Morphological Analysis of 13 LMNA Variants Identified in a Cohort of 324 Unrelated Patients With Idiopathic or Familial Dilated Cardiomyopathy

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Background—Mutations in the LMNA gene, encoding lamins A/C, represent a significant cause of dilated cardiomyopathy. We recently identified 18 protein-altering LMNA variants in a cohort of 324 unrelated patients with dilated cardiomyopathy. However, at least one family member with dilated cardiomyopathy in each of 6 pedigrees lacked the LMNA mutation (nonsegregation), whereas small sizes of 5 additional families precluded definitive determinations of segregation, raising questions regarding contributions by those variants to disease.

Methods and Results—We have consequently expressed, in COS7 cells, GFP-prelamin A (GFPLaA) fusion constructs incorporating the 6 variants in pedigrees with nonsegregation (R101P, A318T, R388H, R399C, S437Hfsx1, and R654X), the 4 variants in pedigrees with unknown segregation (R89L, R166P [in 2 families], I210S, R471H), and 3 additional missense variants (R190Q, E203K, and L215P) that segregated with disease. Confocal immunofluorescence microscopy was used to characterize GFP-lamin A localization and nuclear morphology. Abnormal phenotypes were observed for 10 of 13 (77%) variants (R89L, R101P, R166P, R190Q, E203K, I210S, L215P, R388H, S437Hfsx1, and R654X), including 4 of 6 showing nonsegregation and 3 of 4 with uncertain segregation. All 7 variants affecting coil 1B and the lamin A-only mutation, R654X, exhibited membrane-bound GFP-lamin A aggregates and nuclear shape abnormalities. Unexpectedly, R388H largely restricted GFP-lamin A to the cytoplasm. Equally unexpected were unique streaked aggregates with S437Hfsx1 and giant aggregates with both S437Hfsx1 and R654X.

Conclusions—This work expands the recognized spectrum of lamin A localization abnormalities in dilated cardiomyopathy. It also provides evidence supporting pathogenicity of 10 of 13 tested LMNA variants, including some with uncertain or nonsegregation. (Circ Cardiovasc Genet. 2010;3:6-14.)

Key Words: dilated cardiomyopathy ■ genetics ■ lamin A/C

The lamins are a family of intermediate filament proteins, which localize to the inner nuclear envelope in which they perform a number of crucial functions involving nuclear architecture, gene expression, mitosis, DNA replication, apoptosis, and signaling (reviewed in references 1 and 2). The major A-type lamins, lamins A and C, are produced through differential splicing of the 12 exon LMNA gene (1q21.2-q21.3). Because the alternative splice site is located in exon 10, lamin A and C transcripts encode proteins that differ along only a short C-terminal segment. The N-termini represent a series of α-helical coiled-coil domains necessary for lamin polymerization.3 The C-terminal tail domain, in contrast, adopts an immunoglobulin (Ig)-like structure and house-binding sites for DNA, chromatin, and other lamin-associated polypeptides.4 Together, these domains permit the array of interactions necessary for maintaining integrity of the lamina.

Clinical Perspective on p 14

Production of a mature lamin A polypeptide (664aa) necessitates a series of posttranslational modifications targeted to the prelamin A C-terminal CaaX motif, which is lacking in the truncated lamin C (572aa). Although the precise functions of prelamin A processing remain unknown, the sequential modifications may facilitate interactions with other lamin A proteins or with the nuclear membrane.5 LMNA mutations have been implicated in at least 8 distinct clinical phenotypes (laminopathies). Although recognized as unique entities, reports of patients6 or families7 exhibiting features of >1 disease and of individual mutations resulting in multiple laminopathies8,9 suggest that these diseases may be better considered as a phenotypic spectrum. Forms of muscular dystrophy with or without cardiac involvement (autosomal dominant Emery Dreifuss muscular dystrophy, limb-girdle muscular dystrophy type 1B), diseases of adipose
tissue and fat deposition (familial partial lipodystrophy—
Dunnigan type, mandibuloacral dysplasia), restrictive der-
mopathy, Charcot-Marie-Tooth disease type 2, and premature
aging syndromes, such as Hutchinson-Gilford progeria syn-
drome and atypical Werner syndrome, are all part of the
spectrum. LMNA mutations additionally represent the most
frequent known genetic cause of dilated cardiomyopathy
(DCM), occurring with a prevalence of ≈5% to 10% (famil-
ial) and 2% to 5% (sporadic).10 Missense mutations predom-
inate; however, rare deletions, insertions, frameshifts, and
nonsense mutations have all been reported.

Clinically, LMNA-related DCM is characterized by much
inter- and intrafamilial variability in onset and severity but
typically manifests as left ventricular enlargement and re-
type systolic function preceded by significant conduction
system disease (CSD), particularly atrioventricular block and
supraventricular arrhythmias. Sudden cardiac death is also
common and can represent the initial sign of disease (see
references 11 and 12 for review).

We recently identified 18 unique protein-altering LMNA
variants in 19 probands from a cohort of 324 unrelated
patients with idiopathic (nonischemic DCM of unknown
cause) or familial DCM.10 Identified variants showed usual
patterns of age-dependent segregation with disease in many
of the larger families (segregation pedigrees), supporting the
pathogenicity of these mutations. However, the small sizes of
other families precluded definitive assessments of segrega-
tion (unknown segregation pedigrees). We additionally
observed that in 6 of the 19 families (32%), at least 1 family
member with clinically evident DCM lacked the putatively
causative LMNA variant (nonsegregation pedigrees), raising
questions regarding the contribution of these variants to
disease.10 A large number of studies have demonstrated
abnormalities in nuclear morphology and lamin A/C local-
ization in cells expressing LMNA variants.13–21 Therefore, to
better determine their pathogenic potential, we generated
GFP-pref lamin A fusion constructs corresponding to 13 of the
18 identified LMNA variants (including variants lacking
definitive segregation data) and constitutively expressed each
in COS7 cells. These studies complement available molecular,
family, and/or clinical data10,22,23 by providing evidence
supporting pathogenicity for 10 of the 13 analyzed LMNA
variants.

Methods

Plasmid Construction

Full-length human prelamin A (664 amino acids) was generated from
HEK293 total RNA extract using the Transcriptor High Fidelity
cDNA Synthesis Kit (Roche Applied Science, Indianapolis, Ind.) and
was cloned into the pAcGFP1-C1 fluorescent protein expression
vector (Clontech, Mountain View, Calif.) using manufacturer’s
protocols for the In-Fusion 2.0 Dry-down Kit (Clontech). The
In-Fusion Primer Design Tool (http://bioinfo.clontech.com/infusion/
convertPerPrimerInit.do) was used to design sense (5'-GGACT-
CAGATCTCGACTGCCTGGCATGGAGAC-3') and antisense (5'-
GATCCCCTGCGTAGCCGTCTGGAGGAT-3') primers. Vector linearization was accomplished using Kpn1 and Xho1. The
cloning reaction was performed with a vector:insert molar ratio of
1:2, and the resulting wild-type GFP-pref lamin A fusion construct
(GFPLA-A-WT) was transformed into One Shot TOP10 Escherichia
coli (Invitrogen, Carlsbad, Calif.). Construct fidelity was confirmed
first by restriction analysis with BglII and BamHI and subsequently
by dye-terminator sequencing using the ABI 3100 Automated
Capillary DNA Sequencer (Applied Biosystems, Foster City, Calif.).

Mutagenesis

Thirteen mutant constructs were generated from GFPLA-A-WT fol-
lowing manufacturer’s protocols for the QuikChange II XL Site-
Directed Mutagenesis Kit (Stratagene, La Jolla, Calif.). Mutations
and their flanking sequences were confirmed as described earlier.
Constructs generated included the 6 variants in pedigrees with
nonsegregation (R101P, A318T, R388H, S437Hfsx1, and
R654X) and an additional 7 variants (R89L, R166P, R190Q, G203K,
I210S, L215P, and R471H) representing all remaining missense
variants in the cohort. To focus research efforts analyses were restricted
to these 13 variants because the remaining 5 variants were considered
likely to cause significant disruption of the LMNA gene (splice site
variant [35->1G>T] caused the loss of exon 2).10 Two nonsense
mutations [R225X and Q234X] were a frameshift mutation
[R225X and Q234X], 1 was a frameshift mutation
[35->1G>T] caused the loss of exon 2) 10 were nonsense
mutations [R225X and Q234X] and 1 was an insertion [G474D175insE] mutation,
and clinical data were consistent with their pathogenicity.10

Cell Culture, Transfection, and Confocal

Immunofluorescence Microscopy

COS7 (African green monkey kidney) cells were cultured in Dul-
becco modified eagle’s medium+GlutaMAX (Gibco, Carlsbad,
Calif.) supplemented with 10% fetal bovine serum and 1% antibiotic/
antimycotic (Gibco) at 37°C in a 5% CO2, water-jacketed incubator.
The COS7 cell line was selected for its high transfectability and
demonstrated utility in similar lamin A/C morphological studies.21
Because WT lamin A overexpression has been shown to form
aggregates similar to those observed in some mutant samples,14,17
transfection conditions were optimized to minimize aggregation of
overexpressed GFP-lamin A in GFPLA-A-WT samples before assess-
ment of mutant constructs. Transfections were conducted using
Lipofectamine 2000 reagent (Invitrogen) scaled for culture in 35-mm
diameter, 10 mm-well microglass-bottom dishes (MatTek Corpora-
tion, Ashlord, Mass.). Twenty-four hours before transfection,
400 000 cells were seeded in growth medium lacking antibiotic and
microcyst and were cultured overnight to 80% to 90% confluence.
Plasmid DNA (0.5 µg) was complexed with lipofectamine in a ratio
of 1:2.5. Transfection proceeded for 24 hours at 37°C.

The same investigator (J.C.) completed all experiments. A second
investigator (D.L.) assigned unique identifiers before transfection,
blinding the first investigator to all sample identities. The first
investigator remained blinded until all images were acquired, qual-
ified, and analyzed.

Before image acquisition, overnight culture media was aspirated
and replaced with 1-mL phenol red-free and antibiotic/antimycotic-
enriched Dulbecco modified eagle’s medium and supplemented with
10 µL Hoechst 33258 (0.2 mmol/L) nuclear stain. Images were acquired
with a Zeiss LSM510/UV confocal microscope outfitted
with a C-Apochromat 63x/1.2 W Corr water immersion objective
lens and separate UV (351 nm) and GFP (488 nm) emission filters.
During acquisition, fluorescent nuclei were qualitatively classified
before image analysis.

Statistical Analyses

The number of aggregate-containing and abnormally shaped nuclei
for each variant LMNA construct were compared with corresponding
WT counts using 2-tailed χ² (all cell counts > 5) or Fisher exact tests
(any cell count ≤5). InStat 3 (Graphpad Software, Inc., La Jolla,
Calif.) statistical software (http://www.graphpad.com/) was used for
all analyses.

Results

Clinical Data

Clinical and pedigree data from clinically affected individuals
with confirmed or obligate LMNA mutations previously

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published by our group\textsuperscript{10,22,23} are summarized for each family (Table 1, Figure 1).

### Morphological Data

Full-length WT and mutant prelamin A cDNAs were transiently expressed as GFP fusion constructs in COS7 cells. Each construct was analyzed by confocal immunofluorescence microscopy to determine nuclear morphology and GFP-lamin A localization. The results are summarized (Tables 2 and 3), and representative images are provided (Figure 2).

Nearly all nuclei in cells transfected with GFPLaA-WT exhibited homogenous GFP-lamin A localization throughout the nuclear periphery (95%). Rarely, nuclei contained small, nuclear envelope-associated aggregates (5%). Transfection with the 13 mutant constructs resulted in a number of GFP-lamin A distribution patterns, were seen with the remaining constructs. Of these, rates of aggregation were lowest for GFPLaA-L215P (family I, 64%) and significantly higher (>95%) for GFPLaA-R89L, -R101P, -R166P, and -I210S (families A, B, D/E, and H, respectively). GFPLaA-R388H (family N), GFPLaA-S437Hfx1 (family P), and GFPLaA-R654X (family S) were most intriguing. GFPLaA-R388H was predominantly and markedly restricted to the cytoplasm, as a diffuse, low-fluorescence veil, or as highly saturated aggregates. Only rarely was nuclear localization observed, and in these few instances, GFP-lamin A was as likely to form small aggregates as to remain homogenously distributed. Equally unexpected was the finding of unique streaked aggregates (43%) in the nuclei of S437Hfx1 expressing cells, and the presence of giant aggregates in S437Hfx1 (50%) and R654X (5%) expressing nuclei. Smaller aggregates were also prominent.

Consistent with previous reports\textsuperscript{14–16,19,20,25,26} all coil 1B variants exhibited variable levels of aggregation. Compared with cells expressing GFPLaA-WT, significantly higher levels of mild to gross nuclear shape abnormalities, including nuclear envelope blebbing, were additionally observed. The extent and relative severity of these abnormalities are summarized in Table 3 and Figure 2.

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### Table 1. Clinical Characteristics of Clinically Affected LMNA Variant Carriers

<table>
<thead>
<tr>
<th>Individuals*</th>
<th>n</th>
<th>Family</th>
<th>Amino Acid</th>
<th>Nucleotide</th>
<th>Disease Onset</th>
<th>AV Block (n, %)</th>
<th>AFib (n, %)</th>
<th>CSD Other (n, %)</th>
<th>PC/M (n, %)</th>
<th>ICD (n, %)</th>
<th>DCM (n, %)</th>
<th>HF (n, %)</th>
<th>HrtTx (n, %)</th>
<th>CSD†</th>
<th>DCM‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.3</td>
<td>1</td>
<td>A</td>
<td>R89L</td>
<td>266G</td>
<td>31</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>+</td>
<td>+++ +++++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.8*</td>
<td>1</td>
<td>B</td>
<td>R101P</td>
<td>302G</td>
<td>36</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>+ +++++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D.3</td>
<td>1</td>
<td>D</td>
<td>R166P</td>
<td>497G</td>
<td>40</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>+ +++++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.3</td>
<td>1</td>
<td>E</td>
<td>R166P</td>
<td>497G</td>
<td>42</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>+ +++++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.6, F.7, F.8</td>
<td>3</td>
<td>F</td>
<td>R190Q</td>
<td>569G</td>
<td>A</td>
<td>26</td>
<td>45</td>
<td>1 (33)</td>
<td>0</td>
<td>2 (67)</td>
<td>0</td>
<td>1 (33)</td>
<td>1 (33)</td>
<td>1 (33)</td>
<td>+ +++++</td>
</tr>
<tr>
<td>G.3, G.5, G.8</td>
<td>11</td>
<td>G</td>
<td>E203K</td>
<td>607G</td>
<td>A</td>
<td>30</td>
<td>46</td>
<td>8 (73)</td>
<td>4 (36)</td>
<td>5 (45)</td>
<td>3 (27)</td>
<td>0</td>
<td>3 (27)</td>
<td>2 (18)</td>
<td>1 (9)</td>
</tr>
</tbody>
</table>

CDD, sudden cardiac death; HF, heart failure; AVB, atrioventricular block; AF, atrial fibrillation; IDC, idiopathic dilated cardiomyopathy; FDC, familial dilated cardiomyopathy; LVE, left ventricular enlargement; CSD, conduction system disease; DCM, pacemaker.

\*No. of clinically affected individuals. For the 6 families showing LMNA variant nonsegregation, individuals marked with a * represent LMNA mutation carriers who are also at risk for carrying a possible second causative genetic variant.

†+ + + indicates severe CSD characterized by ICD/PM placement or SCD; ++, any degree AVB, Aflut, or AFib; +, any other conduction system abnormality.

‡+ + + indicates severe IDC/FDC characterized by HF or transplantation; + +, IDC/FDC without HF or transplantation; +, LVE or systolic dysfunction not meeting criteria for IDC/FDC.\textsuperscript{24}
Figure 1. Partial pedigrees for families with LMNA variants. Numbering is consistent with tables and figures in this study and with past reports of these families (see references 10, 22, and 23 for clinical data). Probands are indicated with an arrow. Solid symbols represent idiopathic dilated cardiomyopathy with or without heart failure. Shaded symbols represent any other cardiovascular abnormality. Open symbols represent unaffected individuals. Mutation carrier status is shown by a (+) (presence), (−) (obligate), or − (absence). Absence of any symbol for mutation carrier status indicates lack of available DNA for analysis. Question marks (?) denote insufficient clinical data. A, Pedigrees with nonsegregation. B, Pedigrees with segregation or unknown segregation.
GFPLaA-R399C (family O) was uniquely notable for retraction of DNA from a clearly GFP-lamin A-demarcated nuclear lamina in rare transfected nuclei (Figure 2). Although occurring in only a few cells, this phenotype was observed across multiple transfections and was considered to be an abnormal finding. Nevertheless, the majority of nuclei expressing GFPLaA-R399C, as well as all nuclei expressing GFPLaA-A318T (family L) and GFPLaA-R471H (family Q), were indistinguishable from WT.

Abnormal nuclear phenotypes were ultimately observed for 10 of 13 (77%) LMNA constructs (Table 3), including 4 of 6 (67%) incorporating variants from pedigrees with nonsegregation and 3 of 4 (75%) incorporating variants from pedigrees with uncertain segregation, collectively supporting pathogenicity of these variants.

Morphological data for 3 LMNA variants (A318T, R399C, and R471H) were less revealing, with each showing phenotypes comparable with WT, despite various associated manifestations of cardiovascular disease in carrier families. Families L (L.3) and Q (Q.2) exhibited severe CSD and/or DCM (Table 1). Two at-risk individuals in family Q (Q.4 and Q.5) additionally showed DCM/CSD of extremely early onset, with Q.5 notably suffering sudden cardiac death at 18 years of age. Conversely, no symptoms were present in the mutation-positive mother of the proband in family O (O.9), whereas a maternal grandfather (O.3), who also carried the R399C mutation, exhibited only CSD. Paternally inherited DCM/CSD of unknown cause, however, was noteworthy, as was extremely aggressive, early onset DCM (requiring transplantation at 15 years) in the proband (O.11), who was also at risk of carrying a putative second, paternally inherited, causative genetic variant. These data suggest that the R399C variant may represent a low-risk allele acting in concert with an unknown paternal factor to cause the severe DCM of the proband. This mutation has been previously reported in a female patient with familial partial lipodystrophy—Dunnigan type27; however, no evidence of DCM (or any other laminopathy) was present in that individual.

Discussion

To further delineate pathogenicity of LMNA variants previously identified in our DCM cohort,10 we assessed nuclear morphology and GFP-lamin A localization in 13 variants. These included 6 variants in pedigrees, termed “nonsegregation” pedigrees, in which 1 or more family members with DCM did not carry the family variant, as well as 4 additional LMNA variants for which small family size (only 1 subject with DCM available for genetic analysis) precluded determination of segregation. These latter pedigrees were termed “pedigrees with unknown segregation.” Of the 13 variants, 10 showed abnormal GFP-lamin A localization and/or nuclear morphological abnormalities. Considered alongside clinical diagnoses of CSD and/or DCM in mutation carriers, these data provide evidence for pathogenicity of 3 of the 4 variants identified in families with unknown segregation and 4 of the 6 variants identified in families with nonsegregation. The absence of the variants observed in the nonsegregation pedigrees in 150 unrelated controls or in LMNA mutation databases (http://www.dmd.nl and http://www.umd.be:2000/IFAM.shtml), considered with the current morphological studies and prior molecular and pedigree data,10 argues for the existence of a second, unidentified, causative factor in clinically affected, but LMNA mutation negative, family members. Whether these additional factors are genetically determined or environmentally imposed remains to be determined. However, the apparent heritable nature of DCM in some of the affected subjects who do not carry the LMNA variants (eg, pedigrees N, S) suggests that a second genetic cause of DCM

<table>
<thead>
<tr>
<th>LMNA Construct</th>
<th>Total Cells, n*</th>
<th>No. Nuclei With Aggregates (% n)</th>
<th>P; Test</th>
<th>Abnormal Nuclei With</th>
<th>No. Nuclei With Abnormal Shape, % (n)</th>
<th>P; Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (WT)</td>
<td>415</td>
<td>22 (5)</td>
<td>NA</td>
<td>67 (16)†</td>
<td>28 (20)</td>
<td>0.4158; CS</td>
</tr>
<tr>
<td>R399L</td>
<td>108</td>
<td>106 (99)</td>
<td>&lt;0.0001; FE</td>
<td>32 (30)</td>
<td>11 (10)</td>
<td>0.1935; CS</td>
</tr>
<tr>
<td>R101P</td>
<td>84</td>
<td>83 (99)</td>
<td>&lt;0.0001; FE</td>
<td>55 (65)</td>
<td>38 (27)</td>
<td>0.0078; CS</td>
</tr>
<tr>
<td>R166P</td>
<td>95</td>
<td>92 (97)</td>
<td>&lt;0.0001; FE</td>
<td>52 (55)</td>
<td>38 (27)</td>
<td>0.0078; CS</td>
</tr>
<tr>
<td>R190Q</td>
<td>142</td>
<td>55 (39)</td>
<td>&lt;0.0001; CS</td>
<td>36 (24)</td>
<td>72 (52)</td>
<td>&lt;0.0001; CS</td>
</tr>
<tr>
<td>E203K</td>
<td>151</td>
<td>25 (17)</td>
<td>&lt;0.0001; FE</td>
<td>36 (24)</td>
<td>72 (52)</td>
<td>&lt;0.0001; CS</td>
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<tr>
<td>I210S</td>
<td>138</td>
<td>134 (97)</td>
<td>&lt;0.0001; FE</td>
<td>75 (49)</td>
<td>75 (49)</td>
<td>&lt;0.0001; CS</td>
</tr>
<tr>
<td>L215P</td>
<td>154</td>
<td>99 (64)</td>
<td>&lt;0.0001; CS</td>
<td>75 (49)</td>
<td>75 (49)</td>
<td>&lt;0.0001; CS</td>
</tr>
<tr>
<td>A318T</td>
<td>136</td>
<td>4 (3)</td>
<td>0.3529; FE</td>
<td>27 (20)</td>
<td>0.3863; CS</td>
<td></td>
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<tr>
<td>R388H*</td>
<td>NA</td>
<td>N/A</td>
<td>NA</td>
<td>N/A</td>
<td>N/A</td>
<td>NA</td>
</tr>
<tr>
<td>R399C</td>
<td>143</td>
<td>8 (6)</td>
<td>0.8934; CS</td>
<td>28 (20)</td>
<td>0.4158; CS</td>
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</tr>
<tr>
<td>S437Hfsx1</td>
<td>105</td>
<td>105 (100)</td>
<td>&lt;0.0001; FE</td>
<td>11 (10)</td>
<td>0.1935; CS</td>
<td></td>
</tr>
<tr>
<td>R471H</td>
<td>115</td>
<td>9 (8)</td>
<td>0.4258; CS</td>
<td>25 (22)</td>
<td>0.2068; CS</td>
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<tr>
<td>R654X</td>
<td>114</td>
<td>108 (95)</td>
<td>&lt;0.0001; CS</td>
<td>15 (13)</td>
<td>0.5259; CS</td>
<td></td>
</tr>
</tbody>
</table>

CS indicates χ² test; FE, Fisher exact test.

*Pooled data were derived from at least 2 independent experiments. The predominant cytoplasmic localization of GFPlaA-R388H

LMNA construct is indicated by subscript H.
may be present in these families. Collectively, these data suggest a more complex basis for DCM in some multiplex pedigrees than has been previously appreciated (see also Ref. 10).

As previously described for this cohort,10 a number of mutation-positive individuals with no evidence of disease were present in several families (eg, pedigrees G, I, and N), showing age-dependent penetrance. Because LMNA-related DCM shows age-dependent penetrance (median, 40.9 years at the time of assessment) rather than nonpathogenicity of the familial variant. We distinguish these individuals who show incomplete, age-dependent segregation (a LMNA mutation-positive individual who has not yet manifested LMNA cardiomyopathy) from those subjects affected with DCM who do not carry the LMNA pedigree mutation, the latter of which are termed “nonsegregants” in this and the prior10 work.

Because a number of previous studies have indicated that C-terminal mutations are significantly less likely to result in aggregation,19,20,26 the lack of an abnormal R399C nuclear phenotype does not preclude pathogenicity of this variant, nor of the similarly expressed A318T (family L) and R471H (family Q) variants. C-terminal missense mutations resulting in lamin A aggregation16,17 have, nevertheless, been described. Caution is, consequently, necessary when considering results generated for these variants.

R388H, which borders the C-terminus of coil 2B and downstream nonhelical regions, was unique among the missense variants studied. Unexpectedly, expressing cells predominantly showed cytoplasmic GFP-lamin A localization and aggregation. The proximity of position 388 to the nuclear localization signal at 416 to 42328 may offer some explanation for the dramatic localization defects; however, the fact that the R399C variant resulted in nuclear import indicates that at least a portion of the region proximal to the nuclear localization signal has no influence on this process. An alternative explanation is loss of stable association with critical binding partners, such as lamin-associated polypeptides, DNA, or chromatin. This hypothesis is consistent with the work of Strelkov et al,29 which proposes that coil 2B is critical binding partners, such as lamin-associated polypeptides, DNA, or chromatin. This hypothesis is consistent with the work of Strelkov et al,29 which proposes that coil 2B is critical binding partners, such as lamin-associated polypeptides, DNA, or chromatin. This hypothesis is consistent with the work of Strelkov et al,29 which proposes that coil 2B is critical binding partners, such as lamin-associated polypeptides, DNA, or chromatin.

Table 3. Nuclear Morphology and GFP-Lamin A Localization Patterns for Confocally Imaged Wild Type and Variant LMNA Constructs

<table>
<thead>
<tr>
<th>LMNA Construct</th>
<th>Family</th>
<th>Nuclear Morphology</th>
<th>Lamin A Localization</th>
<th>Supports Mutation Pathogenicity?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (WT)</td>
<td>NA</td>
<td>Smooth-edged, circular NE (≈85%), Mild nuclear shape abnormalities (≈15%)</td>
<td>Homogenous along nuclear periphery (95%), small NE-associated aggregates (5%)</td>
<td>NA</td>
</tr>
<tr>
<td>R89L</td>
<td>A</td>
<td>Mild to moderate abnormal nuclear shapes (≈30%)</td>
<td>Small NE-associated aggregates (98%), homogenous along nuclear periphery (2%)</td>
<td>Yes</td>
</tr>
<tr>
<td>R101P</td>
<td>B</td>
<td>Moderate to grossly abnormal nuclear shapes (≈65%)</td>
<td>Moderate-sized NE-associated aggregates (99%), homogenous along nuclear periphery (1%)</td>
<td>Yes</td>
</tr>
<tr>
<td>R166P</td>
<td>D/E</td>
<td>Mild to moderate abnormal nuclear shapes (≈55%)</td>
<td>Small NE-associated aggregates (97%), homogenous distribution along nuclear periphery (3%)</td>
<td>Yes</td>
</tr>
<tr>
<td>R190Q</td>
<td>F</td>
<td>Mild to moderate abnormal nuclear shapes (≈25%)</td>
<td>Homogenous along nuclear periphery (61%), Small- to moderate (rare)-sized NE-associated aggregates (39%)</td>
<td>Yes</td>
</tr>
<tr>
<td>E203K</td>
<td>G</td>
<td>Comparable with WT</td>
<td>Homogenous along nuclear periphery (83%), NE-associated aggregates (17%)</td>
<td>Yes</td>
</tr>
<tr>
<td>I210S</td>
<td>H</td>
<td>Moderate to grossly abnormal nuclear shapes (≈50%)</td>
<td>Moderate-sized NE-associated aggregates (97%), homogenous along nuclear periphery (3%)</td>
<td>Yes</td>
</tr>
<tr>
<td>L215P</td>
<td>I</td>
<td>Moderate to grossly abnormal nuclear shapes (≈50%)</td>
<td>Moderate-sized NE-associated aggregates (64%), homogenous along nuclear periphery (36%)</td>
<td>Yes</td>
</tr>
<tr>
<td>A318T</td>
<td>L</td>
<td>Comparable with WT</td>
<td>Comparable with WT</td>
<td>No</td>
</tr>
<tr>
<td>R388H</td>
<td>N</td>
<td>Comparable with WT</td>
<td>(1) Diffuse, cytoplasmic (2) Large, nonspherical, signal-saturated cytoplasmic aggregates (3) Intermediate to (1) and (2) (4) Diffuse, cytoplasmic with NE-associated aggregates (rare)</td>
<td>Yes</td>
</tr>
<tr>
<td>R399C</td>
<td>O</td>
<td>Comparable with WT</td>
<td>Comparable with WT</td>
<td>No</td>
</tr>
<tr>
<td>S437Hfsx1</td>
<td>P</td>
<td>Comparable with WT</td>
<td>Variable aggregates phenotypes (100%); giant aggregates (≈50%), streaked aggregates (≈43%), small aggregates (≈35%)</td>
<td>Yes</td>
</tr>
<tr>
<td>R471H</td>
<td>Q</td>
<td>Comparable with WT</td>
<td>Comparable with WT</td>
<td>No</td>
</tr>
<tr>
<td>R654X</td>
<td>S</td>
<td>Comparable with WT</td>
<td>Small to giant aggregates (95%), giant aggregates in 5%, homogenous along nuclear periphery (5%)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

NE indicates nuclear envelope; NA, not applicable.
novel C-terminal S437Hfsx1 insertion and frameshift unmasks a premature stop codon at position 438 leading to truncation of lamin A and lamin C by 227 and 135 amino acids, respectively. COS7 cells expressing this variant showed a variety of defects, often within the same nucleus. Although 35% of nuclei exhibited small membrane-associated aggregates similar to those observed for the coil1B variants, unique streaked aggregates (~43%) and giant aggregates (~50%) were also prominent. The streaked aggregates, which varied in size, shape, and number between nuclei, are a novel finding. Giant aggregates have been observed in cells expressing exogenous mutant lamin C,13,15 but have not, to our knowledge, been reported for lamin A.

The second lesion, a R654X nonsense mutation, truncates prelamin A by 11 amino acids, removing the conserved CaaX motif and elongating the mature protein by 7 amino acids. This mutation was previously reported in a patient with Hutchinson-Gilford progeria syndrome and a homozygous null ZMPSTE24 mutation,31 and, notably, the mother and brother of that individual both displayed no signs of laminopathy, despite carrying the nonsense mutation. This variant seemed to be pathogenic in our cohort (pedigree S), with carriers showing significant CSD and DCM, including instances of sudden cardiac death, pacemaker/implantable cardiac defibrillator placement, and heart failure. Ninety-five percent of expressing nuclei exhibited a variety of GFP-lamin A aggregates, including giant aggregates similar to those seen for S437Hfsx1 (~5%).

Although our study did not address pathogenic mechanisms, we can hypothesize that loss of critical binding domains for DNA, chromatin, and lamin-associated polypeptides may have resulted in the abnormal S437Hfsx1 aggregate phenotypes. Furthermore, the inability of the truncated S437Hfsx1 and R654X transcripts to be posttranslationally modified may also have contributed to disease, possibly through failure to translocate to the nuclear envelope5 and/or through accumulation of a toxic, incompletely processed precursor, as is seen in Hutchinson-Gilford progeria syndrome.32

Two laminopathy pathogenicity models have been proposed, each attempting to recognize both the phenotypic breadth of the laminopathies and the web of interactions existing between the lamins, lamin-associated proteins, DNA, and chromatin. The “structural” model proposes that LMNA mutations increase cellular susceptibility to mechanical strain through impairment of interactions critical for nuclear and cytoskeletal stability.33,34 Because normal functioning places significant mechanical strain on individual muscle cells, the structural model has been particularly attractive for studying LMNA-related cardiomyopathy and muscular dystrophy. The alternative “gene-expression” model proposes that LMNA mutations impair critical signaling pathways through influence on gene expression at the nuclear periphery. This hypothesis is...
supported by abundant literature documenting heterochromatin loss or redistribution in patient fibroblasts for many of the laminopathies. These 2 hypotheses are not necessarily mutually exclusive, in which capacities and to what extent abnormalities in nuclear architecture and/or gene expression determine particular laminopathic phenotypes are important questions and the subject of current experimentation.

**Limitations**

Although this in vitro heterologous cell system has been shown to be a useful and sensitive tool for determining the potential pathogenicity of novel LMNA variants in this and other research studies, negative results for 3 variants carried by families with manifestations of cardiovascular disease suggest that more sophisticated approaches may reveal more subtle abnormalities. Additional nuclear morphological studies, gene transfer experiments into small animals, or studies with human pluripotent cells harboring LMNA variants may help to further clarify the potential impact of LMNA variants of uncertain pathogenicity.

**Conclusions**

LMNA mutations, a significant cause of genetic DCM, were assessed with nuclear morphology and GFP-lamin A localization studies. Analyses of the LMNA variants in nonsegregation pedigrees identified in our DCM cohort support pathogenicity of 4 of 6 and argue for the existence of a second, unidentified causative factor in these families. In addition, demonstration of abnormal GFP-lamin A localization in 3 of 4 pedigrees for which segregation is uncertain indicate that nuclear morphological studies may also be of value in cases in which LMNA-related DCM is suspected, but pedigree data are lacking.

**Acknowledgments**

We thank the many families and referring physicians for their participation in the Familial Dilated Cardiomyopathy Research Program, without whom these studies would not have been possible.

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**Disclosures**

None.

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**CLINICAL PERSPECTIVE**

Dilated cardiomyopathy (DCM) is a remarkably heterogeneous genetic disease. From 20% to 50% of DCM cases may have an underlying genetic cause. Mutations in >30 genes have been associated with DCM but only explain an estimated 25% to 30% of overall familial cases. Mutations in LMNA, encoding lamin A/C, have been detected in 5% to 10% of families with DCM. That >30 genes are involved (locus heterogeneity) greatly complicates investigative strategies. Further, almost all DCM mutations are “private” or specific to a family (allelic heterogeneity). This fact makes it necessary to sequence all coding exons of each gene to detect the variants relevant for disease, but even if a novel genetic variant is detected that changes the coding of an amino acid, the additional challenge is to verify that the variant truly is disease causing. This study examined 13 of 18 LMNA variants detected in the DNAs from 324 DCM probands, the largest series reported to date. The aim of this study was to gain further evidence that the identified variants were indeed pathogenic, particularly those identified in families too small to assess segregation of the variant with disease, and variants associated with nonsegregation within pedigrees. After expression in a cell system, confocal microscopy–based studies of abnormal nuclear lamina architecture were conducted in cells expressing the LMNA variants. In most cases, the LMNA variants seemed to be disease causing. This study illustrates the use and need for sensitive methods to determine the potential pathogenicity of genetic variants.
Morphological Analysis of 13 LMNA Variants Identified in a Cohort of 324 Unrelated Patients With Idiopathic or Familial Dilated Cardiomyopathy

Jason Cowan, Duanxiang Li, Jorge Gonzalez-Quintana, Ana Morales and Ray E. Hershberger

_Circ Cardiovasc Genet_. 2010;3:6-14; originally published online November 17, 2009; doi: 10.1161/CIRCGENETICS.109.905422

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