Autosomal Dominant Dilated Cardiomyopathy With Atrioventricular Block: A Lamin A/C Defect-Related Disease

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OBJECTIVES
We investigated the prevalence of lamin A/C (LMNA) gene defects in familial and sporadic dilated cardiomyopathies (DCM) associated with atrioventricular block (AVB) or increased serum creatine-phosphokinase (sCPK), and the corresponding changes in myocardial and protein expression.

BACKGROUND
It has been reported that familial DCM, associated with conduction disturbances or variable myopathies, is causally linked to LMNA gene defects.

METHODS
The LMNA gene and myocardial ultrastructural and immunochemical changes were analyzed in 73 cases of DCM (49 pure, 15 with AVB [seven familial, eight sporadic], 9 with increased sCPK), four cases of familial AVB and 19 non-DCM heart diseases. The normal controls included eight heart donor biopsies for tissue studies and 107 subjects for LMNA gene studies.

RESULTS
Five novel LMNA mutations (K97E, E111X, R190W, E317K, four base pair insertion at 1,713 cDNA) were identified in five cases of familial autosomal dominant DCM with AVB (5/15: 33%). The LMNA expression of the myocyte nuclei was reduced or absent. Western blot protein analyses of three hearts with different mutations showed an additional 30-kDa band, suggesting a degrading effect of mutated on wild-type protein. Focal disruptions, bleb formation and nuclear pore clustering were documented by electron microscopy of the myocyte nuclear membranes. None of these changes and no mutations were found in the nine patients with DCM and increased sCPK or in the disease and normal controls.

CONCLUSIONS
The LMNA gene mutations account for 33% of the DCMs with AVB, all familial autosomal dominant. Increased sCPK in patients with DCM without AVB is not a useful predictor of LMNA mutation. (J Am Coll Cardiol 2002;39:981–90) © 2002 by the American College of Cardiology Foundation

Lamins A and C are major constituents of the nuclear lamina, the proteinaceous meshwork underlying the inner nuclear membrane (1). Defects in the lamin A/C (LMNA) gene (2) have been causally linked to four different diseases with 42 reported mutations (3–18) (Fig. 1):

- dilated cardiomyopathy (DCM) with conduction system disease (3,4) or variable myopathy (7),
- limb girdle muscular dystrophy (LGMD) (8),
- autosomal dominant variant of Emery-Dreifuss muscular dystrophy (EDMD) (8–13),
- autosomal dominant partial lipodystrophy (15–17).

Together with emerin defects (19), lamin A/C defects constitute the first entries in the new field of nuclear laminopathies in which, with the exception of two familial DCMs (5,6) and partial lipodystrophy (15–17), atrioventricular conduction disease is a major cardiac marker (3,4).

On the basis of the cardiac phenotypes reported as being associated with LMNA defects, we hypothesized that atrioventricular disturbances or increased serum creatine-phosphokinase (sCPK) potentially related to subclinical myopathy may be a useful guide for LMNA analysis in patients with DCM.

The aims of this study were: 1) to assess the prevalence of LMNA gene defects in patients with DCM, associated or not with atrioventricular block (AVB) or increased sCPK, and patients with familial AVB without DCM; and 2) to characterize the pathological changes and protein expression in the myocardium of patients carrying LMNA defects.

METHODS

Clinical and control series. Our Institutional Review Committee approved the study, and the patients and relatives gave their written informed consent.
The disease series consisted of:

- 15 patients with DCM and \( \geq \) first degree AVB: seven familial (five autosomal dominant and two sib pairs) and eight sporadic. Dilated cardiomyopathy was defined as familial when two or more members were found to be affected after clinical, electrocardiographic and echocardiographic evaluation of all of the informed and consenting relatives who accepted the Familial DCM Screening Program (20) or when clinical and pathological records were provided for deceased affected members.

- nine patients with DCM, no AVB, but persistent \( \text{sCPK} \geq 190 \text{ mU/ml} \) (two or more resting determinations) unrelated to dystrophin, emerin or dystrophin-associated glycoprotein defects and without clinically overt myopathy. Increased \( \text{sCPK} \) levels were considered a possible marker of “subclinical” myopathy.

The disease control series included:

- 49 patients with familial (\( n = 25 \), eight with left bundle branch block [LBBB]) or sporadic (\( n = 24 \), eight with LBBB) DCM without AVB or increased \( \text{sCPK} \);

- four patients with familial AVB, without DCM in the probands or relatives;

- nine patients with valvular heart disease and 10 with ischemic heart disease, four with and 15 without AVB.

The normal control series included:

- 107 blood donors with normal electrocardiograms and \( \text{sCPK} \) (for LMNA gene screening);

- eight endomyocardial biopsies (EMB) from normal donor hearts before transplantation (for immunohistochemical and Western blot LMNA analysis).

Dilated cardiomyopathy was diagnosed on the basis of World Health Organization criteria (21). The informed and consenting relatives of all of the DCM patients underwent a clinical examination, 12-lead electrocardiography, echocardiography and \( \text{sCPK} \) determination (20). The neuromuscular assessment of probands and relatives was based on a detailed clinical history, physical examination and \( \text{sCPK} \).
and serum lactate determinations. Clinical and pathological records were obtained for deceased affected relatives.

A Fisher exact test was used to compare the proportion of the mutations among phenotype groups and to assess the statistical significance of the results.

**Mutation screening.** The 12 exons of the LMNA gene were amplified from peripheral blood-derived genomic DNA by means of polymerase chain reaction, using primers derived from intronic sequences (14). The polymerase chain reaction fragments were analyzed by means of denaturing high-performance liquid chromatography (DHPLC) (Transgenomic, San Jose, California) using the Wave DNA Fragment Analysis System (22). The fragments with a heteroduplex conformation were purified (QIAquick Kit, Qiagen, Santa Clarita, California) and then sequenced using a BigDye-terminator cycle sequencing system (ABI PRISM 377, Applied Biosystem, Foster City, California).

**Endomyocardial biopsy.** Endomyocardial biopsy was performed in all patients according to the standard procedure (23), with three to four samples being assigned to light microscopy and routine immunostaining for inflammatory cells, dystrophin and the dystrophin-associated glycoprotein complex, emerin, LMNA, actin and desmin (24,25). Two samples were processed for ultrastructural study and analyzed using a Zeiss EM902 electron microscope.

**Light microscopy immunostaining for LMNA and emerin.** Formalin-fixed paraffin-embedded biopsy sections were reacted with antibodies against LMNA (Chemicon International, Inc., Temecula, California, mouse anti-human lamin A + C monoclonal Ab, 1:50) and emerin (Novocastra, Newcastle, United Kingdom, mouse anti-emerin monoclonal Ab, 1:100) using the avidin-biotin complex-peroxidase method, diaminobenzidine tetrahydrochloride as chromogen and 4,6 diamidino-2-phenylindole for nuclear staining (23,25). The anti-lamin antibody binds to an epitope between amino acids 464 and 572, which is shared by both lamin A and C. The positive controls included EMBs from normal heart donors and patients with DCM known to be free of LMNA defects, and heart samples from transplanted patients with valvular and ischemic disease. The negative controls consisted of sections incubated without the primary antibody. All of the reactions were performed using control procedures.

**Western blot.** One-dimensional electrophoresis was performed on proteins extracted from three hearts excised at transplantation (patients A-II-1, A-II-2 and B-III-1) (25). The protein-loading concentration was determined using the bicinchoninic acid protein assay kit (Sigma Aldrich, St. Louis, Missouri) (26), and 25 μg of each sample was run on 10% polyacrylamide gel (SDS PAGE). After electrophoresis, the gels were transferred to nitrocellulose sheets that were immunostained, first with anti-lamin A/C monoclonal primary antibody and then with a secondary antibody conjugated with alkaline phosphatase (25).

**RESULTS**

**Genetic studies.** A unique sequence variant was identified in five unrelated probands with familial autosomal dominant DCM and AVB (5/15: prevalence 33%) (Table 1, Fig. 2). No sequence variants were found in the two sib-pairs or the eight cases of sporadic DCM with AVB, in the nine cases of DCM with increased sCPK (mean: 521.5 ± 254.27 μU/ml; range: 218 to 994) or any of the disease or normal controls (p < 0.001). Each variant was independently confirmed by means of automated sequencing, restriction-enzyme digestion for Lys97Glu (-Sty I) and Glu111Stop (-Mnl I), and DHPLC. None of these five variants was found in 107 normal subjects, thus excluding the possibility that they are common polymorphisms. All five variants affect amino acids shared by lamin A and C: Lys97Glu and Glu111Stop are encoded in exon 1 (col Ib, rod domain), Arg190Trp in exon 3 (col Ib, rod domain), Glu317Lys in exon 6 (col 2, rod domain); the ctgc insertion at 2869cDNA in exon 9 (globular tail) predicts a protein of 525 normal followed by 34 new amino acids. Glu111Stop predicts a truncated soluble protein of 110 amino-acids, with a molecular weight of 12 KD and an average hydrophobicity of −0.756364.

The five sequence variants were considered to be disease-causing mutations because:

- They were found in affected family members but not in unaffected relatives more than 40 years of age. Given the late penetrance of the defects (phenotype onset ranging from 27 to 44 years), the age of 40 was considered a reasonable upper limit for the expected expression of the mutation.
- They changed the charges (n = 3) and predicted major rearrangements (n = 2) of the mutated protein.
- They all affected highly conserved residues.

**Clinical data.** The clinical data, disease evolution and follow-up of the probands and relatives are shown in Table 1. Of the 33 family members with available clinical data, 16 were affected by DCM with AVB, three had AVB (n = 2) or left ventricular dilatation (n = 1), and 14 were unaffected. DNA was available for 25 subjects (23 living, two dead) but not for six dead, one living patient and for one healthy family member. Eighteen of the 25 tested subjects are heterozygous for LMNA mutations, including nine affected by DCM with AVB, one with asymptomatic left ventricular dilatation and two with an isolated AVB (one requiring pacemaker implantation). Of the remaining six heterozygous subjects, three children age 7, 12 and 13 years are free from signs and symptoms, and three young adults age 25, 29 and 39 years have premature ventricular beats.

The natural history of the disease in the five families with mutations was assessed on the basis of the clinical monitoring program for familial cardiomyopathies. Of the 18 living family members tested, seven have been followed up for 33.71 ± 8.77 months (Table 1). In the probands, the onset
of the disease occurred in early middle age (mean: 37 years; range: 27 to 44). The AVB was documented as preceding the DCM in 3/5 probands and their relatives and diagnosed at the same time as the DCM in two. Of the eight living patients, four have undergone, and one is waiting for, a heart transplantation; two are stable in New York Heart Association (NYHA) class II and one in NHYA class III.

The clinical evolution in the five families was different (Table 1). The living patients of family B had rapidly evolving disease; one underwent transplantation within three years of onset, and one is waiting for transplantation three months after onset. In the patients of families A, C and E, there was a long interval between the onset of AVB and DCM. Sudden death occurred in only one patient of family E (but at the age of 70 years), and life-threatening ventricular arrhythmias were recorded only in the proband

<table>
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<tr>
<th>Patient</th>
<th>Mutation</th>
<th>Onset, Evolution, Follow-Up (age)</th>
<th>sCPK (mU/ml)</th>
<th>Electrocardiogram</th>
<th>PQ (m/s)</th>
<th>LVEDD/ESD (mm)</th>
<th>LVFS (%)</th>
<th>LVEDV/ESV (ml)</th>
<th>LVEF (%)</th>
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<td>E111X</td>
<td>Onset: 43 y</td>
<td>Nt Sinus bradycardia</td>
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<td>III° AVB→PM</td>
<td>73/61</td>
<td>16</td>
<td>20</td>
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<td></td>
<td></td>
<td>55 y 70</td>
<td>PM</td>
<td>56 y 68</td>
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<td>5</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td>53 y 51</td>
<td>PM</td>
<td></td>
<td>74/67</td>
<td>9</td>
<td>315/240</td>
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<td>14</td>
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<td></td>
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<td></td>
<td>+ LBBB→PM</td>
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<td>51 y 132</td>
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<td></td>
<td></td>
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<td>PM</td>
<td></td>
<td>II° AVB + LBBB</td>
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<td>Increased</td>
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<td>160</td>
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<td>PM</td>
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<td>74</td>
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<td>Baseline: 34 y</td>
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<td>PM</td>
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<td>56/40</td>
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<td>Baseline: 31 y</td>
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<td>51/33</td>
<td>34</td>
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<td>34 y 207</td>
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<td>R190W</td>
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<td>180 I° AVB + RBBB + LAFB</td>
<td>42 y 185</td>
<td>= Atrial flutter</td>
<td>239</td>
<td>60/53</td>
<td>11</td>
<td>250/178</td>
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<td>42 y 185</td>
<td>= Atrial flutter</td>
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<td>33</td>
<td>82/25</td>
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<td>E317K</td>
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<td>180 I° AVB + RBBB + LAFB</td>
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<td>I° AVB + LBBB</td>
<td>331/182</td>
<td>40</td>
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<td>E-II-2</td>
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<td>Onset: 44 y</td>
<td>64 y 90</td>
<td></td>
<td>I° AVB</td>
<td></td>
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<td>—</td>
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<td>170</td>
<td>43/31</td>
<td>36</td>
<td>110/40</td>
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*LVEDD > 112% of the predicted value corrected for age and body surface area; †from autopsy specimens.

AVB = atrioventricular block; Ins: ctgc, 2,869 = insertion of ctgc at position 2,869; LAFB = left anterior fascicular block; LBBB = left bundle branch block; LVEDD/ESD = left ventricular end-diastolic/end-systolic diameter; LVEDV/ESV = left ventricular end-diastolic and end-systolic volume; LVEF = left ventricular ejection fraction; LVFS = fractional shortening; NA = not available; Normal = the cardiological report; Nt = not tested; PAC = premature atrial complexes; PM = pacemaker; PVC = premature ventricular complexes; RBBB = right bundle branch block; sCPK = serum creatine-phosphokinase; y = year.
Figure 2. 1) Family A: mutation E111X; 2) Family B: mutation K97E; 3) Family C: CTGC insertion at 2,869 of the cDNA; 4) Family D: mutation R190W; 5) Family E: mutation E317K. Filled symbols = patients; open symbols = non-affected subjects; question marks within symbols = non-proven affected subjects. Small squares within symbols = atrioventricular block (upper left), left ventricular dilation (upper right). Diagonal lines = death. The probands are indicated by arrows. The presence (+) or absence (−) of the mutation is indicated for the genetically tested family members. Chromatograms below each pedigree demonstrate the heterozygous mutations.
of family C. In family D, the proband developed the disease at the age of 41 years, and his affected mother died at age 47.

The neuromuscular assessment excluded myopathy in all of the probands and affected relatives, who had normal serum lactate levels (11.70 ± 3.19; range: 19.50 to 7.20). The levels of sCPK were minimally high (245, 341, 260 and 213 mU/ml, respectively) in one proband and three non-penetrant carriers.

Pathological studies. Light microscopy of the EMBs revealed aspecific myocyte damage and interstitial fibrosis. The ultrastructural study showed “delamination,” focal ruptures, blebs and nuclear pore clustering of the myocyte nuclear membranes (Fig. 3). These defects were not observed in the EMBs of the non-mutated DCM patients.

Immunostaining with anti-LMNA antibodies showed normal staining of interstitial and vascular cell nuclei (within sample control) and the loss of staining in a number of myocyte nuclei (Fig. 4). Anti-emerin antibodies regularly immunostained nuclear membranes but no cytoplasmic compartments.

The atrioventricular junction of the three hearts excised at transplantation showed fibrosis, fatty infiltration and degenerative myocyte changes (Fig. 5). Western blot analyses of the proteins extracted from the hearts of patients carrying the E97K and E111X mutations showed reduced protein content (particularly lamin C) and an additional 30 kDa band specifically immunostained by the antibody (Fig. 6, lanes 2, 3 and 5), which was absent in the normal and disease controls (Fig. 6, lanes 1 and 4).

**DISCUSSION**

We documented a high prevalence of LMNA gene defects in our DCM patients with AVB (5/15: 33%), all of whom had familial autosomal dominant diseases. The corresponding immunopathological phenotype was characterized by defective LMNA immunostaining, an abnormal 30 kDa LMNA-positive band and disruption of the nuclear membrane of cardiac myocytes. Our hypothesis that increased
sCPK might be a marker of LMNA defects in DCM patients without AVB was not confirmed.

**Pathological and immunochemical findings.** To the best of our knowledge, this is the first description of ultrastructural nuclear membrane damage associated with LMNA gene defects in DCM patients with AVB. Similar nuclear envelope disruptions, discontinuities of the inner face of the nuclear envelope, and nuclear pore clustering have been observed in patients with X-linked EDMD (27), lamin A null mice (28) and the Drosophila lamin Dm0 mutant (29). According to Hutchison et al. (30), the nuclear membrane damage and lamina fragility could develop into physical cell disruption leading to myocyte death and tissue damage. In skeletal muscle, it is likely that only a large number of altered nuclei can compromise individual multinucleated fibers, whereas in cardiac muscle, the loss of individual mono-nucleated myocytes in adults is cumulative and eventually leads to AVB and heart failure when the number of affected myocytes is sufficient to cause the phenotypes (30).

The extension of myocyte damage in nodal versus left ventricular myocardium could partly explain the earlier occurrence of AVB than left ventricular dysfunction observed in three of our five probands. The defective LMNA immunostaining of myocyte nuclear membranes associated with LMNA mutations contrasts with the normal immunostaining of the nuclei of interstitial and vascular cells. This different pattern could be related to stage-and tissue-specific LMNA expression and fits with the most intriguing and unresolved issue concerning patients with LMNA defects, that is, the selective involvement of muscular, cardiac and fat cells (30). Furthermore, the low molecular weight fragment (about 30 kDa) found only in the patients carrying mutated (K97E) or truncated (E111X) versions of the protein suggests that abnormal LMNAs may induce degradation of the normal allele product. This mechanism may contribute to the dominant negative effect of the mutated on the wild-type LMNA. Dominant negative proteins have been shown capable, in vitro, of disrupting preformed LMNA-types (31). The absence of lamin immunolabeling in cardiac myocytes and the presence of a low molecular weight fragment suggest that mutated LMNAs increase degradation of the wild-type proteins. Interestingly, cells that are not terminally differentiated and are actively cycling, such as fibroblasts, endothelial and smooth muscle cells, still contain enough LMNAs to be detected by immunostaining. A-type-lamin protein levels increase with the terminal differentiation and growth arrest, while the corresponding mRNA levels significantly decrease, suggest-

**Figure 4.** Lamin A/C immunostain (a, c, e) and corresponding DAPI nuclear stain (b, d, f). (a, b) Normal heart sample. (c, d) Control dilated cardiomyopathy without (LMNA) mutation; all myocyte and interstitial-vascular nuclei are immunostained by the anti-LMNA antibody. (e, f) Endomyocardial biopsy from patient A-II-1; note the absence of LMNA immunostain in some myocyte nuclei and positive interstitial-vascular cell nuclei (Avidin-Biotin Complex, DAPI nuclear stain; a and b, 40×; c to f, 110×).
ing a role for transcriptional and post-transcriptional mechanisms in controlling LMNA levels (32). If the mutated LMNA induces an increased degradation of the wild-type protein, terminally differentiated cells such as cardiac myocytes will be progressively depleted of type-A-lamins. Furthermore, the low LMNA mRNA levels present in those cells will not be able to sustain a proportional increase in translation of LMNA mRNA to compensate for the increased degradation rate. On the contrary, in cycling cells, the higher mRNA levels (32) will partially compensate the increased degradation of the protein induced by the mutant.

**Genotype-phenotype correlation.** The genotype-phenotype correlation seems to be quite complex: neither the involvement of given protein domains nor the type of mutation characteristically recurs in DCM with conduction disease. One of our five mutations (E111X in coil 1b) predicts a very small (12 kDa) truncated soluble peptide. Two of the other four mutations (K97E and R190W) are in coil 1b, which is part of the region necessary for lamin B-LMNA interactions and tetramer formation (27). One (E317K) is in a segment of coil 2 that does not interact with proteins or chromatin, and one (insertion ctgc 2869 cDNA) is in a tail domain of the protein essential for LMNA-emerin interactions (30). The observed differences in clinical manifestations and disease evolution in our families (the mildest phenotype is in family E with the E317K LMNA mutation) suggest that the

**Figure 5.** Atrioventricular junction of patients A-II-1 and B-III-1: fibrosclerosis and fibrofatty degeneration (Movat Pentachrome stain, 10×).

**Figure 6.** Western blot showing lamin A and lamin C bands and the additional 30 kDa immunoreacting band corresponding to an abnormal small peptide comprising the epitope recognized by the antibody. This band is identical in patients with different lamin A/C (LMNA) mutations (lane 2: A-II-1; lane 3: B-III-1; lane 5: A-II-2) but absent in normal controls (lane 1) and patients with dilated cardiomyopathy unrelated to LMNA defects (lane 4).
impaired nuclear lamina interactions may play a role in the phenotype.

The hypothesis of a complex genotype/phenotype correlation is strengthened by the informative family #1 described by Bonne et al. (14), in which the same mutation co-segregated with both DCM and EDMD, and by the family described by Brodsky et al. with coexistent LGMD, EDMD and DCM (7). In our series, none of the nine DCM patients with increased sCPK, but without AVB, had LMNA gene defects; the B-II-1 proband, and subjects C-II-1, C-II-2 and D-III-4, who had slightly raised sCPK levels, had no contractures and did not show any clinical signs of myopathy. The size of the pedigrees does not explain these inconsistencies. For example, of the four sons of EDMD patient III-11 reported by Bonne et al. (14), two had EDMD (IV-13 and 14), and two had DCM (IV-15 and 16). It is therefore likely that factors other than LMNA mutations influence the phenotype, such as tissue-specific or systemic modifier gene expression, and the late and variable penetrance of the defects.

Role of AVB in molecular diagnosis. In the cardiological setting, the AVB associated with DCM is a reliable marker for LMNA gene molecular screening (3,4). In our series, none of the DCM patients without AVB had any LMNA defects, and none of their EMBS showed the ultrastructural and immunostaining defects observed in the five patients with LMNA gene mutations. The absence of LMNA mutations in our four families with isolated AVB does not exclude the indication for LMNA screening, as AVB preceded DCM diagnosis in three of our five families (Families A, C and E).

Genetic counseling. A major issue in genetic counseling is the risk of sudden death in carriers of LMNA mutation (3,4,33). In our series, sudden death was recorded in only one affected family member of the oldest patient (Family E), but life-threatening arrhythmias were recorded in Patient C-I-2. The currently available data do not clarify whether mutated subjects with normal electrocardiograms and ventricular function risk sudden death. This information would be essential for counseling and, potentially, for preventing fatal events.

In summary, LMNA gene mutations accounted for 33% of our cases of DCM with AVB, all of which were familial autosomal dominant DCMs. Increased sCPK levels in DCM patients without AVB were not useful in predicting LMNA mutations. The cardiac immunopathological phenotype was characterized by abnormal/defective nuclear LMNA expression and nuclear membrane disruption.

Nucleotide numbers: cDNA sequence GeneBank Accession No #M13451 (lamin C mRNA), L12399, Version GI:12399.3-6960369 (LMNA DNA exon 1), L12400 (LMNA DNA exon 2), L12401 (DNA exons 3 to 12), M13452 (lamin A, mRNA). Neutral polymorphisms found in our overall series: T995C, T1472C, C1832T (known) and C185T, G1314A (novel).

Acknowledgments
The authors are indebted to the family members for their participation and collaboration and would like to thank Dr. Claudia Specchia, Department of Health Sciences, University of Genova, Italy, for statistical analysis.

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