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Genetic and ultrastructural studies in dilated cardiomyopathy patients: a large deletion in the lamin A/C gene is associated with cardiomyocyte nuclear envelope disruption

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Abstract

Major nuclear envelope abnormalities, such as disruption and/or presence of intranuclear organelles, have rarely been described in cardiomyocytes from dilated cardiomyopathy (DCM) patients. In this study, we screened a series of 25 unrelated DCM patient samples for (a) cardiomyocyte nuclear abnormalities and (b) mutations in *LMNA* and *TMPO* as they are two DCM-causing genes that encode proteins involved in maintaining nuclear envelope architecture. Among the 25 heart samples investigated, we identified major cardiomyocyte nuclear abnormalities in 8 patients. Direct sequencing allowed the detection of three heterozygous *LMNA* mutations (p.D192G, p.Q353K and p.R541S) in three patients. By multiplex ligation-dependant probe amplification (MLPA)/quantitative real-time PCR, we found a heterozygous deletion encompassing exons 3–12 of the *LMNA* gene in one patient. Immunostaining demonstrated that this deletion led to a decrease in lamin A/C expression in cardiomyocytes from this patient. This *LMNA* deletion as well as the p.D192G mutation was found in patients displaying major cardiomyocyte nuclear envelope abnormalities, while the p.Q353K and p.R541S mutations were found in patients without specific nuclear envelope abnormalities. None of the DCM patients included in the study carried a mutation in the *TMPO* gene. Taken together, we found no evidence of a genotype–phenotype relationship between the onset and the severity of DCM, the presence of nuclear abnormalities and the presence or absence of *LMNA* mutations. We demonstrated that a large deletion in *LMNA* associated with reduced levels of the protein in the nuclear envelope suggesting a haploinsufficiency mechanism can lead to cardiomyocyte nuclear envelope disruption and thus underlie the pathogenesis of DCM.

Keywords

Lamin A/C; Thymopoietin; Mutation; Dilated cardiomyopathy; Cardiomyocyte; Nucleus ultrastructure

Introduction

Dilated cardiomyopathy (DCM) is characterized by dilatation of cardiac chambers and impaired contraction. Severity of symptoms and age of onset are highly variable. To date,

mutations in 28 genes have been associated with autosomal dominant DCM [3, 9–11, 18, 19, MIM#115200]. *LMNA*, which encodes lamin A/C, is one of the most commonly implicated genes in DCM. Mutations in *LMNA* are associated with a high risk of dysrhythmia, sudden death and heart failure [4, 35]. Furthermore, symptomatic DCM patients carrying *LMNA* mutations display a worse prognosis than DCM patients carrying a mutation in another DCM-associated gene [2, 17, 19, 26, 44, 46, 48].

The A-type lamins A and C, alternatively spliced products from *LMNA*, are type V intermediate filament proteins expressed in terminally differentiated somatic cells. They are components of a thin filamentous mesh-work—the nuclear lamina—underlying the nucleoplasmic side of the inner nuclear membrane, and are also present in the nucleoplasm [8, 20, 28, 30]. Lamins have traditionally been considered key components in providing structural support to the nucleus and anchoring chromatin and nuclear pore complexes to the nuclear envelope [24, 40, 52]. Intranuclear lamins have proposed functions in DNA replication [20, 25, 28, 29], transcription [41] and chromatin organization [38, 50]. In order to carry out their functions, lamins interact with a number of nuclear envelope proteins, chromatin and transcription factors. Among the genes encoding the lamin A/C interacting proteins, *TMPO*, the gene encoding nucleoplasmic thymopoietin alpha [also called lamina-associated polypeptide 2 (LAP2) alpha] has been shown to harbour a mutation in DCM patients [47]. The identified mutation occurred in the C-terminal domain of thymopoietin alpha, a region known to interact with lamin A/C (residues 319–566) [9]. Three thymopoietin isoforms, alpha, beta and gamma are encoded by *TMPO* [6]. The three isoforms share an identical amino terminal sequence but have divergent carboxy terminal sequences [9]. Thymopoietin is thought to play an important role in maintaining nuclear architecture through its binding with lamins and other proteins.

Cardiomyocytes from DCM patients with *LMNA* mutations exhibit the following characteristics: (a) reduced lamin A/C expression in myocyte nuclei on immunofluorescence/immunohistochemistry studies and (b) two types of ultrastructural changes, minor nuclear envelope damage, such as focal disruptions, blebs and nuclear pore clustering, and major morphologic alterations, including a complete loss of the nuclear envelope and accumulation of mitochondria, glycogen and/or lipofuscin in the nucleoplasm [2, 7, 13, 44, 51]. To date, these major nuclear envelope defects were only found in a DCM patient carrying the p.D192G *LMNA* mutation [7, 13, 44]. However, in non-genotyped DCM patients as well as in heart failure patients, minor nuclear defects such as irregular nuclear envelope, enlarged and bizarre shaped nuclei, indentations, giant and multiple nucleoli and irregularly distributed chromatin are commonly found [1, 4, 34, 37]. There is considerable heterogeneity in the abnormalities observed from one cardiac sample to the next as well as within one particular sample [1, 37].

The aim of the present study was to ascertain whether: (1) major nuclear envelope abnormalities are a common feature in patients with DCM and (2) *LMNA* and/or *TMPO* mutations are associated with nuclear envelope defects in cardiomyocytes from a series of 25 unrelated DCM patients.

Materials and methods

Patient recruitment

Written informed consent was obtained from all patients in accordance with study protocols approved by the hospital ethics committees. Diagnosis of DCM was established based on WHO/ISFC criteria modified by Mestroni and colleagues [27] that included severe LV systolic dysfunction (LV dilatation exceeding 117% of normal value corrected for age and body surface area) and LV ejection fraction (LVEF) ≤45% measured in angiography, without significant coronary artery disease (>50% lumen diameter reduction of one of the main coronary arteries). Exclusion criteria included hypertension, or acquired or congenital heart disease. All patients underwent clinical examination, evaluation of functional status according to NYHA classification, ECG study, two-dimensional echocardiography with Doppler and/or coronary angiography to exclude coronary artery disease. Left ventricular enlargement was calculated according to the method of Henry. A total of 25 index cases recruited in Canada and Poland were enrolled in the study.

Cardiac tissue collection, electron microscopy and immunostaining

Cardiac tissue samples were collected from endomyocardial biopsies performed on clinical indication or from explanted heart tissue. Samples were immediately processed in 1.6–3% gluteraldehyde. Fixed heart tissues were processed into thick and thin sections according to the standard methods. Tissue sections were examined with a Hitachi 7100 or JEM 1200EX electron microscope.

For indirect immunofluorescence examinations, 8 μm cryostat sections were stained with two monoclonal antisera against lamin A/C (NCL-LAM-A/C) (Novocastra Laboratories, Newcastle, UK) and A4, kindly provided by Dr. Hutchison (Department of Biological Science, University of Dundee, UK). A4 antibody detects only lamin A as its recognition site lies after amino acid 572. NCL-LAM-A/C recognizes both lamin A and lamin C. In brief, antibodies diluted 1:10 were applied to tissue sections for 60 min. Sections were rinsed with PBS and incubated for another 60 min with the appropriate Rodamine Red X conjugated goat anti-rabbit IgG secondary antibody diluted 1:30 with PBS. After being washed with PBS, the sections were mounted in gelmount and viewed with an Opton Zeiss standard LAB16 microscope with epifluorescence optics.

Screening of *LMNA* and *TMPO* coding sequences

Genomic DNA was isolated from white blood cells (Qiagen Flexigene kit). To screen for somatic mutations, DNA was extracted from cardiac tissues using a Qiamp Mini DNA extraction kit (Qiagen).

Intronic oligonucleotide primers flanking each of the exons were designed based on published sequences (Gen-bank accession number: L123399, L12400 and L12401 for *LMNA* and UCSC genome bioinformatics database for *TMPO*). All DNA samples were subjected to PCR and direct sequencing (ABI Prism Big Dye, ABI PRISM 3100 genetic analyzer). If there was any suspicion of genomic variation in a given patient, another sample of DNA collected independently was systematically double-strand sequenced (Table 1).

Sequences were compared to two control samples from individuals without known cardiovascular disease. Each sample's electropherogram was analyzed by two independent investigators.

Multiplex-ligation dependant probe amplification (MLPA) analysis was performed to screen for deletions and duplications in the *LMNA* gene coding sequence according to the manufacturer's instructions (SALSA MLPA KIT P048 LMNA, MRC Holland). The probe mix contained probes for 10 of the 12 coding exons of *LMNA*, but not for exons 5 and 9 because of the close proximity of exons 4 and 5, and exons 8 and 9. The probe mix also contained 13 probes for other human genes. Two of these probes recognize genes localized on chromosome 1. One of these probes was located 94 kb upstream of *LMNA* exon 1 and another 100 kb downstream of *LMNA* exon 12. MLPA amplification products were analyzed on an ABI model 3130XL Genetic Analyzer (Applied Biosystems) with the GeneMapper software V.3.7, using Genescan 500LIZ internal size standard (Applied Biosystems). Each patient's electropherogram was compared to three controls. We used the Coffalyzer MLPA DAT (MRC-Holland) software to analyze MLPA data. Resulting normalized ratios were ~1.0 for every wild-type peak, 0.5 for heterozygous deletions and 1.5 for heterozygous duplications.

Quantitative real-time PCR was used to confirm the deletion found in *LMNA* (Roche LightCycler 2.0) [36]. Primers were designed for *LMNA* exon 9 (forward: 5′-ggagcgctggggtaagtgtc-3′; reverse: 5′-ctcgtccagcaagca gcccag-3′). PCR was set up in capillaries in a total volume of 20 μl; each PCR mixture contained 2.5 mM $MgCl_2$ (2.5 mM), 2 μl of sybr green mix, 0.6 μM of mixture of forward and reverse primers and 20 ng of DNA. Standard curves for both the housekeeping gene (β2-microglobulin) and target gene were generated. Two different types of sample DNA (i.e., one sample from the DCM patient carrying the deletion and one sample from a DCM patient without the deletion) were utilized to ensure that the gene dosage ratio of the target to the housekeeping gene was disrupted only when a deletion was present. Using the slope and *y*-intercept of the standard curve, the LightCycler system calculated the concentrations (M) of sample and control DNA for both the reference gene (β2 microglobulin) and the target gene (Lamin A/C). These concentrations were then used to calculate the gene dosage ratio using the following equation:

$$R = \frac{M_{\text{target}}\,(\text{patient})/M_{\text{reference}}\,(\text{patient})}{M_{\text{target}}\,(\text{control})/M_{\text{reference}}\,(\text{control})}$$

where $R = 0.5$ (0.4–0.7) indicates deletion, $R = 1$ (0.8–1.2) indicates normal copy number and $R = 1.5$ (1.3–1.7) indicates duplication

Statistical analysis

LVEF and LVEDD in percentage values were compared between patients with major cardiomyocyte nuclear abnormality and patients with non-specific cardiomyocyte nuclear abnormality using a two-tailed Student's *t* test (level of significance 5%).

Results

Ultrastructural characteristics of endomyocardial samples

Ultrastructural analysis of endomyocardial samples enabled us to distinguish two groups of DCM patients. One group consisted of eight individuals exhibiting major nuclear envelope abnormalities on electron micrographs (Fig. 1) and the second consisted of 17 patients with minor and non-DCM specific abnormalities.

Among the patients with nuclear envelope defects, the proportion of defective cells ranged from a small percentage to as much as 30% (patients 2 and 6). The most obvious abnormality, occurring in four of eight patients, was intrusion and accumulation of mitochondria and other cytoplasmic organelles into the nuclear matrix (patients 1, 2, 5 and 7) (Fig. 1a, d). The membranes of these organelles appeared discontinuous from the nuclear envelope (Fig. 1a, d). The nuclear envelope was usually irregular (Fig. 1a–c) and partially disrupted or totally missing in six of eight patients (patients 1, 2, 4, 5, 6 and 7) (Fig. 1a, c, d). Chromatin disorganization was also observed (patients 2 and 6). In contrast, samples from the 17 patients in the second group displayed modest and non-specific nuclear membrane alterations, such as nuclear irregularity, that are commonly found in DCM patients regardless of the presence of *LMNA* mutations (Fig. 1e, f).

Clinical characteristics of the patient cohort

Clinical characteristics of the 25 DCM patients (19 males and 6 females) included in our study are shown in Table 2. There was considerable heterogeneity in terms of the clinical characteristics and severity of DCM across the cohort of 25 patients.

In the group of eight patients who exhibited nuclear envelope abnormalities, age at disease onset ranged from 14 to 63 years, four patients had a family history of DCM (Table 2, Fig. 2, [44]), while none had a suspicion of muscular dystrophy. Disease severity ranged from mild DCM to progressive heart failure and LVEF values ranged from <10 to 50%. Various arrhythmias were present in all eight patients; two patients required ICD (patients 1 and 7) and one required pacemaker (patient 5). Three patients had received a heart transplant (patients 4, 5 and 6) and two patients had died of heart failure (patients 2 and 3). In addition, patient 5 presented with hypothyroidism and patient 7 with mild mental retardation. No other phenotypic abnormality was identified within this population.

A similar degree of heterogeneity was also observed among the 17 patients with no major nuclear envelope abnormalities: age at disease onset ranged from 12 to 61 years, 10 patients had a family history of DCM (Table 2, Fig. 2, [44]), 1 patient had documented skeletal myopathy (patient 10) and 1 had elevated CPK values [up to 600 U/I (patient 18)]. LVEF values ranged from 10 to 38%; conduction defects were present in all but two patients (data were not available for four patients), four patients required ICD and two had pacemakers. Twelve patients had received heart transplants and two patients had died of heart failure (patients 13 and 21).

We compared mean LVEF and LVEDD in percentage values between patients exhibiting major nuclear envelope abnormalities and patients with minor and non-DCM-specific

abnormalities. No significant correlation between the severity of the phenotype and the presence of nuclear envelope defect was detected ($p > 0.05$).

Screening of *LMNA* and *TMPO* coding sequences

We screened the complete coding sequence as well as the intron–exon boundaries of *LMNA* (12 exons) and *TMPO* (8 exons) for mutations using direct sequencing of DNA extracted from white blood cells of all 25 patients. We identified three patients with *LMNA* mutations, one in the group with major nuclear envelope defects (patient 2, p.D192G) and two in the group with non-specific nuclear envelope abnormalities (patient 9, p.R541S and patient 10, p.Q353K). Patient 2 and 9 and their *LMNA* mutations have been described previously [7, 44]. All these variations were absent in DNA from more than 100 controls. No mutation was found in *TMPO*. Previously reported polymorphisms in both *LMNA* (synonymous polymorphisms rs538089, rs505058, rs4641) and *TMPO* (non-synonymous polymorphism rs17459334) were detected. We also found a c.C1341A synonymous polymorphism in *LMNA* exon 6, which has not been previously reported, in two control individuals. Mutations in the corresponding codon have been previously reported in patients with limb-girdle muscular dystrophy 1B (p.R377H, [31]; p.R377L, [21]).

Sequencing analysis cannot detect large heterozygous deletions or duplications. In an attempt to ascertain whether the defects resulted from deletions or duplications in *LMNA*, we performed multiplex ligation-dependant probe amplification (MLPA) on the *LMNA* coding sequence in all 25 DCM patients. One DCM patient (Table 2, patient 1) with major nuclear abnormality displayed a MLPA normalized ratio peak area of ~0.5 for exons 3–12 (Fig. 3). This indicated the presence of a heterozygous deletion encompassing exons 3 to 12 (>4,704 bp). The remaining probes, including those for exons 1 and 2, displayed a normalized ratio of ~1.0 indicating normal copy number (Fig. 3). We confirmed the presence of the deletion using qPCR (R value of 0.5 for the patient with the deletion and 1 for the patient without the deletion) (Fig. 4).

Lastly, to assess whether the nuclear envelope defects in the remaining patients could result from somatic mutations, we screened for *LMNA* and *TMPO* mutations in the heart from sporadic cases with nuclear envelope defect for which cardiac tissue was available (patients 5, 6) as well as in heart from patients without defective nuclear envelope (patients 19, 20, 21, 22, 23, 24 and 25). This analysis did not reveal any somatic mutations.

In summary, a large degree of clinical heterogeneity was evident in the cohort of DCM patients studied regardless of the presence of nuclear envelope abnormalities, *LMNA* mutation or family history of DCM.

Immunostaining results

To gain further insight into the functional significance of the heterozygous deletion of exons 3–12 of *LMNA* observed in patient 1, we performed indirect immunofluorescence analysis of endomyocardial samples from this patient as well as a control patient without *LMNA* mutation, using antibodies directed against both lamin A and C. All cardiomyocyte nuclei were immunostained with antibodies directed against lamin A and C epitopes (Fig. 5). The

immunostaining was reduced in patient 1 as compared to the control patient indicating significant attenuation of lamin expression (Fig. 5).

Discussion

Among heart samples from 25 unrelated DCM patients, we identified major cardiomyocyte nuclear abnormalities in 8 individuals and non-specific nuclear abnormalities in the remaining patients. The presence of nuclear abnormalities, along with the fact that all patients presented with dysrhythmias and/or severe heart failure leading to cardiac transplantation, prompted us to analyze the sequence of *LMNA* and *TMPO*, two DCM-causing genes involved in maintaining nuclear envelope architecture.

Direct sequencing allowed the identification of three heterozygous *LMNA* genomic variations (p.D192G, p.R541S and p.Q353K) in three patients. Since sequencing analysis cannot detect large heterozygous deletions or duplications, we performed multiplex ligation-dependant probe amplification (MLPA) on the *LMNA* coding sequence in all 25 DCM patients. We found a heterozygous deletion encompassing exons 3–12 of the *LMNA* gene. This deletion was confirmed using quantitative real-time PCR. All variations were absent in DNA from more than 100 controls. The occurrence of *LMNA* mutation in our population is high probably due to fact that the studied population presented with cardiac condition requiring undergoing endomyocardial biopsies or cardiac transplantation. Moreover, various arrhythmias were present in all but two patients. Since mutations in *LMNA* are usually associated with a worse prognosis than any other DCM-associated gene mutation [2, 17, 18, 26, 44, 46, 48] as well as with dysrhythmia [4, 35], our population was probably biased toward *LMNA* mutations carriers. None of the patients carried a mutation in the *TMPO* gene.

While rare findings of small deletions in the lamin A/C gene in patients with laminopathies have been reported previously, to our knowledge, this study is the first to document a large deletion encompassing most exons of the lamin A/C gene in a DCM patient. Walter et al. [53] found a 15 amino acid deletion (−3 to 12 nucleotides) in the 5′ end of the *LMNA* gene in a patient with Emery–Dreifuss muscular dystrophy (EDMD) that resulted in loss of the translation initiator codon. van Tintelen et al. [49] reported a 674 bp deletion encompassing exon 1 and the adjacent non-coding exon in a patient with myocardial fibrosis. The presence of the exon 1 p.Q6Stop *LMNA* mutation resulting in reduced levels of lamin A/C proteins has also been reported in a family with EDMD [5].

Most *LMNA* mutations that cause DCM are heterozygous point mutations, only some of which have been associated with cardiomyocyte nuclear envelope defects [2, 7, 13, 44, 51]. It has been suggested that mutations causing such abnormalities have a dominant-negative effect on the functions of normal lamin protein. Impaired lamin integration into the nuclear lamina due to the dominant-negative effects of a mutation may cause disruption of the lamina and ultimately compromise nuclear envelope integrity. Our results suggest that, apart from the dominant-negative effects of mutant lamin A/C protein, lamin haplo-insufficiency can also cause nuclear envelope defects and underlie the pathogenesis of DCM. Since the deletion encompasses *LMNA* exon 3 to 12, it is likely that the observed nuclear

abnormalities are not due to the expression of a putative truncated protein composed of exons 1–2 only. It was shown that the potential truncated protein resulting from the Y259X nonsense mutation (exon 4) was not detectable in patient fibroblasts [33]. Similarly, in the mouse model with the *LMNA* exon 8–11 deletion, the 54-kDa truncated protein resulting from this deletion was not detected [43]. Furthermore, immunostaining revealed reduced lamin A/C in the patient's cardiomyocyte nuclei. That lamin haploinsufficiency can cause such defects is further corroborated by the fact that a heterozygous $LMNA^{+/-}$ mouse model in which cardiac lamin A/C levels were diminished by 50% compared to wild-type, demonstrated early-onset conduction system disease and late-onset DCM [54]. Furthermore, misshapen cardiomyocyte nuclei and heterochromatin clumping were also observed [54].

The *LMNA* deletion of exons 3–12 was observed in a patient presenting with major nuclear envelope abnormalities—notably broken nuclear envelope and accumulation of mitochondria within and around the nuclei. This suggests that haplo-insufficiency is the mechanism underlying the observed nuclear abnormalities. Among their many functions, lamins play an integral role in maintaining the mechanical stability of the nucleus; hence we can speculate that the nuclear envelope defects observed in the patient carrying this *LMNA* deletion results from the reduced levels of lamin A/C protein.

Apart from the observed deletion, one of our patients with nuclear envelope defects carried a p.D192G point mutation in the *LMNA* gene. When expressed in COS-7 cells, this mutation resulted in a discontinuous nuclear lamina due to the formation of multiple lamin aggregates [44, 45]. These results therefore corroborated the pathological findings from the patient with the mutation.

In our study, not all *LMNA* mutations were found to cause nuclear envelope abnormalities in patients' cardiomyocytes. Indeed, two patients with *LMNA* point mutations demonstrated no specific nuclear envelope abnormalities in the samples taken from explanted heart tissue: one patient carried a p.Q353K mutation, while the second carried the p.R541S mutation. It is possible that the number of abnormal nuclei was so low in these two patients that it resulted in a false-negative finding. Patient age at the time of tissue collection and the protein domain affected by the mutation were considered as possible explanations for the observed discrepancy in nuclear envelope phenotype. Age at the time of tissue collection was 38 and 12 years for the patients carrying mutations p.Q353K and p.R541S, respectively, both of which were associated with normal nuclear envelope ultrastructure. The patients with *LMNA* deletion and p.D192G mutation, both of whom demonstrated nuclear envelope abnormalities, were 39 and 26 years of age, respectively. Hence, there was no apparent relationship between age and observed nuclear envelope phenotype. It was also considered possible that the presence of nuclear envelope abnormalities may depend upon the protein domain affected by each mutation [12, 32]. The p.D192G mutation, which was associated with major nuclear envelope abnormalities, is located in the central α-helical rod domain at the distal end of coil 1B. This highly conserved region of the lamin A/C protein is critical for the formation of the α-helical coiled-coil dimer, the basic building block for the construction of lamin filaments. In contrast, the p.Q353K mutation in exon 6 affected a highly conserved residue localized in the central rod domain coil 2B fragment. Based on the coil 2B fragment crystal structure, mutations in this domain are unlikely to alter the dimer structure but may

interfere with essential molecular interactions occurring in later stages of filament assembly, lamina formation and/or chromatin interaction [42]. Similarly, the p.R541S mutation is located in highly conserved residues in a region of gene shared by lamin A and C isoforms within the carboxyl-terminal end. The buried side chain of R541 participates in the stabilization of the carboxy-terminal β-sandwich through hydrophobic contacts with the core of the domain [22]. Mutations in this domain could therefore destabilize the three-dimensional structure of the C-terminal domain of lamin A/C. This domain also carries sites for many lamin A/C interacting proteins. Therefore, both p.Q353K and p.R541S mutations could theoretically disrupt multiple functions of lamin A and C, including the maintenance of nuclear architecture. Unexpectedly, the ultrastructural phenotype associated with these mutations was indistinguishable from controls. Hence, our results do not support the presence of a relationship between the position of the mutation and the resulting ultrastructural phenotype.

The six patients demonstrating nuclear ultrastructural defects without *LMNA* or *TMPO* mutations showed that germ-line mutations in these genes are not a prerequisite for nuclear envelope defects. However, we could not discount the possibility that some of these individuals possessed somatic *LMNA* or *TMPO* mutations restricted to diseased cardiac tissue. The fact that only a percentage of observed cardiomyocytes from these patients displayed an abnormal nucleus lends support to this hypothesis. Indeed, somatic mutation occurring in a selective subpopulation of progenitor cell lineage is one of the molecular mechanisms leading to such tissue mosaicism. Furthermore, in other cardiovascular diseases such as idiopathic ventricular tachycardia or idiopathic atrial fibrillation, both germ-line and somatic missense mutations have been linked to the disease [14, 23]. We therefore screened for the presence of *LMNA* and *TMPO* mutations in DNA extracted from heart tissues of patients who did not have familial DCM, but were unable to identify any somatic mutations. Another possibility is that mutation in genes encoding lamin A/C-binding proteins are responsible for the major cardiomyocyte nuclear abnormalities observed in these patients [15, 16, 39, 55–57].

We showed here that there is no clear correlation between the presence or position of the *LMNA* mutation and either clinical phenotype with respect to DCM onset or severity or cardiomyocyte ultrastructural phenotype. We also found no apparent correlation between genotype and either severity of the ultrastructural aberration (i.e., the degree of nuclear envelope disruption), or the proportion of observed cells displaying abnormal nuclei. Furthermore, there was no significant correlation between the severity of the disease and the presence of ultra-structural nuclear envelope defects. Although a quantitative analysis was not possible due to the small sample size, overall, we found no evidence of a relationship between the onset and the severity of DCM, the presence of nuclear abnormalities and the presence or absence of *LMNA* mutations.

Taken together, our results suggest that lamin A/C haplo-insufficiency, documented by reduced protein level in the patient's cardiomyocyte nuclear envelope caused by a large deletion in the *LMNA* gene, can lead to nuclear envelope disruption and underlie the pathogenesis of DCM. However, the finding that two patients without cardiomyocyte nuclear abnormalities had *LMNA* mutations indicates that *LMNA* mutations may not

necessarily lead to major cardiomyocyte nuclear envelope defects. Furthermore, patients with major nuclear envelope abnormalities may not have *LMNA* or *TMPO* mutation. This demonstrates that patients with a clinical suspicion of laminopathy and marked abnormality of cardiomyocyte nuclei can be free of both *LMNA* and *TMPO* mutations and may carry a mutation in another gene encoding a protein involved in the maintenance of the nuclear architecture.

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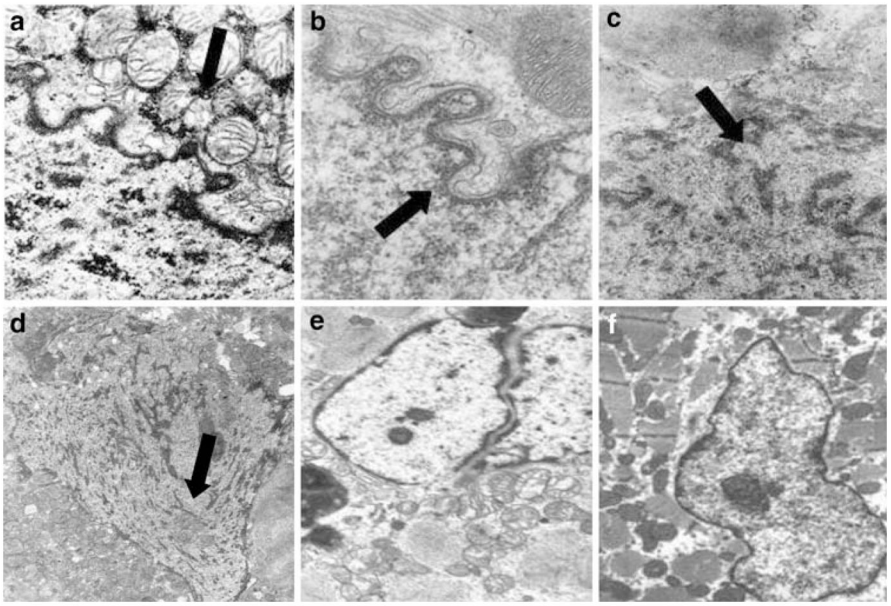

Fig. 1.

Electron micrographs of cardiomyocytes from dilated cardiomyopathy patients with or without *LMNA* mutation. **a**, **b**, **c** and **d** Major nuclear abnormalities in, respectively, patient 1, 3, 4 and 5 (Table 2). Patient 1 carried the *LMNA* deletion exons 3–12, while patients 3, 4 and 5 had no *LMNA* nor *TMPO* mutation; **a** accumulation of mitochondria around the nuclear envelope; **b** blebbing of the nuclear envelope and separation of the inner and the outer nuclear membrane; **c** extrusion of nucleoplasm from the cardiomyocyte nucleus into the cytoplasm; **d** accumulation of cytoplasmic organelles in the nucleoplasm; **e** non-specific nuclear alteration in patient 10 (Table 2) carrying the p.Q353K *LMNA* mutation; **f** non-specific alterations in dilated cardiomyopathy patient 14 (Table 2) with wild-type *LMNA* and *TMPO* (original magnifications: **a**, 20,000×; **b**, 18,000×; **c**, 12,000×; **d**, 10,000×; **e**, 4,000×; and **f**, 4,000×)

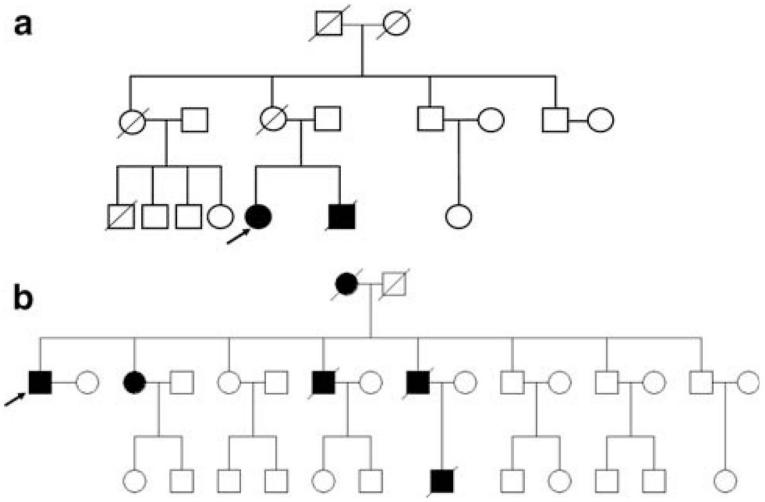

Fig. 2.

Pedigree of the families with *LMNA* mutations. *Arrow* indicates proband; *black filled symbol* dilated cardiomyopathy patient; *open symbol* asymptomatic individual. **a** Deletion of exons 3–12; **b** p.Q353K

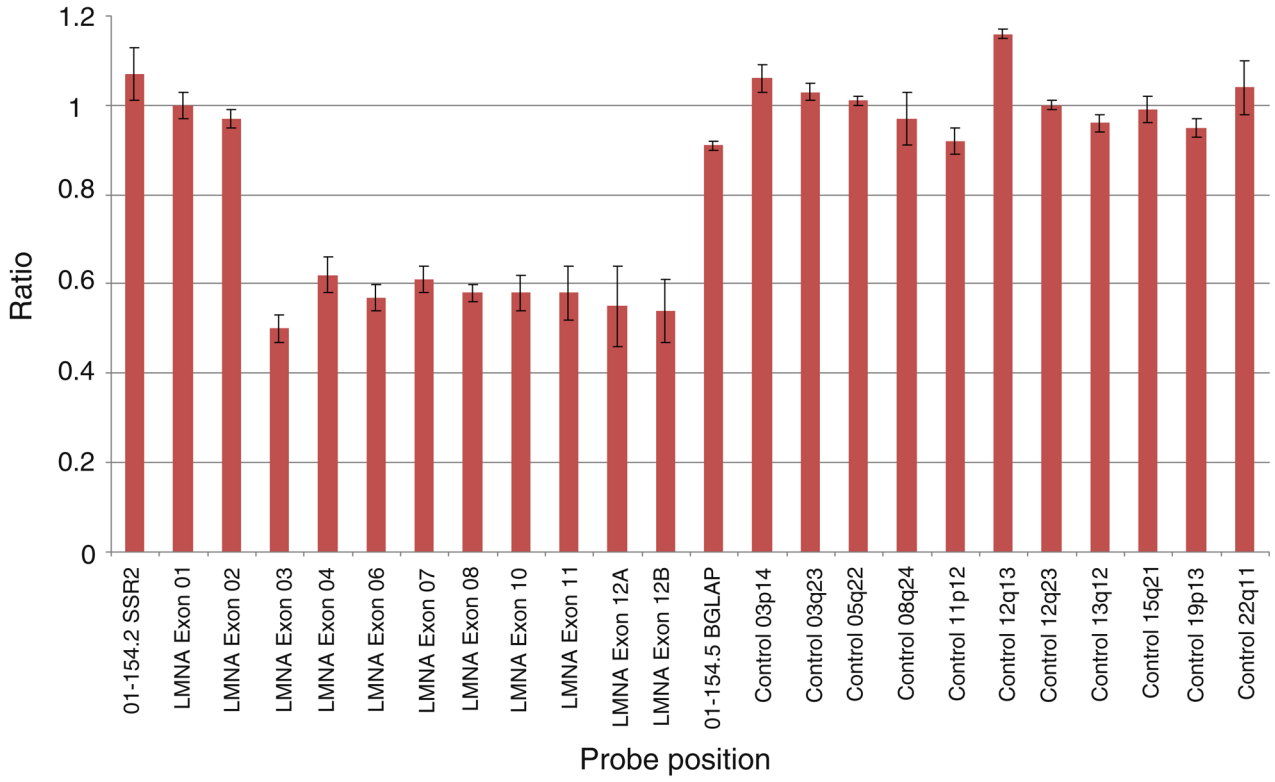

Fig. 3.

MLPA analysis graph showing the heterozygous *LMNA* deletion of exons 3–12 in patient 1. Exons 3–12 display a normalized ratio of ~0.5; indicating a loss of genetic materiel. Exons 1, 2 and the remaining control probes display a normalized ration of ~1.0; indicating normal copy number. The control probes are targeted within the same chromosome and to different chromosomes as indicated by their locus

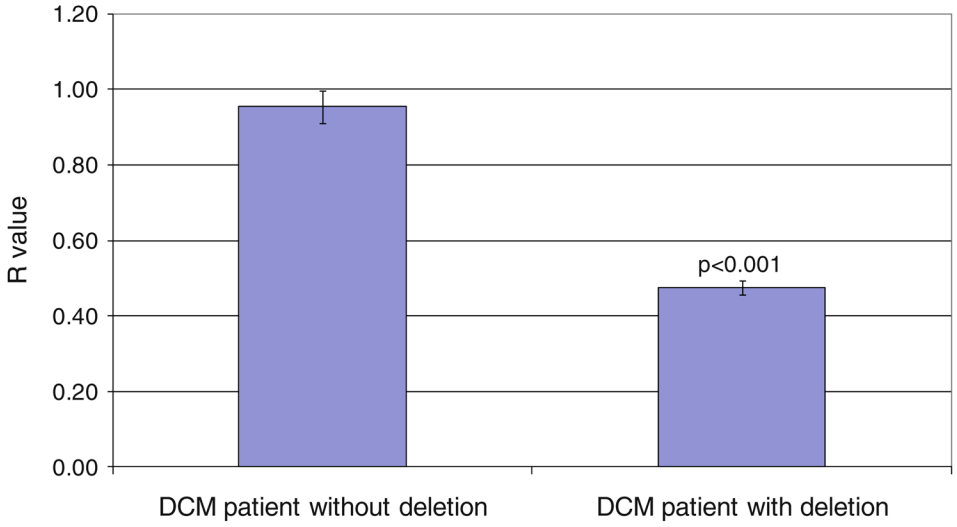

Fig. 4.

Confirmation of the deletion of exons 3–12 in *LMNA* in patient 1 using qPCR. *R* values were calculated as described in the "Materials and methods". Patient 14 without the deletion had a *R* value of 0.955, which indicates a normal copy number of the exon. The *R* value for patient 1 is 0.476, which indicates the presence of only one copy of the exon ($n = 3$)

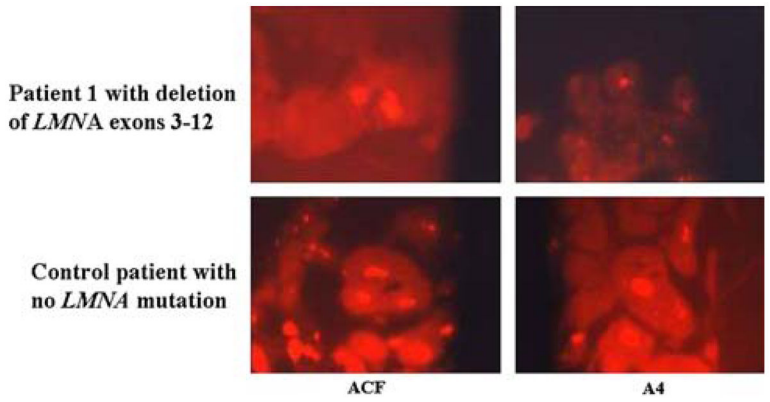

Fig. 5.

Indirect immunofluorescence analysis of endomyocardial biopsy from patient 1 carrying the *LMNA* heterozygous exon 3–12 deletion and from a control patient with no *LMNA* mutation. Endomyocardial biopsy was taken from the right ventricle. Immunostaining was performed using anti-lamin A+C monoclonal antibodies ACF and antibody A4 which detects lamin A only (see “Materials and methods”) and the goat anti-rabbit IgG secondary antibody (original magnification 1,050×)

Table 1

Primer pairs and annealing temperatures used to amplify *LMNA* and *TMPO* coding regions

		Primer sequences (5′ to 3′)		PCR annealing temperature (°C)
LMNA	Exon	Forward	Reverse	
	1A	TCTGTCCTTCGACCCGAG	GTAGACCGCCAAGCGATC	51
	2A	GGAGGACCTGCAGGAGCT	GCCCTCTCACTCCCTTCC	50
	3	CCTTCAAGTTCTTGTGTTCTGTGAC	CCTAGCCCAGCCCAAGTCTGTC	58
	4	GGCCTCCCAGGAACTAATTCTG	CTCCCTGCCACCATCTGC	63
	5	GCTGTAGCAGTGATGCCCAAC	CCAAAGCCCTGAGAAGTGAAG	62
	6	ATCCTGGAGAGAGTAGCCAG	TCTAGTCAAGGCCAGTTGCC	60
	7	CCCCACTTGGTCTCCCTCTCC	CCCTGATGCAGCTGTATCCCC	60
	8	GAGGCCTCAATTGCAGGCAGGC	GAAAAGGACACTTACCCCAGC	62
	9	GGAGCGCTGGGGTAAGTGTC	CTCGTCCAGCAAGCAGCCAG	60
	10	GTAAGCAGCAGGCCGGACAAAG	GATGCCATGGAATATTCCTGTG	58
	11A	GGTCAGTCCCAGACTCGC	ACCAGATTGTCCCCGAAG	55
	11B	GTCACTCGCAGCTACCGC	CCACCTCGTCCTACCCCT	52
	12	CTTGTCTGAGCCCCAGACTGGAG	AGGGAAAAGGAAGGGAGGAGAAAT	65
TMPO	1	GTCTAAGGGGAAGGGTGGAG	CAGACCCACACGTCAAAGAAC	60
	2	CCAATTTGGTAGTGAGTTTGCA	TAATTTGGGGTCCTGCTTCA	60
	3	TGAGCTGCATCCTAAATGAAAC	TGAGCTGCATCCTAAATGAAAC	57
	4	GCTTTGTCTACCAGGGCAAATC	GGCAGCCATCTTCACTCATC	60
	5	AAACCAGGGTTCCCGATTA	TGGATTAGTGTTGTCAGGAGGTT	60
	6	CCAGTATGGCCGTTATTAAAGTA	CTCCCTCCCACTCCAAAAA	60
	7	AAGAGAGCCTTGGAAGCATGTG	ACCATTGTACCTGGCTCCAAA	60
	8	TCAGGGAATGTGCTTGGAAT	GCAGTTTTTATTCAGCAGAGAA	60

Table 2

Patients' clinical characteristics at the time of the heart sampling, *LMNA* mutation status and nuclear envelope defect status

Patient	*LMNA* mutation	Family history	Nuclear envelope defect	Sex/age at onset (years)	NYHA class	Echocardiography	Weight (kg)/ height (cm)/ LVEDD (%)	Dysrhythmias	Clinical status
Major nuclear envelope defects and *LMNA* mutation									
1	Deletion exons 3–12	Yes	Irregular and broken nuclear envelope; accumulation of mitochondria within and around the nuclei	F/39	II	LVEDD 54 mm, LVEF 50%	57/164/120.0	nsVT, couplets, frequent VE, ICD	Mild progressive HF No MD
2*	p.D192G (c.575A > G)	Yes	Complete loss of nuclear envelope, accumulation of mitochondria, glycogen and/ or lipofuscin in the nucleoplasm, chromatin disorganization	M/26	IV	LVEDD 60 mm, LVEF 20%	67/175/127.2	1 AVB, LAFB	Died at 27 while awaiting for a heart transplant No MD
Major nuclear envelope defects and no *LMNA* mutation									
3	No	Yes	Blebbing of nuclear envelope, separation of inner and outer nuclear membrane	M/63	III	LVEDD 64 mm, LVEF 26%	71/180/136.5	SVT, VT	Died of progressive HF, 4 years after diagnosis No MD
4	No	Yes	Extrusion of nucleoplasm from the cardiomyocyte nucleus into the cytoplasm	M/21	III	LVEDD 75 mm, LVEF 25%	80/176/153.7	nsVT	HTx at 24 No MD
5	No	No	Irregular nucleus border; lack of nuclear membrane, presence of mitochondria within nuclear matrix	M/29	III	LVEDD 59 mm, LVEF 30%	84/176/120.5	RBBB, LPFB, nsVT, Pacemaker at 32	Progressive HF, HTx at 40 No MD
6	No	No	Local disruption of nuclear envelope, chromatin disorganization	F/14	IV	LVEDD 64 mm, LVEF < 10%	55/152/144.7	Recurrent VT/ VF	HTx at 14 No MD
7	No	No	Misshapen nuclei, local disruption of nuclear envelope, penetration of mitochondria into nucleus	M/14	III	LVEDD 71 mm, LVEF 20%	57/168/155.6	nsVT, couplets, frequent VE, ICD at 20	Progressive HF No MD
8	No	No	Irregular shape of nuclei	M/18	III	LVEDD 68 mm, LVEF 20%	47/160/156.0	LBBB, nsVT	Progressive HF No MD
Non-specific nuclear envelope defects and *LMNA* mutation									
9*	p.R541S (c. 1621C >A)	Yes	No	M/12	IV	LVEDD 64 mm, LVEF 25%	49/152/147.3	NA	HTx at 13 No MD
10	p.Q353K (c.1057C > A)	Yes	No	M/38	IV	LVEDD 62 mm, LVEF 22%,	59/163/138.1	ICD	HTx at 38

Patient	*LMNA* mutation	Family history	Nuclear envelope defect	Sex/age at onset (years)	NYHA class	Echocardiography	Weight (kg)/ height (cm)/ LVEDD (%)	Dysrhythmias	Clinical status
									Skeletal myopathy
Non-specific nuclear envelope defects and no *LMNA* mutation									
11	No	Yes	No	M/27	II	LVEDD 60 mm, LVEF 38%	100/186/117.2	Permanent AF, single VE	Improved after AF ablation, LVEF 45% No MD
12	No	Yes	No	M/26	III	LVEDD 75 mm, LVEF 20%	73/183/154.8	Single VE	Stable HF No MD
13	No	Yes	No	M/40	NA	LVEDD 77 mm, LVEF 19%	73/168/164.3	ICD, pacemaker	Died at 59, poor candidate denied for HTx No MD
14	No	Yes	No	F/14	IV	LVEDD 58 mm, LVEF 14%	37/155/139.4	No	HTx at 14 No MD
15	No	Yes	No	M/36	II	LVEDD 78 mm, LVEF 25%	94/185/154.9	Single Vex	Stable HF No MD
16	No	Yes	No	M/27	III	LVEDD 78 mm, LVEF 10%	72/186/160.7	Sinus tachycardia	HTx within several months No MD
17	No	Yes	No	M/34	IV	LVEDD 78 mm, LVEF 20%	73/178/163.1	I-degree AVB	Fulminant HF leading to HTx within several months No MD
18	No	Yes	No	M/35	III	LVEDD 58 mm, LVEF 15%	80/170/121.1	Frequent sVT	Atrial septal aneurysm; HTx at 39 No MD CPK value up to 600 U/I
19	No	No	No	F/13	NA	LVEDD 69 mm, LVEF 10%	41/164/160.3	NA	HTx at 13 No MD
20	No	No	No	F/61	NA	LVEDD 69 mm, LVEF 12%	88/165/145.3	ICD	Pericarditis, HTx at 62 No MD
21	No	No	No	M/54	III	LVEDD 88 mm, LVEF 20%	109/175/175.1	Pacemaker	Died at 60, waiting for HTx No MD
22	No	No	No	M/40	III–IV	LVEDD 68 mm, LVEF 27%	71/160/147.8	No	HTx at 62 No MD
23	No	No	No	M/27	NA	LVEDD 96 mm, LVEF 13%	70/170/203.8	ICD	HTx at 57 No MD

Patient	*LMNA* mutation	Family history	Nuclear envelope defect	Sex/age at onset (years)	NYHA class	Echocardiography	Weight (kg)/ height (cm)/ LVEDD (%)	Dysrhythmias	Clinical status
24	No	No	No	F/12	NA	LVEF 13%	42/153/NA	NA	HTx at 12 No MD
25	No	No	No	M/20	NA	LVEF 13%	86.5/197/NA	NA	HTx at 22 No MD

Clinical characteristics and mutation where previously described for patients 2 and 9 [7, 40]

AF atrial fibrillation, *AVB* atrioventricular block, *HF* heart failure, *HTx* heart transplantation, *ICD* implantable cardioverter defibrillator, *LAFB* left anterior fascicular block, *LPFB* left posterior fascicular block, *LVEDD* left ventricle end diastolic diameter, *LVEF* left ventricle ejection fraction, *MD* muscular disease, *NA* not available, *RBBB* right bundle branch block, *VEX* ventricular extrasystole, *VE* ventricular ectopy, *VT* ventricular tachycardia, *nsVT* nonsustained ventricular tachycardia, *SVT* supraventricular tachycardia, *VT/VF* ventricular tachycardia/ventricular fibrillation