the age of 12. In addition, the patient developed cardiac conduction system disease requiring pacing at the age of 11 and severe dilated cardiomyopathy in the early teens. Despite pacing, he had several syncopal episodes attributed to ventricular dysrhythmias. As these resemble the cardiac features of patients with the autosomal dominant variant of EDMD, we examined the lamin A/C gene, identifying a de-novo mutation in the propositus. The second patient had a cardioskeletal myopathy, similar to his mother who had died more than 20 years previously. Because of the dominant family history, a laminopathy was suspected and a mutation in exon 11 of the LMNA gene was identified. This mutation, however, was not present in his mother, but instead, surprisingly, was identified in his virtually asymptomatic father. Unusual accumulations of desmin found in the cardiac muscle of the propositus prompted us to examine the desmin gene in this patient, and in so doing, we identified a desmin mutation, in addition to the LMNA mutation in the propositus. These cases suggest that separate mutations in related proteins that are believed to interact, or that represent different parts of a presumed functional pathway, may synergistically contribute to disease severity in autosomal dominant EDMD. Furthermore, digenic inheritance may well contribute to the clinical severity of many other neuromuscular disorders.

Keywords: muscular dystrophy; dilated cardiomyopathy; lamin; desmin; emerin

Abbreviations: CSD = conduction system disease; EDMD = Emery Dreifuss muscular dystrophy; PCR = polymerase chain reaction


Introduction

Mutations in genes encoding proteins associated with the nuclear envelope, lamins A/C and emerin, both result in Emery-Dreifuss muscular dystrophy (EDMD), characterized by weakness of the scapuloperoneal muscles, restriction of
movements at the elbows, Achilles tendons and spine, and, invariably, cardiac conduction system disease (CSD) (Emery and Dreifuss, 1966; Bione et al., 1994; Bonne et al., 1999). The two conditions are clinically similar; however, the X-linked variant due to mutations in the emerin gene (EMD, XL-EDMD) is milder. Patients affected by XL-EDMD remain ambulant for life, and the associated CSD can be readily corrected by pacemaker insertion (Bonne et al., 2003).

EDMD due to dominant mutations in the lamin A/C gene (LMNA, AD-EDMD) on the other hand has a much wider spectrum of severity, ranging from congenital onset in children who are never able to walk (Mercuri et al., 2004, 2005) to milder variants with onset in adolescent or adult life (Bonne et al., 2000, 2003). The cardiac involvement in AD-EDMD is significantly more severe than XL-EDMD, as progression to dilated cardiomyopathy and cardiac failure in AD-EDMD occurs in up to 35% of cases and often requires cardiac transplant (Becane et al., 2000; Brodsky et al., 2000; Sanna et al., 2003). Furthermore, the conduction defects in AD-EDMD are not limited to the atrioventricular conduction system; ventricular tachyarythmias also occur, often resulting in sudden death despite pacing (Becane et al., 2000; van Berlo et al., 2005).

Mutations in the LMNA gene also result in a range of other conditions, some with associated skeletal muscle involvement such as limb-girdle muscular dystrophy 1B (Muchir et al., 2000), or with isolated cardiac involvement in the form of dilated cardiomyopathy with conduction defects (Fatkin et al., 1999). Others affect the peripheral nervous system (autosomal-recessive axonal neuropathy or Charcot–Marie–Tooth disease, CMT2B1) (De Sandre-Giovannoli et al., 2002), adipocytes (Dunnigan-type familial partial lipodystrophy) (Shackleton et al., 2000) and the skeletal system (mandibuloacral dysplasia) (Novelli et al., 2002), or result in the progeric syndromes Hutchinson–Gilford progeria (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003) and atypical forms of Werner syndrome (Bonne and Levy, 2003; Caux et al., 2003; Chen et al., 2003). The latest addition to the long list of allelic disorders caused by mutations in the lamin A/C gene is restrictive dermopathy, also known as ‘tight skin contracture syndrome’, characterized by early neonatal lethality (Navarro et al., 2004).

The association between individual LMNA mutations and different phenotypes is not fully understood. Rare mutations tend to be associated with one specific clinical entity; most familial partial lipodystrophy cases, for example, carry dominant mutations affecting codon R482, the recessive R298C mutation is associated with CMT2B1, the recessive R527H mutation results in mandibuloacral dysplasia and different mutations at codon 608 result in Hutchinson–Gilford progeria [reviewed in Worman and Courvalin (2004)]. However, in AD-EDMD, identical mutations can result in extreme variability in severity (Mercuri et al., 2004, 2005), highlighting the role of genetic background. Here, we report two patients with unusual clinical and pathological features, in whom digenic inheritance was identified.

Case reports
Family 1

This is a family with XL-EDMD with three affected males in two generations. Two males, a maternal uncle and a maternal first cousin of the index case, followed a typical course, with onset of skeletal symptoms in the second decade of life, followed by atrioventricular CSD by the end of the third decade of life, corrected by pacing. The index case, however, was much more severe; he presented at 2.5 years of age with frequent falls and difficulty in rising from the floor. Over the next few years he lost the ability to run. A muscle biopsy at age 4 showed advanced dystrophic features (Fig. 1A). At 5, he required surgical elongation of one Achilles tendon but had no other significant contractures; by 8, he could only walk for short distances and had difficulty getting up from the floor because of progression of muscle weakness, and contractures that affected both Achilles tendons and, to a lesser extent, his elbows and lumbar spine. The distribution of weakness was more generalized and his serum creatine kinase (CK) more elevated (>10 times normal) than is usual in XL-EDMD. At age 11, he developed first-degree atrioventricular block and runs of atrial tachycardia and atrial standstill for which a pacemaker was inserted. The following year, reduced left ventricular fractional shortening (FS; 25%) was recorded. At 13, he lost the ability to walk because of progression of weakness, in particular of his hip extensors, and due to his Achilles tendon and hip flexion contractures. His dilated cardiomyopathy had progressed (FS; 18%), requiring the use of angiotensin converting enzyme (ACE) inhibitors. Recently, at 15, he had several syncopal episodes, secondary to intermittent ventricular dysrhythmias.

A nonsense mutation in the emerin gene (Y105X) was present in the maternal uncle and maternal first cousin (but not in unaffected males of this family), and in three obligate carriers (Fig. 2). This mutation was corroborated by the absence of emerin in the proband’s skeletal muscle (Fig. 1B and C). We reported this unusually severe XL-EDMD case previously (Muntoni et al., 1998) before his additional atypical cardiac features prompted further investigation.

Family 2

A young man and his mother were affected by a cardioskeletal myopathy. The mother, aged 22, presented with dizzy spells. One year later she gave birth to her only child, but developed complete heart block for which a pacemaker was inserted. At 31, she developed muscle weakness with difficulty in walking and bilateral foot drop. She had generalized muscle wasting and no significant contractures. Both her weakness and heart condition deteriorated rapidly and she died at the age of 35 following a respiratory infection, which aggravated her cardiomyopathy. Shortly before death, examination showed moderate weakness of the neck flexors and lower limbs, both proximally and distally, with relative preservation of upper limbs. Serum CK levels were normal.
Her son had no complaint until age 14, when an incidental ECG suggested left ventricular hypertrophy, confirmed as a mild increase in wall thickness for his age on echocardiography. At 17, while still asymptomatic, a repeated ECG showed signs of marked ventricular hypertrophy, QRS widening and widespread repolarization changes. The echocardiogram showed marked concentric left ventricular hypertrophy (20 mm), with no intraventricular gradient. Ventricular function was abnormal with reduced systolic wall thickening and a restrictive mitral filling on Doppler. At 19, he underwent a needle muscle biopsy (Fig. 3A) and a right ventricular biopsy (Fig. 3B and C). He also underwent a muscle MRI that showed mild involvement of the sartorius and adductor magnus at the thigh levels and of the soleus and medial gastrocnemius at the calf level (Fig. 4A and B). At 21, he developed signs of cardiac failure with severe breathlessness (New York Heart Association, NYHA, functional class III) and peripheral oedema requiring urgent hospitalization. He was started on diuretics and ACE inhibitors and underwent cardiac transplantation. On examination, he had mild neck and knee flexor weakness, but serum CK activity was normal. He had no significant contractures. He recovered well from cardiac transplantation, but later developed cellular rejection, which was resistant to treatment and he died after 7 months with cardiac dysfunction secondary to acute rejection-related coronary vasculitis. The formalin-fixed explanted heart was available for examination (Fig. 5). This patient has been described as part of a series of individuals carrying the same exon 11 LMNA mutation [Case 2 in Mercuri et al. (2005); c.1930C>T, resulting in R644C]. We have since been able to obtain a DNA sample and perform a detailed clinical examination of his father, including echocardiography, 24 hours Holter ECG and muscle MRI. Surprisingly, this 54-year-old man was also found to have the R644C LMNA mutation. His serum CK was normal and muscle MRI showed only mild involvement of the paraspinal and biceps femori muscles (Fig. 6). Cardiac studies that included an echocardiography, an ECG and a 24 h Holter ECG monitoring were normal.

**Methods**

Informed consent for the genetic studies was obtained from both families.

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**Fig. 1** (A) Haematoxylin and eosin staining of a muscle biopsy from Patient 1, showing advanced dystrophic features, including wide variation in fibre size, fibrosis and increased numbers of internal nuclei. (B) Indirect immunocytochemistry for emerin, showing absent immunoreactivity in the patient, while in the control (C), the muscle nuclei show positive staining.

**Fig. 2** The pedigree of Family 1, showing a clear X-linked inheritance of the XL-EDMD trait. The index case is III-5. The STA mutation (Y105X) was confirmed in individuals I-1, II-1, II-2, II-5 and III-3, III-5, but was not found in III-4 and III-6. Note that individual III-5 but not his parents (II-5 and II-6) carried the S326T LMNA mutation.
**Family 1**
The coding region of *LMNA* was analysed by denaturing high-performance liquid chromatography (DHPLC). The 12 exons were amplified by polymerase chain reaction (PCR) (Bonne et al., 1999). For DHPLC, Wavemaker software (Transgenomic, San José, California, USA) was used to calculate specific melting curves for each PCR fragment and to determine the optimal temperature for heteroduplex separation.

**Family 2**
Genomic DNA was used as the template for PCR assay of the desmin gene, with the primers and conditions described previously (Dalakas et al., 2000). The resultant DNA fragments were purified by QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and directly sequenced using the BigDyeTerminator™ sequencing protocol on an automated 3100 ABI Prism® Genetic Analyzer (Applied Biosystems, Foster City, CA). Data was extracted and analysed using Sequencing Analysis software (Applied Biosystems).

The *LMNA* mutation identified in this family has been reported previously [Case 2 in Mercuri et al. (2005); c.1930C>T, resulting in R644C]. Two PCR assays to study the frequency in the general population of both the *LMNA* and the desmin changes were also devised.

**Pathological specimens**
The muscle biopsy of the proband in Family 1 was processed for indirect immunocytochemistry with antibodies against emerin (a kind gift of GE Morris, Oswestry) at a dilution of 1 : 5

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**Fig. 3** (A) Indirect immunocytochemistry for desmin revealed mild accumulation of the protein towards the centre of rare skeletal muscle fibres. (B) In the cardiac muscle, however, there was more significant desmin accumulation; this was confirmed on electron microscopy of cardiac tissue (C), which showed accumulation of granulomatous electron dense material.

**Fig. 4** Muscle MRI of the propositus of Family 2, showing the thigh (A) and calf muscles (B). There is mild involvement of the sartorius and adductor magnus at the thigh levels and of the soleus and medial gastrocnemius at the calf level. Abbreviations: Thigh: RF: rectus femoris; VI: vastus intermedius; VM: vastus medialis; VL: vastus lateralis; SA: sartorius; B: biceps; AM: adductor magnus; SM: semimebranosus; ST: semitendinosus; G: gracilis. Calf: TA: tibialis anterior; ED: extensor digitorum longus; P: peroneus; PO: popliteus; GM: gastrocnemius medialis; GL: gastrocnemius lateralis; SO: soleus.
The muscle and cardiac biopsies obtained from the proband in Family 2 (age 19) were processed for indirect immunocytochemistry with antibodies against desmin at a dilution of 1:100 (clone D33, Dako). All primary antibodies were applied for 1 hour and revealed with an appropriate biotinylated secondary antibody (Amersham 1:200) for 30 mins, followed by streptavidin conjugated to Alexa 594 (Molecular Probes) for 20 mins. All dilutions and washings were made in phosphate-buffered saline. Control sections were labelled without primary antibodies and all sections were compared with control samples from other neuromuscular disorders, and with normal muscle.

Mapped tissue blocks from a mid-ventricular slice of the formalin-fixed heart were routinely processed to paraffin wax. Sections 4 microns thick were cut and stained with haematoxylin-eosin for cell morphology, Masson’s trichrome (MT) for myocyte architecture and interstitial connective tissue content and Mallory’s phosphotungstic acid haematoxylin for myofibrillary structure.
Disease severity in dominant Emery Dreifuss

Analysis of the desmin gene revealed a c.1503G>A change resulting in p.V469M. The V469M mutation is located in the non-conserved tail domain of desmin; functional studies performed in SW13 cells transfected with this mutant allele did not compromise the desmin intermediate filament network (data not shown). This mutation was not found in the patient’s father, nor in 250 controls of European descent. Unfortunately, there was no more DNA from his deceased mother to confirm the probable inheritance of this desmin mutation.

Given evidence of incomplete penetrance in regard to the R644C LMNA mutation in Family 2, we extended the study of this change to 250 Caucasian control individuals. None of the disease controls had the R644C change. We also studied 150 patients affected by other neuromuscular disorders. Interestingly, we found that two cases with ptosis carried the R644C change.

Results

Family 1

In view of the severity of the skeletal myopathy and the superimposed ventricular tachyarrhythmias, we suspected a concurrent laminopathy in the propositus. DHPLC profiles identified both heteroduplexes and homoduplexes in his LMNA exon 6 and sequencing analysis showed a c.976T>A change resulting in p.S326T. Neither parents carried this mutation, suggesting that it had occurred de novo. The S326T LMNA mutation was not identified in 100 healthy controls, but we have recently detected it in three other patients, one affected by late-onset scapular myopathy with CSD and two by isolated Dilated cardiomyopathy with conduction system disease (DCM-CD) (personal observation).

Family 2

The rapidly progressive hypertrophic cardiomyopathy in the proband, and the finding of the same LMNA mutation in his asymptomatic father, prompted further investigation: immunohistochemistry of the skeletal muscle biopsy showed desmin accumulation towards the centre of occasional fibres (Fig. 3A), whilst cardiac muscle had marked desmin accumulations (Fig. 3B). Electron microscopy confirmed the accumulation of granulomatous electron dense material in the cardiac biopsy (Fig. 3C).

Analysis of the explanted heart showed features consistent with hypertrophic cardiomyopathy. There was hypertrophy of septum (25 mm thick) with posterior left ventricular thickness of 14 mm and poor development of papillary muscle giving a ‘spongy’ appearance to the inner left ventricle (Fig. 5A). On microscopy, there was extensive fibrosis of both ventricles (both replacement and interstitial patterns) affecting the middle and outer thirds of the myocardium (Fig. 5B), with relative sparing of the subendocardial myocardium. The myocytes of the residual myocardium were hypertrophied with enlarged nuclei; there was myocyte disarray (Fig. 5C) in the middle third of the myocardium affecting both the lateral free wall and the septum. Many myocytes both within and outside the areas of myofibre disarray showed disruption of myocyte cytoplasm by amorphous aggregates of material that, using the PTAH stain, were shown to be disrupted myofibrils (Fig. 5D) and positive immunohistochemically for desmin intermediate filaments (Fig. 5E). This finding has been previously noted in myocyte disarray associated with hypertrophic cardiomyopathy (Francalanci et al., 1995), which was considered to be the morphological diagnosis on the explanted heart for propositus 2.

Discussion

Our findings suggest that variability in clinical severity of EDMD patients can be influenced by independent mutations in functionally interacting proteins. Coincidental double mutations in muscular dystrophy have been reported in a boy with Duchenne muscular dystrophy and myotonic dystrophy (Dubrovsky et al., 1995), and in two brothers with separate mutations in the TRIM32 and FKRP genes, each associated with different forms of limb-girdle muscular dystrophy (Frosk et al., 2005). In these examples though, exacerbation of clinical severity caused by mutations in two separate genes was not observed, probably because the respective proteins have unrelated functions and sub-cellular localizations in muscle. In another example, a single patient with Becker muscular dystrophy who followed an unusually severe course was found to carry (in addition to the expected in-frame dystrophin gene deletion) a missense mutation in the myogenic regulatory factor Myf6 (Kerst et al., 2000). The authors speculated that this latter mutation might have affected the regeneration potential of the BMD patient, resulting in a more severe phenotype. More recently, a family with McLeod syndrome who followed a relatively severe phenotype were found to be heterozygous carriers for a calpain 3 mutation, suggesting that the heterozygote state for this form of muscular dystrophy could have influenced the severity of the McLeod myopathy (Starling et al., 2005).

The cases reported here, however, have important and novel clinicopathological features. In XL-EDMD, loss of ambulation in childhood is not reported and dilated cardiomyopathy is seen only exceptionally, after decades of follow-up (Bialer et al., 1991). Ventricular dysrhythmias (despite pacing) have also never been reported previously in XL-EDMD, although they are common in AD-EDMD. Indeed in AD-EDMD, severe skeletal involvement is more common, and recent management guidelines suggest the need to implant defibrillators instead of pacemakers.
(Bushby et al., 2003; van Berlo et al., 2005), hence further highlighting the greater severity and importance of primary CSD in the dominant form of the disorder. De-novo dominant LMNA mutations as in Family 1 are not uncommon in AD-EDMD; indeed we previously reported this finding in 83% of AD-EDMD-affected patients (Bonne et al., 2000).

The second patient developed an unusual and rapidly progressive hypertrophic cardiomyopathy, which is not a feature of AD-EDMD. His father was also subsequently found to carry the same LMNA mutation, but remains asymptomatic in his 50s. The same mutation was independently reported in one patient with isolated DCM (Genschel et al., 2001) and in another with atypical progeria (Csoka et al., 2004), suggesting an important role for the genetic background of the individual. Unfortunately, the inheritance of this mutation was not studied by Csoka et al. (2004).

We believe it is extremely unlikely that R644C is a rare polymorphism, as we have excluded it from 250 healthy controls. Moreover, it was identified in four unrelated EDMD-affected individuals described in Mercuri et al. (2005). Furthermore, more recently, the same mutation has been identified in another patient with a severe EDMD variant (H Jungbluth, personal observation). We strongly believe it to be pathogenic but of low penetrance. It is, however, possible that variability in age of onset could account for the fact that the father of the propositus in Family 2 is apparently unaffected, even though his muscle MRI is not entirely normal, suggesting mild subclinical skeletal muscle involvement. Non-penetrant LMNA mutations have been previously reported; Di Barletta et al., reported a family with incomplete penetrance in which a male patient with very mild AD-EDMD carried the missense mutation R336Q. The same mutation was detected in one of the proband’s sisters, in the mother and in the grandmother, who were all neurologically and cardiologically healthy (Raffaele Di Barletta et al., 2000). More recently, Vytopil et al. (2002) reported three additional families with heterozygous LMNA mutations showing incomplete penetrance and suggested that this phenomenon might be a common feature of mutations in this gene. These authors further commented that other genes interacting with lamin ‘could have contributed to the phenotype of these patients’ (Vytopil et al., 2002).

Since the hypertrophic cardiomyopathy and the desmin accumulation particularly in the cardiac muscle of the son of Family 2, together with the distal skeletal involvement in his mother, are features of desmin accumulation myopathies (Dalakas et al., 2000; Selcen et al., 2004), we considered the desmin gene as a possible candidate for the digenic inheritance in this family. The missense change in the desmin tail domain that we identified (which regulates interaction with other cytoskeletal proteins) is also likely to be pathogenic; the fact that this mutant failed to disrupt desmin assembly in vitro is not unexpected, as only rod domain mutations influence filament assembly (Dalakas et al., 2000; Selcen et al., 2004). Mutations in the tail domain have nonetheless been linked to cardiomyopathy (Li et al., 1999).

What could be the reason for the unusual disease course observed in these two patients? Emerin and lamin interact via the tail domain of lamin A/C (Clements et al., 2000) and it has been suggested that emerin mislocalization and improper function underlies EDMD’s pathogenesis (Fairley et al., 2002). Emerin, however, directly interacts with proteins involved in transcriptional regulation and is implicated in signalling via a lamin-independent pathway (Lammerding et al., 2005). Absence of lamin A/C causes increased nuclear fragility and abnormal nuclear mechanics leading to impaired signalling responses following mechanical strain, and cytokine stimulation as indicated by the attenuated transcription of NF-kB, a mechanical stress-responsive transcription factor that has anti-apoptotic role (Lammerding et al., 2004). In contrast, emerin-deficient cells display preserved NF-kB signalling, but reduced activation of several transcription regulation pathways including those involving BAF, GVC and YTH21-B. These transcription factors directly bind emerin and are not correctly localized in emerin-deficient cells (Lammerding et al., 2005). These observations suggest that at least part of the function of emerin is independent of lamin A/C, potentially accounting for the synergistic effect of the mutations detected in the propositus of Family 1.

Regarding the patient with the R644C change, Toth et al. (2005) recently studied fibroblasts from a patient with this change affected by progeria. The patient’s fibroblasts did not show an accumulation of prelamin A, as observed in progeroid patients with other mutations, but nevertheless the R644C nuclei often had abnormal shape, probably as the result of the mutant lamin A. Incubation with a drug that inhibited the farnesylation of lamin A, a post-translational modification of this protein, improved the nuclear shape in the R644C fibroblasts. This might be due to the effect of this drug in preventing the biogenesis of mature mutant lamin A (Toth et al., 2005).

Desmin and lamin A/C are believed to be indirectly connected via other intermediate filament proteins (Dalakas et al., 2000). A direct interaction between lamin B and desmin has been demonstrated (Cartaud et al., 1995) and is enhanced in the presence of lamin A (Georgatos and Blobel, 1987). In addition, lamin A/C null mice clearly show disorganization of desmin-nuclear attachments (Nikolova et al., 2004), suggesting their functional association. The very rapidly progressive hypertrophic cardiomyopathy we observed in the propositus of the second family (with desmin and lamin A/C mutations) is not a common finding in conditions caused by desmin mutations alone (Dalakas et al., 2000; Selcen et al., 2004), and was not observed in his mother, probably affected by desminopathy only. Furthermore, the propositus’ father, with only the lamin A/C mutation, was asymptomatic. However, mutations in both interacting proteins appear to have resulted in an unusually severe phenotype in the propositus.

In conclusion, these two cases indicate that the unusual clinical course in these two patients was caused by the digenic inheritance of mutations in genes whose products are

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functionally linked; they therefore further emphasize the significance of these functional interactions in protecting striated muscles from degeneration. Our results have important implications for genetic counselling; mutations occurring simultaneously in two different genes are infrequently studied and digenic inheritance may well contribute to the increased clinical severity of AD-EDMD and many other monogenic disorders.

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