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Mutation Glu82Lys in lamin A/C gene is associated with cardiomyopathy and conduction defect

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Abstract

Dilated cardiomyopathy is a form of heart muscle disease characterized by impaired systolic function and ventricular dilation. The mutations in lamin A/C gene have been linked to dilated cardiomyopathy. We screened genetic mutations in a large Chinese family of 50 members including members with dilated cardiomyopathy and found a Glu82Lys substitution mutation in the rod domain of the lamin A/C protein in eight family members, three of them have been diagnosed as dilated cardiomyopathy, one presented with heart dilation. The pathogenic mechanism of lamin A/C gene defect is poorly understood. Glu82Lys mutated lamin A/C and wild type protein was transfected into HEK293 cells. The mutated protein was not properly localized at the inner nuclear membrane and the emerin protein, which interacts with lamin A/C, was also aberrantly distributed. The nuclear membrane structure was disrupted and heterochromatin was aggregated aberrantly in the nucleus of the HEK293 cells stably transfected with mutated lamin A/C gene as determined by transmission electron microscopy.

Keywords: Dilated cardiomyopathy; Lamin A/C gene; Mutation

Dilated cardiomyopathy (DCM) is a form of heart muscle disease characterized by impaired systolic function and ventricular dilation of the left, or both ventricles, representing the most common heart-failure eventually requiring heart transplantation [1]. This condition is also associated with a high rate of sudden death due to ventricular arrhythmias and a high mortality rate of 15–50% at 5 years [2]. The prevalence of DCM in the US population, using diagnostic criteria for advanced disease, was estimated to be 36.5 per 100,000 persons [3]. The etiology and the pathogenetic mechanisms are still largely unknown in approximately half of the patients [4]. Several prospective studies have clearly demonstrated the existence of genetic transmission of the disease detectable in at least 25% of DCM patients [5,6].

Familial DCM is suggested by different patterns of inheritance, with autosomal trait prevailing, and variable clinical features. Clinical and molecular genetic studies have resulted in the identification of 26 candidate disease loci and 22 genes responsible for familial DCM. One of the genes is the lamin A/C gene, that encodes for major structural components of the lamina network that underlies and supports the nuclear envelope. Mutations in lamin A/C have been associated with nine different inherited diseases including DCM, Emery–Dreifuss muscular dystrophy (EDMD), and Hutchinson–Gilford progeria syndrome (HGPS) [7]. A new term, laminopathies, has been coined to describe the growing number of disorders caused by mutations in nuclear lamins and lamin-binding proteins.

The mechanism responsible for laminopathies is presently not well defined. The lamin A/C gene has been

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mapped on the long arm of chromosome 1 (1q21.2–q21.3) and encodes two main isoforms by alternative splicing [8], lamins A and C. The lamins are the major proteins comprising the nuclear lamina, which belongs to the V intermediate filament family and forms a molecular interface between the inner nuclear envelope membrane and chromatin. Lamins are classified as A and B types depending on their primary sequence, behavior at mitosis, and tissue-specific expression patterns [9,10]. Lamins A and C are encoded by single lamin A/C gene, whereas lamins B1 and B2 are encoded by two different lamin B1 and lamin B2 genes. B-type lamins are constitutively expressed in all embryonic and somatic tissues, whereas A-type lamins are expressed exclusively in differentiated non-proliferating cells and developmentally regulated [11,12]. In addition to supporting the nuclear envelope and providing anchorage sites for chromatin, the nuclear lamins are also involved in a number of other functions including nuclear envelope assembly, DNA replication, RNA transcription, cell cycle regulation, cell development and differentiation, and apoptosis [13].

Lamin A/C plays a crucial role in many cellular activities, but it is poorly understood why and how different mutants cause such diverse phenotypes in specific tissues, but yet other tissues are apparently unaffected [9]. We found a novel mutation in lamin A/C gene in a large Chinese pedigree with familial DCM and conduction defect, and investigated the function of the mutant form of this gene.

Materials and methods

Family data. The study was approved by the review board of Cardiovascular Institute, Chinese Academy of Medical Sciences. A large Chinese pedigree with dilated cardiomyopathy was collected, including four patients with DCM and 46 relatives (Fig. 1). All members entered a diagnostic protocol that consisted of clinical examination, electrocardiography (ECG), and two-dimensional and Doppler echocardiography. DCM was diagnosed on the basis of World Health Organization criteria [1]. The control group consisted of 60 healthy volunteers who showed no abnormalities on physical and electrocardiographic examinations. All patients and volunteers gave their informed consent.

Mutation detection. The genomic DNA was isolated from peripheral blood leukocytes by proteinase–phenol–chloroform extraction method. Mutation in lamin A/C gene was screened with single PCR assays. Intronic sets of primers flanking each of the exons from 1 to 12 of the lamin A/C gene were designed according to the lamin A/C gene reference sequence. Each of the exons of the gene was amplified with PCR in a GeneAmp PCR system 9700 (Perkin-Elmer Corp., California, USA). Each PCR was performed in a total volume of 50 μl containing 250 ng of genomic DNA, 20 pmol of each primer, 200 μM dNTPs, 50 mM KCL, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, and 1 U Taq DNA polymerase (Sino-American Biotechnology Company, Beijing, China), which was denatured at 94 °C for 4 min, followed by 30–35 cycles of denaturation at 94 °C for 30 s, annealing at 58–67 °C for 30–45 s, and extension at 72 °C for 30 s with final extension at 72 °C for 10 min. The PCR products were sequenced on ABI PRISM 377 (Applied Biosystems/Perkin-Elmer) according to the manufacturer's instructions. Heterozygous mutations were detected using ABI PRISM data analysis software by comparison of the coding sequence in affected individuals with the sequences obtained from unaffected individuals and the reference sequences.

Plasmid construction. Human wild-type full-length lamin A/C gene cDNA was subcloned into eukaryotic expression vector pTracer-CMV, a gift from Dr. Colin (Cancer and Development Biology Laboratory, ABL-Basic Research Program, Frederick, USA). Point mutation was introduced into the cDNA using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All sequences were confirmed by DNA sequencing.

Cell culture. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), 100 μg/ml of penicillin, and 100 μg/ml of streptomycin, and kept in an atmosphere of 95% air, 5% CO_2 in a 37 °C humidified incubator.

Transfection and selection. Lamin A/C wild type and mutant were transfected into HEK293 cells, respectively, with the LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 1 μg of pTracer-CMV vector containing mutated or wild-type lamin A/C gene cDNA was incubated with 50 μl of OPTI-MEM I Medium (Invitrogen, Carlsbad, CA, USA) at room temperature for 5 min; 2 μl of LipofectAMINE 2000 reagent was added; and the mixture was incubated at room temperature. After 20 min, 70% confluent cells were washed twice with OPTI-MEM I Medium and then incubated with DNA-LipofectAMINE 2000 reagent complexes at 37 °C in a humidified incubator containing 5% CO_2 for 5 h. After transfection, the mixture was aspirated, and cells were cultured in DMEM with 10% FBS for an additional 24 h. Subsequently, cells were incubated with complete

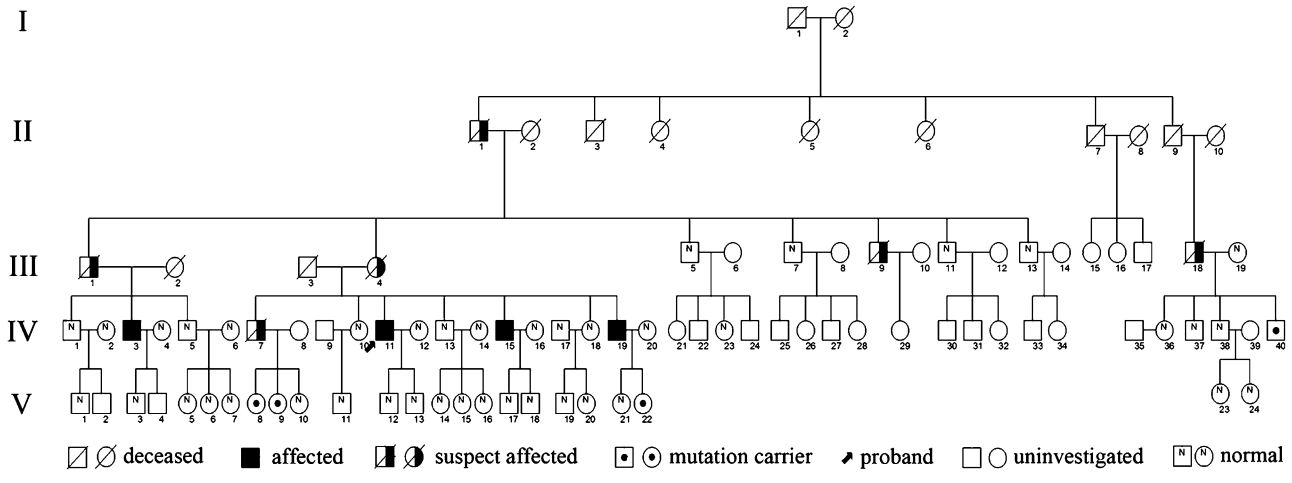

Fig. 1. The pedigree map of the Chinese dilated cardiomyopathy family.

medium containing 400 μg/ml Zeocin (Invitrogen, Carlsbad, CA). After 4 weeks, stable transfected cell clones resistant to Zeocin were isolated and analyzed.

Transmission electron microscopy. HEK293 cell line and HEK293 stable cell lines transfected with wild-type or mutated lamin A/C gene and empty vector were grown on 6-well plate. When the cells reached 80% confluence, they were fixed with 2% glutaraldehyde in cacodylate buffer (50 mM cacodylate, pH 7.2, 50 mM KCl, and 2 mM $MgCl_2$) at room temperature for 30 min, then treated with 1% OsO_4 in cacodylate buffer at room temperature for 45 min, 2% uranyle acetate in 50% ethanol block stain at room temperature for 10 min, and finally dehydrated through a series of graded ethanol. Ultrathin sections were made on a Reichert ultracut, stained with lead citrate and uranyl acetate. Sections were examined with a Philips EM 400 transmission electron microscope.

Immunofluorescence microscopy. Cells grown on glass coverslips were fixed in methanol at −10 °C for 6 min. Following (PBS) washes, the fixed cells were incubated with the primary antibody for 30 min at 37 °C, washed with PBS, incubated with the secondary antibody at 37 °C for 30 min, washed again with PBS, and mounted in Nikon 50i (Nikon, Japan).The primary antibodies were goat polyclonal lamin A (Santa Cruz, USA), rabbit polyclonal emerin (Santa Cruz, USA). Appropriate dilutions of all antibodies were established by immunofluorescence microscopy. Secondary antibodies (Vector, USA) included tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG and tetramethylrhodamine isothiocyanate-conjugated rabbit anti-goat IgG.

Western blot. Transfected cells grown in 2-ml dishes were trypsinized and harvested after 3 days of transfection. Equal amounts of extract were separated on 10% polyacrylamide gels and transferred to nitrocellulose. Immunostaining with anti-lamin A and anti-emerin was carried out by using ECL-Western blot detection system (Vigorous, Beijing, China).

Results

Molecular genetic findings

Mutation screening found that the proband and other seven familiar members had a variation c.244G>A at exon 1 of lamin A/C gene. The mutation results in change from glutamic acid to lysine at amino acid position 82 (Glu82Lys) (Fig. 2). All affected members were heterozygous for the mutation. The other familiar members of the pedigree and 60 normal controls did not carry this mutation.

Clinical findings

The clinical characteristics of the subjects carrying the mutation Glu82Lys are shown in Table 1. The proband presented with symptoms of dyspnea and heart failure. A two-dimensional echocardiogram showed left ventricular dysfunction (increased end-diastolic diameter). ECG showed complete atrioventricular conduction block (III° AVB) in three affected members with implantation of pace maker. The proband's brother and one of his uncle had the similar clinical manifestations and both died of heart failure at the age of 40s. Three of his brothers had the similar symptom. Other four genetically affected relatives were without symptom, but two of them had abnormal ECG.

The mutation causes the nucleus ultrastructure abnormal

To investigate whether the lamin A mutant could affect structure of the cell nucleus, we transfected HEK293 cells with plasmid expressing wild-type and mutated forms of lamin A and isolated stable cell lines. With electronic microscopy, we found the nuclear membrane structure of HEK293 cells stably transfected with mutated lamin A/C gene to be disrupted, locally collapsed or bulged, and partially fragmented. The heterochromatin was aggregated aberrantly and showed a prominent irregular electron-dense lamina region associated with the inner nuclear envelope membrane in the nucleus presented (Figs. 5C and D). The nuclear membrane of HEK293 cells stably transfected with wild-type lamin A/C gene was smooth, and the distribution of heterochromatin was integrated uniformly in the nucleus (Figs. 5A and B).

The lamin A mutant and emerin have dramatically abnormal intranuclear localization

To determine if the intracellular localization of mutant form of lamin A differed from the wild type, we transiently transfected HEK293 cells with plasmids expressing wild-type and mutated forms of lamin A. In the cells transfected with wild-type lamin A/C, both lamin A and emerin proteins were localized to the nuclear periphery (Figs. 6B and D), whereas cells transfected with mutant lamin A/C showed a decrease in nuclear rim localization, giving a more diffuse, nucleoplasmic staining (Figs. 6A and C). The expression levels of transfected cells were detected with specific antibodies (Fig. 6).

Discussion

A novel mutation Glu82Lys in the lamin A/C gene was associated with familial DCM in a large Chinese family. Eight living subjects were heterozygous for the Glu82Lys mutation. DCM was present in three of them (IV.3, IV.11, and IV.15) and one (IV.19) showed clinical phenotype of heart dilation and all of them associated without skeletal muscular dystrophy. Interestingly, in this family the DCM members (IV.3, IV.11, and IV.15) showed cardiac symptom presented with conduction system disease at their onset age of 32 or 33 years and progressed to higher levels of AV block at their 40s (IV.3 and IV.11), commonly requiring pacemaker implantation (IV.11). In an additional subject (IV.19), the onset of the cardiac phenotype was characterized by heart dilation and atrial fibrillation, but without DCM since left ventricular ejection fraction was preserved 55%. Two members (V.9 and V.22) in this family less than 30 years old presented with sinus arrhythmia and no other cardiac symptoms and other two young subjects (IV.40 and V.8) aged 25 and 27, heterozygous for the mutation had no signs or symptoms of heart disease. Two members (III.18 and IV.7) in the family history were deceased for congestive heart failure and conduction system disease

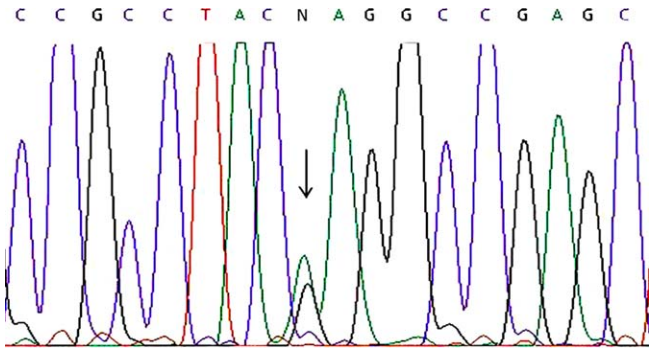

Fig. 2. The G to A nucleotide substitution (arrow) resulted in a missense mutation in the lamin A/C gene (Glu82Lys) in the proband.

at the age of 42 and 48 years. In this family, we observed those subjects carrying Glu82Lys mutation usually presented DCM and associated with conduction system disease at their early clinical onset (32- or 33-year-old) and rapidly progressed to severe cardiac symptom associated with class III AV block. This phenotype was identical with previous reports about the phenotype of lamin A/C mutations characterized by significant conduction defect associated with DCM [14–16]. But it was in contrast with the pure form of DCM, where atrial fibrillation usually followed the occurrence of DCM [17].

Lamin A/C mutations have also been associated with different phenotypes. Other than LV dysfunction and AV block [18], isolated cardiac form phenotype has been previously described as well [19]. Arg541Cys mutation detected in a French family resulted in a particular form of DCM in which myocardial dysfunction occurred at a later stage and apical aneurysm was the initial presentation with ventricular arrhythmia but without AV block [20]. Manuel and his colleagues found that Arg190Trp could also cause isolated left ventricular noncompaction associated with severe forms of DCM with the absent atrioventricular conduction defects [21]. Collectively, these observations suggest the correlation of lamin mutations with phenotypes. Studying these subtle genotype–phenotype correlations may facilitate a better understanding of the mechanisms of disease in DCM and other lamin-associated disorders.

Nuclear lamins are localized at the nuclear envelope, underlying the inner nuclear membrane [22]. They are organized in a meshwork of fibers which sometimes appears as well-organized lattice of near-tetragonally oriented filaments [10]. Nuclear lamins play a critical role in maintaining the structural integrity of the nuclear envelope. The functional role of lamin proteins, expressed in many tissues, has not been fully elucidated. Lamin A/C knockout mice appear normal at birth, but growth is

Table 1
The clinical characteristics of the patients

Patient	Age (years)	Sex	NYHA	ECG	LVEDD (mm)	EF (%)	Cardiac symptoms at onset (age)	Lamin A/C gene mutation
III-18	42[a]	M	IV	AVB(III°) , AF, Pace maker rhythm	70	12	32	
IV-3	43	M	III	AVB(III°)	78	45	33	Glu82Lys
IV-7	48[b]	M	IV	AV junctional escape beats, ST-T change			32	
IV-11	43	M	IV	SVT, AVB(III°), Pace maker rhythm	68	41	32	Glu82Lys
IV-15	38	M	III	AV junctional escape beats	60	43	32	Glu82Lys
IV-19	34	M	II	AF	55	55	32	Glu82Lys
IV-40	25	M	I	Normal	52	66	—	Glu82Lys
V-8	27	F	I	Normal	45	57	—	Glu82Lys
V-9	24	F	I	Sinus arrhythmia	47	59	—	Glu82Lys
V-22	12	F	I	Sinus arrhythmia	41	64	—	Glu82Lys

NYHA, New York Heart Association cardiac function classification; SVT, supraventricular tachycardia; AF, atrial fibrillation; AVB, atrioventricular block; LVEDD, left ventricular end-diastolic dimension; EF, ejection fraction.
[a] Patient died of heart failure at the age of 42 years (without DNA sample).
[b] Patient died of heart failure at the age of 48 years (without DNA sample).

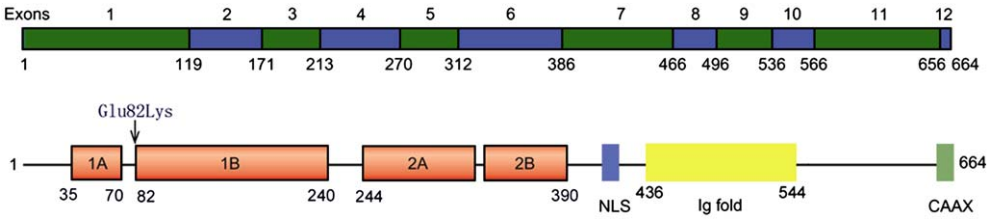

Fig. 3. Primary structural elements in lamin A. The 12 exons are indicated along with their corresponding amino acid residues. The central rod domain is identified by the subdomains 1A, 1B, 2A, and 2B. In addition, the nuclear localization signal (NLS) is shown as a blue box (residues 417–422), the immunoglobulin (Ig) fold is shown as a yellow box (residues 436–544), and a CAAX sequence is shown at the carboxyl terminus (green). The Glu82Lys mutation is located at the boundary of the subdomain 1B (arrow).

severely retarded, muscular dystrophy develops, and die at the age of 6–8 weeks [23].

The mutation described in our report results in a change in an amino acid residue (Gly82Lys) in exon 1 of lamin A/C gene, located in the coil 1B domain of central α-helical rod domain of the lamin A and the lamin C proteins (Fig. 3). This is highly conserved amino acid in IF proteins in various species (Fig. 4). The conserved regions of the rod domain have been shown to play crucial roles in the assembly of intermediate filament dimers into higher order oligomers [10]. Mutations affecting this region of IF proteins may disrupt the interaction between the monomers and are linked to several diseases [24]. We introduced the mutant lamin A/C into HEK293 cells and observed loss of integrity of the nuclear envelope, local collapse or bulge, and partial fragmentation. Members of the IF superfamily are critical mechanical integrators of the nuclear membrane and the cytoskeleton, protecting the cell from repeated mechanical stress. Mutations in the lamin A/C gene may cause cardiomyopathy by weakening nuclei [25,26]. The etiology of EDMD and DCM with conduction system disease might be explained by an accumulation of damaged nuclei as a result of a reduction in load-bearing properties of the nuclear lamina [9].

Similar features had been reported in the skeletal muscle cells of patients with EDMD caused by emerin or lamin A/C gene mutations [9,27]. Introduction of LAΔ50(a point mutation in the lamin A/C gene resulted in a protein lacking 50 amino acid near the C-terminus, denoted LAΔ50) into normal cells by transfection or protein injection causes significant abnormality in nuclei, including loss of peripheral heterochromatin [28]. Nuclear fragility is observed in the nuclei of embryonic fibroblasts obtained from mice lacking lamin A. Xenopus nuclei assembled from egg extracts with the lamin A mutant show nuclear fragility as compared to the nuclei of wild-type cells [23,29]. Increased fragility of nuclei could be particularly harmful to muscle cells and lead to muscle-specific disorders as

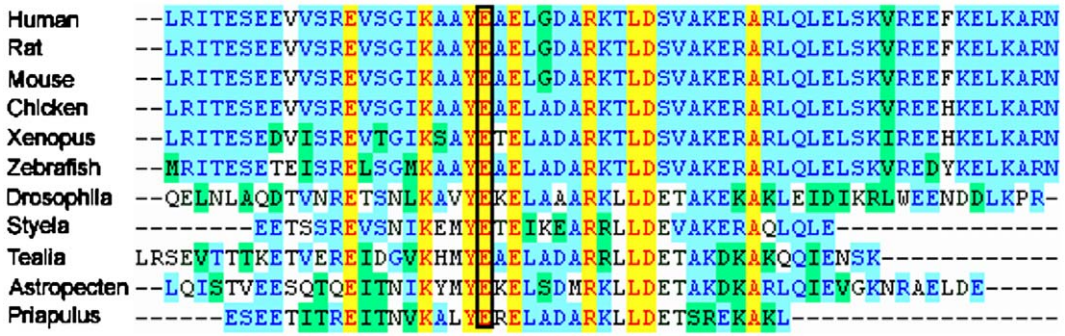

Fig. 4. Multialignment of partial of lamin A protein. Identical amino acids are in red, conservative in blue, and weakly similar in green. The 82th residue glumatic acid (black pane) of lamin A protein was highly conservative in the different species.

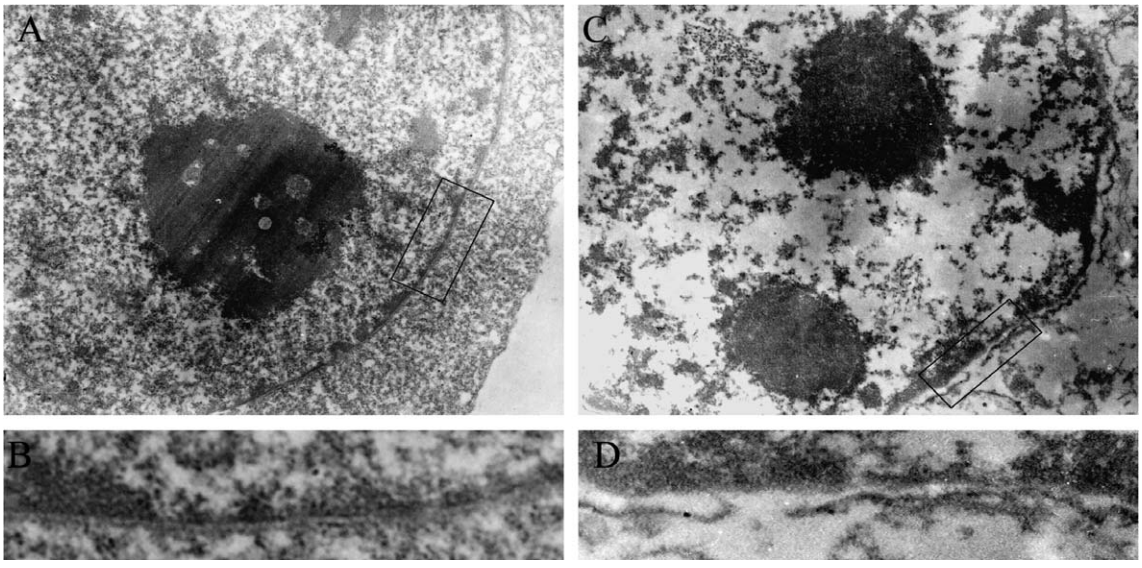

Fig. 5. Electron microscopic examination of nuclear membranes. (A,B) Cells transfected with wild-type lamin A/C gene showed integrated and smooth nuclear membrane, chromatin distributed uniformly in nucleus, (B) was magnified of black pane in (A). (C,D) The outer nuclear membrane of mutant cells showed collapse or bulge and partial fragmentation, aggregation of peripheral heterochromatin, and a prominent electron-dense lamina region associated with the inner nuclear envelope membrane, (D) was magnified of black pane in (C). Magnification, (A,C) (12,000×); (B,D) (24,000×).

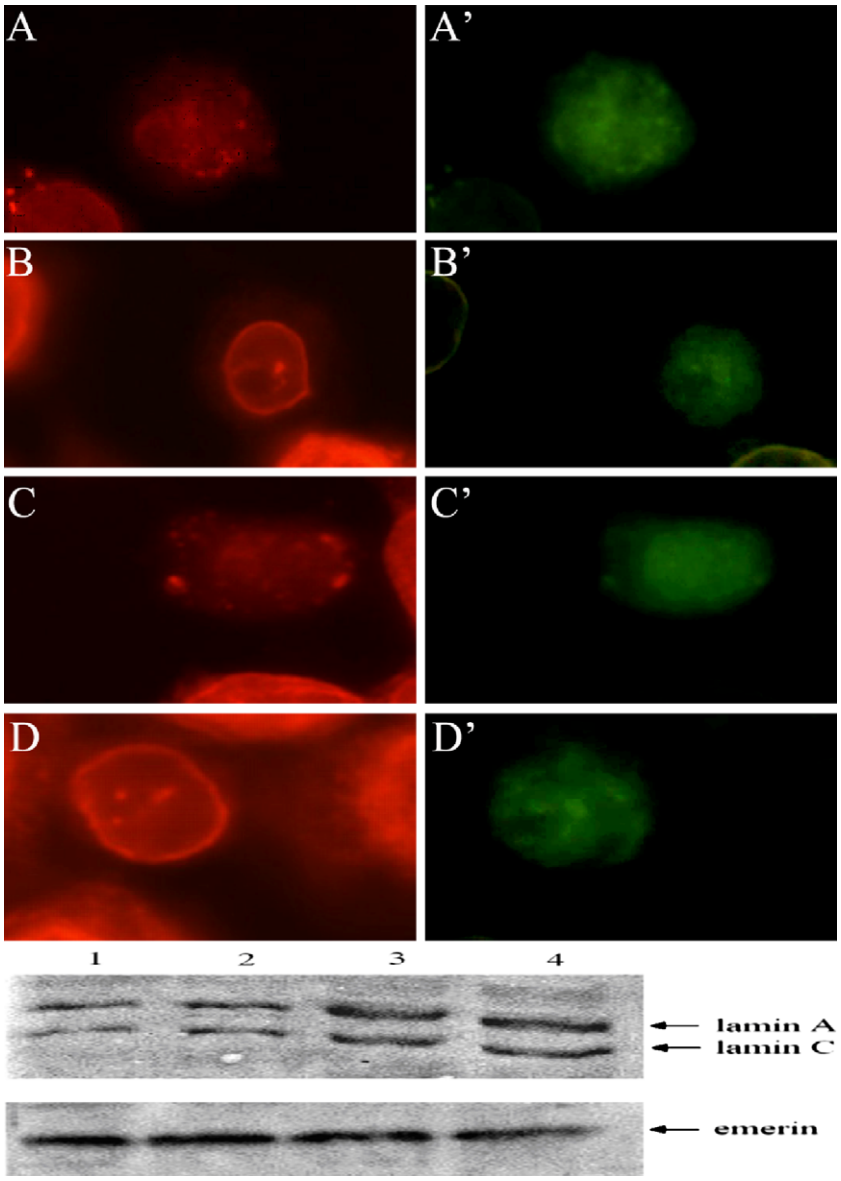

Fig. 6. Cellular localization of wild-type and mutant forms of lamin A and emerin protein. The panels show immunofluorescence microscopy images of HEK293 cells transiently transfected with wild-type lamin A/C (B) or with mutation lamin A/C (A), images (A,D) show emerin protein localization in cells transiently transfected with mutation and wild-type lamin A/C, respectively. Images (A′,C′) and (B′,D′) show GFP of cells transiently transfected with mutation and wild-type lamin A/C, respectively. Antibodies used were polyclonal antibodies against lamin A or emerin recognized by TRITC-conjugated secondary antibodies (red). The mutated lamin A localizes aberrantly (A) and causes the emerin to delocalize (C). Magnification, 400×. The bottom panel shows detection of lamin A/C and emerin protein with specific antibodies. Lanes 1–4 show whole cell extracts from cells when untransfected, transfected with empty vector, wild-type, and mutant lamin A/C, respectively.

EDMD and cardiomyopathy. Forces generated during muscle contraction might potentially lead to preferential breakage of nuclei containing a defective nuclear lamina [9].

Wild-type lamins and emerin protein are co-localized to the inner nuclear membrane in normal cells. However, the mutant lamin A protein introduced into cells is localized aberrantly and emerin was also distributed abnormally with lower localization at nuclear rim and more diffusion in the nucleoplasm.

Some evidence showed that lamin A might be involved in nuclear targeting of emerin. Lamin A has been found to bind to emerin directly in vitro [30]. In cells from mice lacking lamin A, emerin is mislocalized to the cytoplasm [23]. Östlund transfected four lamin A/C gene mutations (Arg60Gly, Leu85Arg, Asn195Lys, and Glu203Gly) which are identified in patients with DCM into C2C12 mouse myoblasts. The Asn195Lys lamin A mutant showed a dramatically aberrant localization in cells and emerin was also not localized properly [31]. The proposed roles for

lamins in replication and transcription are related to the lamins association with chromatin. In addition to directly binding to chromatin, lamins interact with chromatin via their connections to lamin-associated proteins [13,32]. Besides emerin, several other nuclear proteins such as LAP2, BAF were involved in gene expression, either at the level of transcription, splicing or mRNA transport [32,33]. Our findings also showed that mutant lamin A induced into cells caused aberrant heterochromatin aggregation. Lamina exerts a profound influence on the organization of heterochromatin. Heterochromatin is generally silent in terms of transcriptional activity but changes in chromatin organization could lead to changes in gene expression programs with potentially deleterious effects on muscle disorders [12,34].

In summary, lamin A/C gene mutation Gly82Lys is linked to malignant dilated cardiomyopathy, and may be related to the abnormality of nuclear structure. The intrafamily heterogeneity in clinical phenotype by the same mutation still needs to be determined.

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