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Abnormal proliferation and spontaneous differentiation of myoblasts from a symptomatic female carrier of X-linked Emery–Dreifuss muscular dystrophy

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Abstract

Emery–Dreifuss muscular dystrophy (EDMD) is a neuromuscular disease characterized by early contractures, slowly progressive muscular weakness and life-threatening cardiac arrhythmia that can develop into cardiomyopathy. In X-linked EDMD (EDMD1), female carriers are usually unaffected. Here we present a clinical description and *in vitro* characterization of a mildly affected EDMD1 female carrying the heterozygous *EMD* mutation c.174_175delTT; p.Y59* that yields loss of protein. Muscle tissue sections and cultured patient myoblasts exhibited a mixed population of emerin-positive and -negative cells; thus uneven X-inactivation was excluded as causative. Patient blood cells were predominantly emerin-positive, but considerable nuclear lobulation was observed in non-granulocyte cells – a novel phenotype in EDMD. Both emerin-positive and emerin-negative myoblasts exhibited spontaneous differentiation in tissue culture, though emerin-negative myoblasts were more proliferative than emerin-positive cells. The preferential proliferation of emerin-negative myoblasts together with the high rate of spontaneous differentiation in both populations suggests that loss of functional satellite cells might be one underlying mechanism for disease pathology. This could also account for the slowly developing muscle phenotype.

Keywords: Emery–Dreifuss muscular dystrophy; Emerin; *EMD*; Myoblast differentiation; X-inactivation

1. Introduction

Emery–Dreifuss muscular dystrophy (EDMD) is a neuromuscular disease characterized by early contractures, slowly progressive muscular weakness and life-threatening cardiac arrhythmia that can develop into cardiomyopathy. Contractures affecting the elbows, Achilles tendons and post-cervical muscles usually occur as the first clinical manifestation [1]. EDMD is genetically variable and thus far ~50% of patients have been linked to *EMD* (*STA*), *LMNA*, *FHL1*, *SYNE1*, *SYNE2*, *LUMA* and *SUN1* mutations [2]. Over 90% of linked patients have dominant mutations in *LMNA* [3] or recessive mutations in the X-chromosomal *EMD* gene [4]. The *EMD* gene encodes emerin, a 254 amino acid protein that is anchored in the nuclear envelope with a transmembrane span close to its C-terminus [5]. Most *EMD* mutations are predicted to cause loss of the protein, but missense mutations have also been reported. Out of 97 *EMD* mutations reported on http://www.umd.be only 6 mutations (affecting 5 codons) are missense mutations. Various reports have proposed that the disease results from defective emerin function affecting gene expression, cell proliferation and differentiation, or cellular susceptibility to mechanical stress damage [6].

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Female carriers of *EMD* mutations are usually asymptomatic; however, cardiac involvement has been occasionally though rarely described [7]. In all thus far reported cases of symptomatic females the clinical manifestation has been associated with unequal X-inactivation. However, it is also possible that the phenotype in symptomatic carriers could be caused by modifying mutations similar to how modifying mutations have been previously shown to affect disease severity. For example, combinations of *EMD* and *LMNA* mutations [8] as well as *EMD* and *DES* (the gene encoding the muscle intermediate filament desmin) [9] can increase the severity of EDMD. Findings in tissue culture indicate that mutations in *SUN1*, *SUN2* and *SYNE1* also act as severity modifiers in muscular disease [10,11]. The remarkable intra- and inter-familial variability regarding onset and severity of EDMD [12–16] makes it likely that severity modifiers are frequently involved, possibly even involving mutations that on their own do not cause a noticeable phenotype.

Here we report a symptomatic female carrying an emerin mutation that has also been found in her affected father. We have excluded uneven X-inactivation as a causative factor, finding that the majority of muscle as well as blood cells express the emerin wild-type allele. This makes a modifying mutation likely and raises the question of the contribution of each mutation to the disease. Nonetheless, analysis of the growth and differentiation potential of emerin-positive and emerin-negative cells in the population suggests a model whereby the emerin mutation contributes to depletion of a functional satellite cell population. Finally, as the X-linked *EMD* gene would not normally have been sequenced for a female presenting with EDMD this study highlights the importance of extensive analysis of the pedigree when searching for disease-causing mutations.

2. Materials and methods

2.1. Patient and controls

The patient attended the clinic followed by routine diagnostic mutational analysis of the *EMD* (MIM *300384) and *LMNA* (MIM *150330) genes. All materials (blood and muscle biopsies to generate myoblast lines) included in this study were taken with informed consent of the donors and with approval of the local ethics board.

2.2. Mutational analysis and tissue culture

Sanger sequencing was used to sequence the coding areas and exon/intron boundaries of the *LMNA* and *EMD* genes. Myoblasts were gained from a biopsy of biceps brachii muscle performed in the index patient at age 16. These myoblasts as well as myoblasts from an age matched control were grown in tissue culture using skeletal muscle cell growth medium (PromoCell, Heidelberg, Germany). Cells were kept from reaching confluency to avoid differentiation. For differentiation DMEM (containing 0.1% FBS, 5 mg/ml insulin and 5 mg/ml transferin) was used. Cells were grown at 37 °C in a 5% CO_2 incubator.

2.3. Analysis of leucocyte populations by flow cytometry

A typically principally mononuclear leucocyte fraction was isolated from heparinized blood using Histopaque®-1077 (Sigma-Aldrich®) following the provided protocol. To determine composition of the fraction, cells were analysed by flow cytometry using fluorescently labelled antibodies to CD19 (Becton Dickinson, 555413) for B-cells, CD3 (Beckman Coulter, A07746) for T-cells, CD66b (BioLegend, 305102) for granulocytes, and CD14 (Becton Dickinson, 560349) for macrophages and myeloid cells. After staining, cells were analysed on an LSR II flow cytometer (BD Bioscience, UK) equipped with 488 nm and 350 nm lasers and appropriate filters. Cell debris and cell aggregates were excluded from analysis by application of electronic gates and numbers of the live singlet cells in each gate calculated using *FlowJo* software (TreeStar, Inc).

2.4. Immunohistochemistry

Myoblasts were fixed with methanol (−20 °C). As a marker for proliferation Ki-67 (Thermo Scientific, RM-9106-S0), antibody was used [17]. Relocalization from the centrosome to the nuclear envelope of PCM1 has been found to be an early and reliable marker of differentiation [18] and so was used to assess myoblast differentiation. For emerin immunofluorescence staining of myoblasts and biopsy sections MANEM1 antibody was used which recognizes emerin amino acids 89–96 (GYNDDYYE) [19]. For stainings with leucocyte type-specific antibodies the same fluorescently conjugated antibody set that was used for flow cytometry was employed; however, because the laser lines of the flow cytometer did not match the filter sets of the microscope, secondary antibodies were also used. All secondary antibodies were Alexafluor conjugated and generated in donkey with minimal species cross-reactivity. DNA was visualized with DAPI (4,6-diamidino-2 phenylindole, dihydrochloride). To determine if the small fragment encoded by the mutant allele is stably expressed, Western blot analysis optimized for small MW proteins was performed using Millipore Immobilon® P^{SQ} Transfer Membranes and following the manufacturer's instructions. The blot was probed with monoclonal antibody MANEM14 against emerin amino acids 7–14 (LSDTELTT) [19].

2.5. Microscopy and image analysis

Most images were obtained using a Nikon TE-2000 microscope equipped with a 1.45 NA 100× objective or a 20× objective and CoolSnapHQ High Speed Monochrome CCD camera (Photometrics, Marlow, UK). Image analysis was performed using ImageJ software.

2.6. RNA depletion of emerin in control myoblasts

The previously published siRNA against emerin 5′GGUGGAUGAUGACGAUCUUtt-3′ [20] was transfected into myoblasts using jetPRIME® (Polyplus) following the manufacturer's instructions.