Expanding the phenotype of *LMNA* mutations in dilated cardiomyopathy and functional consequences of these mutations

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Aims: Mutations in the lamin A/C gene (*LMNA*) have been reported to be involved in dilated cardiomyopathy (DCM) associated with conduction system disease and/or skeletal myopathy. The aim of this study was to perform a mutational analysis of *LMNA* in a large white population of patients affected by dilated cardiomyopathy with or without associated symptoms.

Methods: We performed screening of the coding sequence of *LMNA* on DNA samples from 66 index cases, and carried out cell transfection experiments to examine the functional consequences of the mutations identified.

Results: A new missense (E161K) mutation was identified in a family with early atrial fibrillation and a previously described (R377H) mutation in another family with a quadriceps myopathy associated with DCM. A new mutation (28insA) leading to a premature stop codon was identified in a family affected by DCM with conduction defects. No mutation in *LMNA* was found in cases with isolated dilated cardiomyopathy. Functional analyses have identified potential physiopathological mechanisms involving identified mutations, such as haploinsufficiency (28insA) or intermediate filament disorganisation (E161K, R377H).

Conclusion: For the first time, a specific phenotype characterised by early atrial fibrillation is associated with *LMNA* mutation. Conversely, mutations in *LMNA* appear as a rare cause of isolated dilated cardiomyopathy. The variable phenotypes observed in *LMNA*-DCM might be explained by the variability of functional consequences of *LMNA* mutations.

Dilated cardiomyopathy (DCM), the most frequent form of cardiomyopathy, is a myocardial disorder characterised by ventricular dilatation and impaired systolic function leading to congestive heart failure and sudden death. The aetiology of the disease is variable but one-third of cases of idiopathic DCM are inherited.^{2–4} Various modes of inheritance have been reported but the autosomal dominant form occurs most frequently and exhibits both clinical variability and genetic heterogeneity. To date, 11 genes, cardiac actin,⁵ desmin,⁶ δ -sarcoglycan,⁷ cardiac troponin T,^{8–9} β -myosin heavy chain,^{8–10} α -tropomyosin,¹¹ titin,¹² the cardiac MyBP-C,¹⁰ metavinculin,¹³ muscle LIM protein,¹⁴ and phospholamban¹⁵ have been associated with autosomal dominant DCM without conduction system disease, skeletal muscle dysfunction, or other characterised phenotypes. Differences in clinical manifestations of DCM are apparent among the different families where inherited mutations within the lamin A/C gene have been described. The first mutation of *LMNA* was identified by Bonne *et al*¹⁶ in a large French pedigree with autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD).¹⁶ Interestingly, in this pedigree, five out of the 17 affected members had typical AD-EDMD with both skeletal and cardiac symptoms and 12 had isolated cardiac involvement characterised by severe atrioventricular conduction defects and sinus dysfunction that resulted in DCM.¹⁷ Mainly, two laboratories have identified several missense *LMNA* mutations; each of these reported mutations was associated with conduction defects such as sinus bradycardia, atrioventricular conduction block, or atrial arrhythmias.^{18–20} A family was also reported with dilated cardiomyopathy with variable skeletal muscle involvement where one patient, out of five affected subjects, displayed an isolated DCM phenotype. Jakobs *et al*²¹ reported two novel mutations in the rod segment in lamin A/C associ-

ated with variable conduction system disease and DCM without skeletal myopathy. *LMNA* mutations in patients with DCM without conduction defects or skeletal muscle dystrophy have been reported by Genschel *et al*,^{22–23} but only in mutation reports without detailed clinical data. More recently, we have identified a missense mutation in a large family associated with DCM with conduction defects and a quadriceps specific myopathy.²⁴ Despite a few publications implying *LMNA* mutations to be responsible for isolated DCM, the exact prevalence of such mutations remains unknown.

Lamins A and C proteins are intermediate filaments, components of the inner layer of the nuclear membrane. They are assumed to play an important role in maintaining the structural integrity of the nuclear envelope and in organising chromatin within the nucleus. However, it remains unknown why defects in these ubiquitous proteins can result in abnormalities of specific and highly differentiated tissues such as skeletal and cardiac muscle composed of differentiated cells. Functional studies have investigated the intracellular localisation of mutant lamin A proteins by an immunofluorescence microscopy approach.^{25–28} Among 15 mutant proteins analysed, four showed an abnormal localisation and partially disrupted the endogenous lamina and three altered the localisation of emerin.

In order to determine the epidemiology of *LMNA* mutations in a large white population with isolated DCM or affected by DCM with conduction defect and/or skeletal muscle myopathy, we screened the entire coding sequence of the lamin A/C gene for mutations in 47 unrelated probands from families and 19 sporadic cases recruited in France. We also examined the functional consequences of identified mutations on heart and muscle biopsies by cell transfection experiments of mutant lamin cDNAs and by standardised RT-PCR.

SUBJECTS AND METHODS

Subjects and clinical evaluation

Patients with at least one first degree relative with documented idiopathic DCM were identified as familial cases (probands). The clinical status was determined in all available first degree relatives after clinical examination including muscular testing, 12 lead ECG, and echocardiography, as previously described.^{29,30} Holter ECG and serum creatine kinase dosages were performed when possible. Clinical status (affected, unknown, or healthy) of subjects was defined according to criteria previously described.^{29,31} Written informed consent was obtained in accordance with the study protocol approved by the local ethical committee. Inheritance in familial forms was reviewed and families with X linked or mitochondrial inheritance were excluded. A total of 66 consecutive and unrelated index cases were included in the study, including 47 from families with autosomal dominant DCM and 19 with sporadic forms of DCM. They were mainly of European origin. Most cases were characterised by isolated DCM (n=56), 3% of patients had DCM with AV block and muscular dystrophy (n=2), and 6% of cases presented a DCM with isolated raised serum creatine level (without clinical muscular dystrophy) (n=4). Finally, four families had frequent atrial fibrillation including one family with early atrial fibrillation preceding DCM (1.5%).

Molecular biology

Genomic DNA was extracted from white blood cells by means of standard procedures, in collaboration with the Genethon Bank (Evry, France). Systematic PCR-SSCP sequencing analysis of the coding sequence of *LMNA* was performed as previously described.^{32,33} Sequencing reactions were performed using the ABI PRISM BigDye Terminator cycle sequencing ready reactions kit and run on a 3100 Genetic Analyzer (Applied Biosystems). Mutations were independently confirmed and the genotypes of family members determined. The 481 G>A (E161K) and 1130G>A (R377H) mutations were identified by direct sequencing on both strands. The presence of the insertion 28insA, which abolishes a *MwoI* (Biolabs) restriction site, was confirmed by *MwoI* digestion of PCR products.

RT-PCR

Lymphoblastoid cell lines were established by Epstein-Barr virus transformation of peripheral blood lymphocytes in collaboration with the Genethon bank. Total RNAs were extracted from these cells using the Trizol reagent following the manufacturer's recommendations (Life Technologies). Reverse transcription was performed at 42°C for two hours on 2 µg of total RNA using the pd(T)12-18 primers (Biolabs) and the Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). The absence of contaminating DNA from each RNA sample was checked by omitting the reverse transcriptase from the RT reaction. When the reaction was complete, 2 µl of the cDNA solution were amplified in a final volume of 50 µl with the following primers: 62F 5'-CTCTGTCCTTCGACCGAG-3' and 1R 5'-AGCCCTGCGTTCTCGTTTC-3'. The PCR fragment obtained (252 bp) contained the 28insA mutation. In order to evaluate the rate of mutated mRNA in comparison to the normal one, we performed a *MwoI* restriction digestion to discriminate between mRNAs transcribed from normal and mutated alleles. As a control, we amplified genomic DNA containing the mutation. Bands were quantified by Molecular Analyst™ software (Bio-Rad).

Recombinant vector constructions

The wild type lamin A cDNA cloned in pcDNA3 vector²⁵ was generously provided by Dr Colin Stewart (Frederick, Maryland, USA). This construct is 5' end tagged with the 12CA5

epitope of influenza haemagglutinin. In order to obtain the lamin cDNAs containing each of the described mutations (28insA, E161K, and R377H), we performed direct in vitro mutagenesis with the QuikChange™ Site-Directed Mutagenesis Kit with the protocol according to the manufacturer's recommendations (STRATAGENE). Mutant cDNA was entirely sequenced using the ABI PRISM BigDye Terminator cycle sequencing ready reactions kit and run on a 3100 Genetic Analyzer (Applied Biosystems).

Cell culture and transfection

COS and C2C12 cell lines (American Type Cell Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) Glutamax supplemented with 10% fetal calf serum (AbCys) and gentamicin at 50 µg/ml. Cells were plated on coverslips at 300 000 cells per 35 mm plates for 12 to 24 hours before transfection. Transient transfections were performed by lipofection with lipofectamine2000 (Invitrogen) as follows: DNA (7.5 µg) and lipofectamine2000 (11 µg) were mixed in a final volume of 1 ml of DMEM without serum and left at room temperature for 15-20 minutes and then incubated with the cells for five hours at 37°C. Then the transfection medium was replaced by 8 ml of DMEM with serum and cells incubated for 24 to 48 hours before analysis.

Pathological studies on heart

We analysed one post-transplant cardiac biopsy from patient III.15 (family A). Sections of paraffin embedded cardiac tissue were stained with haematoxylin/eosin and Sirius red, and morphometric analysis allowed the quantifying of fibrosis by Q-win software (Leica).

Antibodies

The mouse monoclonal antibody against lamin A/C (clone JOL2) and desmin (clone D33) were obtained from Chemicon, the mouse monoclonal antibody against dystrophin (clone Dy4/6D3) and emerin (clone 4G5) from Novocastra Laboratories. The rabbit polyclonal antibody against lamin A was purchased from Cell Signaling Technology and the anti-haemagglutinin-fluorescein isothiocyanate (anti-HA-FITC, clone 12CA5) provided by Roche Diagnosis. For the immunohistochemistry labelling followed by peroxidase reaction, we used the LSAB 2 System, HRP kit (Dako). Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse or anti-rabbit antibodies were purchased from Jackson Immuno-Research Laboratory.

Immunocytochemistry and immunohistochemistry

Cultured cells were rinsed three times with phosphate buffered saline (PBS) 1 × and coverslips were fixed with ice cold methanol/acetone (80/20) at -20°C for seven minutes. After two rinses with PBS, cells were blocked in PBS +10% normal goat serum (NGS) for one hour at room temperature. All primary and secondary antibodies were diluted in PBS +5% NGS. Primary antibodies were diluted at 1:20 for mouse monoclonal anti-lamin A/C, anti-HA, and anti-emerin antibodies, and at 1:40 for the rabbit polyclonal anti-lamin A antibody. Goat anti-mouse TRITC was diluted at 1:300 and at 1:100 for goat anti-rabbit FITC. Coverslips were mounted on vectashield with DAPI (Vector Laboratory). Analyses were performed with fluorescent microscope eclipse E800 (Nikon) with a 40 × oil objective.

Immunohistochemical analyses of lamin A/C, desmin, and dystrophin were performed on 7 mm heart muscle sections as described above: monoclonal antibodies against lamin A/C at a dilution of 1:10, desmin at dilution of 1:20, and dystrophin antibodies were undiluted. For immunohistochemistry labelling, the staining was followed by a peroxidase reaction using the LSAB 2 System, HRP kit (Dako).

Figure 1 Pedigrees of the three families affected by DCM associated with mutations in *LMNA*. Solid symbols, affected members; open symbols, unaffected members; shaded symbols, members of unknown clinical status. For family C, the left half shows heart disease status and the right half, skeletal muscle disease status. Plus signs indicate the presence of a mutation, and minus signs the absence of a mutation.

Statistical analysis

To study the effects of the mutated lamins, COS and C2C12 cells were transfected with normal and mutant lamin cDNA expression vectors and processed for immunocytochemistry 24 to 48 hours later, as described above. The percentage of transfected cells was determined for each coverslip. Data obtained from six independent transfections were analysed. For each construct, 12 coverslips were examined and approximately 8000 cells were scored for transfection efficiency estimation. Data were analysed by Student's *t* test.

The cumulated survival curve in families was constructed according to the Kaplan-Meier method.

RESULTS

Molecular analysis of DCM population

We screened for mutations the 12 exons of the lamin A/C gene by a PCR-SSCP method in 47 probands from families as well as in DNA from 19 sporadic cases. A double stranded sequence was carried out (1) systematically for each type of SSCP profile from patient and control DNA and (2) if there was any suspicion of an abnormal SSCP profile. In order to check the sensitivity of the SSCP analysis, we sequenced all PCR products for exons 1, 2, and 7 from 52 patients, confirming the results obtained with SSCP analysis.

This approach allowed us to identify three mutations including two new mutations: a missense mutation in exon 2 (481G>A, E161K) in the proband of family A, an adenine

insertion in exon 1 (28insA) in the proband of family B, and an already described mutation in exon 6 (1130G>A, R377H) in family C. No other mutation was identified in the remaining probands. In addition to polymorphisms previously described, we detected several new polymorphisms in *LMNA*: one in exon 1 (51C>T, S17S), one in exon 2 (357C>T, R119R), one in exon 6 (1098G>A, K366K), and another one in exon 7 (1299C>T, H433H).

Phenotypic analysis of the three families carrying a *LMNA* mutation

The pedigrees of the families are shown in fig 1 and detailed clinical features of the heterozygous subjects for the mutation are indicated in table 1.

Family A

Two cardiac deaths were reported in the family history (sudden death at 38 years and congestive heart failure at 68 years). Seven living subjects were heterozygous for the E161K mutation (table 1). DCM was present in only two of them (III.15 and IV.28). The onset of the disease was characterised by congestive heart failure and atrial fibrillation (at 29 and 44 years respectively) and led to heart transplantation in both patients (at 34 and 51 years). In three additional subjects (III.16, III.20, IV.22), the onset of the disease was also characterised by atrial fibrillation at 22, 49, and 63 years respectively but without DCM since left ventricular ejection fraction was

Table 1 Phenotype of the heterozygous subjects for a mutation in the *LMNA* gene

Subject	Age/ gender	ECG	LVEDD (mm)	EF (%)	DCM	Muscular abnormality	Comments
Family A, E161K mutation							
III.15	66/F	AF, cLBB	53	10	Yes	No	HT (51 y)
III.16	53/F	AF, cLBB	53	52	No	No	CVA
III.20	67/F	AF	52	52	No	No	PM (67 y)
IV.22	31/M	AF, PVB	55	54	No	No	CVA
IV.28	45/M	AF	54	38	Yes	No	HT (34 y)
V.31	12/F	SR	47	84	No	No	Healthy
V.32	16/M	SR	50	71	No	No	Healthy
Family B, 28insA mutation							
III.7	55/F	AVB(II), AF	58	27	Yes	Muscular dystrophy	PM (46 y)
III.5	47/M	AV (I), AF	NA	NA	No	No	-
III.12	44/F	AVB(I)	50	75	No	No	-
IV.2	34/F	SR	40	61	No	No	-
IV.3	35/F	PVB	58	42	Yes	No	-
Family C, R377H mutation							
II.2	d/F	cLBB, AF	NA	40	Yes	Muscular dystrophy	Died at 78 y (CHF)
III.6	58/F	AVB(III), AF	61	50	No	Muscular dystrophy	PM
III.8	52/M	cLBB, AF	73	47	Yes	Borderline	VT, PM
III.10	d/M	cLBB, AVB(I)	60	53	No	NA	VT, sudden death (49 y)
III.12	d/M	AVB(III), AF	60	53	No	Muscular dystrophy	PM, sudden death (51 y)
III.18	d/M	cLBB, AF	NA	NA	No	NA	VT, death at 45 y (CHF)
III.21	65/F	AVB, AF	62	36	Yes	Muscular dystrophy	PM
IV.8	27/M	SR	54	NA	No	No	Septal hypokinesia
IV.9	24/F	AF, VT	50	60	No	No	-
IV.10	21/F	SR	48	60	No	No	Healthy
IV.14	28/F	AF	57	60	No	No	-
IV.21	30/M	AF	70	NA	No	No	-

Age: age at genetic investigation; d: dead; DCM: dilated cardiomyopathy; LVEDD: left ventricular end diastolic diameter (echography); EF: ejection fraction (echography); SR: sinus rhythm; PVB: premature ventricular beats; cLBB: complete left bundle branch block; AVB: atrioventricular block (grade I, II or III); AF: atrial fibrillation; CVA: cerebrovascular accident; PM: pacemaker; HT: heart transplantation; VT: ventricular tachycardia.

preserved (50 to 54%) and IV dimensions were normal in two subjects. Finally, two young subjects (V.31, V.32) aged 12 and 16 years were heterozygous for the mutation but had no signs or symptoms of heart disease. No significant atrioventricular block was observed in the family, except in one subject (III.20) for whom cardiac pacing was necessary at 67 years because of sinoatrial block coexisting with atrial fibrillation. Complete left bundle branch block was present in only two subjects, and IV dilatation was already present at this stage (according to age and body surface area) but only one had systolic dysfunction. Neuromuscular examination was normal in all subjects in the family. Creatine kinase level (CK) was normal for subjects III.15 and IV.22 and not available for the other family members. Fibrosis, quantified on cardiac biopsy from patient III.15 after red Sirius colouration, was severe in the left ventricle free wall (29%). We performed immunostaining experiments, which showed the organisation of lamin A/C proteins to be normal. Dystrophin and alpha-actinin proteins showed a normal organisation in biopsies. Only desmin had an altered staining compared to control biopsies characterised by loss of cross striated organisation (fig 2). As controls, we used heart biopsies from one patient affected with ischaemic DCM and a second patient affected by a DCM owing to a mutation in another gene.

The phenotype of family A was characterised by early atrial fibrillation preceding or coexisting with DCM, without significant AV block, and without neuromuscular abnormalities.

Family B

Five subjects of family B carried the heterozygous 28insA insertion (table 1). Three additional subjects (III.5, III.8, and II.10) were considered as phenotypically affected with documented DCM but were not available for DNA analysis. Moreover, the history was characterised by three cardiac sudden deaths before 55 years of age (II.3, II.7, and III.3).

DCM was diagnosed in a total of five subjects, including three associated with significant AV block leading to pacemaker implantation, one subject with premature ventricular beats leading to a cardioverter defibrillator implantation, and one subject with a mild form of skeletal muscular dystrophy (mild weakness and wasting of quadriceps muscles, myogen abnormalities on electromyogram). CK level available in three family members was normal. Three additional adults with normal echocardiography carried the mutation, including two subjects with first degree AV block (44 and 47 years), and one subject without any cardiac abnormality (34 years).

The phenotype in family B was therefore characterised by DCM with conduction defect and/or with mild skeletal myopathy (for subject III.7).

Family C

We previously reported the detailed clinical phenotype of this family in Charniot *et al.*²⁴ Briefly, 12 subjects in the family were heterozygous for the R377H mutation, including 11 with cardiac abnormalities (table 1): three patients affected by DCM associated with AV block and pacemakers in two of them, AV block and pacemakers in two additional subjects, one subject with atrial fibrillation and congestive heart failure, subject IV.8 with ventricular tachycardia died suddenly at 49 years, three subjects with atrial fibrillation, and one subject with local left ventricle hypokinesia. Four of these patients also had muscular dystrophy with force defect of the quadriceps muscles. Subject IV.10 carrying the mutation was phenotypically healthy (at 21 years). CK level was normal in all the family.

The phenotype in family C was therefore characterised by DCM with conduction defect or atrial/ventricular arrhythmias and with skeletal muscular dystrophy of the quadriceps muscles.

The prognosis in the three families was relatively poor and was characterised by 11 cardiac deaths and two heart

Figure 2 Distribution of desmin in heart muscle sections. The monoclonal antibody recognised specifically the desmin protein in heart followed by biotin conjugated secondary antibody and then a streptavidin conjugated to horseradish peroxidase. (A) Heart section from patient III.15 from family A. (B) Heart section from a patient affected by DCM resulting from *MYH7* mutation. (C) Control section from patient suffering from ischaemic cardiomyopathy.

transplantations. Cumulative survival at 50 and 65 years was 73% and 48% respectively (fig 3).

Functional analysis of the mutations

Cell transfection experiments

To determine if the intracellular localisation of the mutant form of lamin A differs from that of the wild type, transient cell transfections were performed on the COS cell line or C2C12 mouse myoblasts with wild type or various cDNA plasmids containing the identified mutations 28insA, E161K, or R377H. In transfected cells, exogenous and endogenous wild type lamin A were normally localised to the nuclear periphery both in COS and C2C12 cell lines (not shown). The 28insA and E161K lamin A mutants showed a normal localisation after transfection into cells whereas the R377H lamin A mutant showed a significantly abnormal localisation, accumulating in large intranuclear “foci” in many C2C12 cells.²⁴ Double channel analysis showed a colocalisation between endogenous and

exogenous lamins when cells were transfected with mutant or wild type plasmids (not shown).

Since lamin A protein has been shown to bind emerin in in vitro and ex vivo experiments, we investigated the influence of the lamin mutations on emerin localisation. In C2C12 cells transfected with R377H lamin mutant, emerin was located both at the nuclear envelope and within the cytoplasm, whereas with the two other mutants emerin has the expected perinuclear localisation. Double immunostaining showed that wild type, 28insA, and E161K lamins colocalise with emerin whereas emerin did not colocalise with R377H lamin especially in foci (not shown).

Statistical analysis of cell transfection experiments

Six independent cell transfection experiments were carried out to verify the reproducibility of results. For each construct, we calculated transfection efficacy. We observed a statistically significant decrease of transfection efficiency with the 28insA mutant compared to the control ($p < 0.05$). We expected transfection efficiency to be similar for each cDNA construct. The observed decrease of transfection level could reflect a decrease in RNA expression or stability leading to a reduction of protein.

With regard to the immunostaining results obtained with COS and C2C12 cells, we observed the “foci” phenotypic class described above relevant to the lamin A organisation. No statistical difference was observed for the 28insA and E161K cDNAs compared to the normal lamin A. In contrast, approximately 15% of cells transfected with R377H lamin A exhibited “foci” as against only 1% of the cells transfected with the normal lamin A (fig 4). Differences between the patterns obtained from the wild type and the R377H mutant were all statistically significant ($p = 0.015$).

RT-PCR

The 28insA mutation generates a frameshift in the open reading frame, leading to a stop codon 30 amino acids downstream from the insertion; the results of the transfection studies suggest a decrease of lamin A/C containing the insertion

Figure 3 Kaplan-Meier survival curve for the population of the three families with an *LMNA* mutation.

Figure 4 Statistical analysis of the effect of mutation transfection efficiency and lamin organisation. (A, B) Percentage of transfected cells into COS (A) and C2C12 cell lines (B). (C, D) Percentage of cells with foci after transfection into COS (C) and C2C12 cell lines (D). In all cases, the data represent average values and bars represent the standard deviation. When differences between control and mutant lamins were statistically significant (*), it was with $p < 0.05$ for A, B and $p = 0.015$ for C, D.

mutation. In order to determine if this mutation could lead to a decrease in the mutant mRNA, we performed a standardised RT-PCR analysis. To discriminate between wild type and mutant mRNA, we digested RT-PCR products by *Mwo*I generating a 197 bp DNA fragment for the mutant allele and 148 bp for the normal one. We expected an almost equal amount of both transcripts in patients carrying the mutation as they are heterozygous for the mutation. As a control, we amplified genomic DNA from patients carrying the mutant and checked that the ratio between the two alleles was 50/50. As shown in fig 5A, the band corresponding to the mutant RNA seemed to exhibit a lower intensity compared to the wild type for each heterozygous carrier (III.7, III.10, III.12, IV.2, and IV.3) while for the control DNA the intensity is the same. To confirm this

result, we quantified the signals and calculated the percentages of the mutant allele (fig 5B) taking account of the size of the fragments. For all the heterozygous patients, the percentage was dramatically decreased (between 1% and 18%) except for patient IV.3 who presented a mild decrease to 34% which might be the result of the partial digestion observed. These results indicated that the RNA containing the stop codon (197 bp allele) is less abundant in lymphoid cells than the wild type mRNA species, suggesting a lower transcription rate or stability of the mutated mRNA.

DISCUSSION

The present work is the first investigation of the possible involvement of *LMNA* in a large panel of patients with DCM

Figure 5 Analysis of mRNA by standardised RT-PCR. (A) BET-gel analysis. RNAs from patients of family A were analysed. T1: PCR of genomic DNA from patient heterozygous for the mutation (patient III.7); T2 and T3: PCR of genomic DNA from patients IV.1 and IV.4; T4: PCR from normal DNA control not digested. M: molecular weight marker. (B) Quantifications of the mutant allele. We have represented the percentage of mutant allele for each patient available.

with or without conduction defect or skeletal muscular dystrophy. We analysed probands from 47 families with the DCM phenotype frequently diagnosed in hospital and 19 sporadic cases with different phenotypes associated with DCM. Some cases were characterised by atrial fibrillation (6%), but only in family A did atrial fibrillation occur at an early stage and precede DCM.

Our analysis allowed us to identify three mutations in *LMNA* including two new mutations. Interestingly, the new E161K mutation in *LMNA* was identified in family A presenting a particular phenotype; all five adults carrying the mutation in this family had atrial fibrillation and only two of them had DCM. Atrial fibrillation was therefore the first symptom of the disease in these cases. This phenotype is in contrast with previous reports about the phenotype of *LMNA* mutations characterised by significant conduction defect or skeletal myopathy associated with, or pre-existing, DCM.^{17 18 20 34} It is also in contrast with the usual and pure form of DCM, where atrial fibrillation usually follows the occurrence of DCM.³⁵ Whether or not atrial fibrillation in itself favours the evolution towards DCM in family A remains unknown.

The E161K mutation is localised in exon 2 of *LMNA*, in the central α -helical rod domain of the lamin A and the lamin C proteins, as are four out of the nine mutations reported so far in DCM.¹⁸ These four mutations, R60G, L85R, N195K, and E203G, were described in DCM associated with inherited conduction system disease.¹⁸ Another reported missense mutation R571S was located at the carboxyl-terminal end of lamin C and was associated with DCM and subclinical skeletal muscle disease.¹⁸

The new insertion mutation found in family B (28insA) leads to an aberrant truncated protein of 38 amino acids. A nonsense mutation (Q6X) identified in an AD-EDMD pedigree with isolated DCM-CD has been previously reported.¹⁷ The phenotypes observed in these two families are slightly different from the AD-EDMD pedigree: a DCM with conduction defects and only one patient being affected by a skeletal myopathy and no subjects affected by an AD-EDMD.

The third missense mutation found in the screening of our DCM population has already been discussed in detail.²⁴ No mutation in *LMNA* was found in cases with isolated dilated cardiomyopathy. Previously, only two mutations have been reported in two patients affected with DCM without any associated clinical symptoms: a single nucleotide deletion in exon 8 (codon 466) resulting in a frameshift mutation with a stop codon at residue 479 and a missense mutation in exon 11 (codon 644).^{22 23} This latter missense mutation has been also reported in one unaffected subject.³⁶ In the large population studied, *LMNA* mutations appear therefore very uncommon in isolated forms of DCM, so the usefulness of systematic screening of this gene in families with isolated DCM is questionable. Our data emphasise a wide spectrum of disorders resulting from lamin A/C mutations with considerable heterogeneity of phenotypes. Nevertheless, in laminopathies with striated muscle disorders, cardiac manifestations are always reported in families described with *LMNA* mutations, whereas skeletal symptoms are more variable.

The functional role of lamin A and C proteins, expressed in many tissues, has not yet been elucidated in detail. We made a preliminary approach by RT-PCR and cell transfection experiments. These studies show a decrease of the mutated mRNA, which suggests a lower transcription rate or a specific decrease of mutant mRNA stability. This latter hypothesis, so-called nonsense mediated decay (NMD), is a mechanism controlling gene expression in cells.³⁷ NMD prevents the increase of aberrant protein that could disorganise the physiological process in cells acting as a poison polypeptide. The 28insA mutation could act as a haploinsufficiency mechanism. Cell transfection results allow us to hypothesise that the R377H mutation leads to a mislocalisation of both emerin and lamin proteins and

partially disrupts the nuclear membrane and/or prevents interaction of one or both proteins with others. The results obtained on heart biopsy from patients carrying the E161K mutation are in favour of the hypothesis of a disruption of the integrity of nuclear intermediate filaments with E161K mutated lamins. Direct interaction between desmin and lamin A/C has not yet been shown. Two hypotheses can be put forward: lamin A/C and desmin could interact and the E161K mutation destabilise this interaction, or there is no direct interaction and the mutation prevents an interaction with desmin via another protein. It would be of interest to determine if an interaction exists between lamin A/C and desmin proteins, for example, by cell transfection experiments followed by co-immunoprecipitation or a yeast two hybrid system.

The reason why some mutations within the same gene can lead to such different phenotypes remains to be elucidated. Research on modifier genes associated with *LMNA* mutations could be one way to understand the phenotypic heterogeneity.

In conclusion, based on our results, (1) a specific phenotype characterised by early atrial fibrillation, preceding or coexisting with DCM, should lead to a screening of *LMNA*; (2) the isolated form of DCM without significant conduction defect, skeletal muscle dystrophy, or early atrial fibrillation is unlikely to be related to *LMNA*.

These findings may have important implications for molecular diagnosis strategy in clinical practice. Based on our experience, *LMNA* should be screened only in DCM with conduction defects and/or skeletal myopathy and also in DCM with early atrial fibrillation preceding or coexisting with DCM.

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