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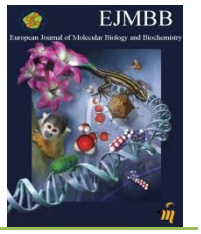

A RARE MUTATION IN LAMIN A GENE IS ASSOCIATED WITH DILATED CARDIOMYOPATHY IN INDIAN PATIENTS

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ABSTRACT
Mutations in lamin A gene (*LMNA*) are associated with a number of genetic diseases that are collectively termed laminopathies. Most *LMNA* mutations cause muscular dystrophies and cardiomyopathies. The incidence of *LMNA* mutations in familial dilated cardiomyopathy (DCM) patients is 5-8% in Caucasian populations. However, there is no large scale study of *LMNA* mutations in Indian DCM patients. Hence, we have carried out sequence analysis of *LMNA* in 239 Indian DCM patients. We have identified a rare non-synonymous mutation c.1873A>T in one patient, which predicted a change in the amino acid serine to cysteine at residue 625. In addition we also identified 20 synonymous single nucleotide polymorphisms in 28 patients. The c.1873A>T mutation was absent in 156 healthy and ethnically matched controls. The serine at position 625 has been earlier identified as a mitotic phosphorylation site in lamin A. Expression of mutant lamin S625C in cultured cells led to a decrease in levels of cyclin dependent kinase inhibitor p21, suggesting compromised cell cycle regulation in these cells. Our study provides important information on the extent of variations in *LMNA* in Indian DCM patients and identifies a rare mutation in *LMNA* that is likely to cause deleterious effects on cellular functions.

INTRODUCTION

The human lamin A gene (*LMNA*) has been mapped to chromosome 1q21.2-q21.3 and encodes two splice variants lamin A and lamin C. Mutations in *LMNA* cause a spectrum of genetic diseases collectively termed laminopathies. Approximately 400 disease-causing mutations have been identified in *LMNA*, which are associated with at least 15 different diseases. Most mutations in *LMNA* cause Emery-Dreifuss muscular dystrophy (EMD) and dilated cardiomyopathy (DCM), while other mutations have been linked to limb-girdle muscular dystrophy (LGMD), lipodystrophy and progeria, and certain mutations are associated with overlapping phenotypes [1-3].

Lamins are components of a filamentous network termed the nuclear lamina that is located beneath the inner nuclear membrane and extends into the interior of the nucleus [4-7]. Lamins belong to the type V family of intermediate filament proteins and are present only in metazoan cells. Lamins are important for maintaining the structural integrity of the nucleus and are involved in chromatin organization and spatial organization of various nuclear functions such as transcription and replication as well as mitotic assembly and disassembly of the nucleus. Lamins are of two types; the A-type lamins, lamin A (664 amino acids) and lamin C (574 amino acids), which are encoded by *LMNA* and alternatively spliced at exon 10, are expressed in most differentiated cells while the B-type

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lamins, lamin B1 and B2 (encoded by separate genes) are expressed in all somatic cells. Most disease-causing mutations have been identified in lamin A; only a few mutations have been reported in the B-type lamins. At the cellular level, mutations in lamin A affect DNA repair, replication and gene regulation, and also trigger proteasomal degradation of key regulatory proteins. Mutations in the central rod domain of the protein affect lamina assembly while mutations in the N-terminal and C-terminal globular domains are likely to hinder binding to specific proteins.

DCM is characterized by ventricular chamber dilation and systolic dysfunction and is associated with heart failure, arrhythmias and sudden cardiac death. Approximately 30-50% of idiopathic DCM cases have been attributed to mutations in any one of ~ 40 genes, which include genes coding for sarcomeric proteins, mitochondrial proteins, cytoskeletal proteins and nuclear proteins [8]. In 1999, Bonne and co-workers first reported that mutations in *LMNA* caused EMD that was characterized by muscle weakness, tendon contractures and dilated cardiomyopathy [9]. Subsequently Fatkin and co-workers have shown the association of certain mutations in *LMNA* with DCM without skeletal muscle involvement [10]. Studies on large cohorts of Caucasian populations indicate that 5-8% of DCM patients harbour mutations in *LMNA* [11-13]. Most families display an autosomal dominant pattern of inheritance, with variable penetrance. DCM due to *LMNA* mutations is usually accompanied by conduction system disease.

There is presently very little information on the prevalence of disease-causing *LMNA* mutations in Indian patients with cardiomyopathy [14, 15]. Identification of patients with *LMNA* mutations would help in early diagnosis and proper clinical management of the disease, especially important as mutations in *LMNA* predict a high risk of sudden cardiac death [16]. Therefore, in the present study, we screened for *LMNA* mutation(s) in 239 DCM patients along with 156 ethnically matched controls. We identified a rare missense mutation and expressed the mutant protein in cultured cells to estimate the levels of key cell cycle regulatory proteins.

MATERIALS AND METHODS

A total of 239 DCM patients from Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India were recruited for the study, after informed consent was obtained. All the patients belonged to Indo-European linguistic group. Individuals were diagnosed with DCM based on clinical examination, which include physical evaluation, echocardiography and family history; based on the NYHA (New York Heart Association, 1994), and WHO (www. who.int/ cardiovascular diseases) guidelines. This study conforms to the principles outlined in the Declaration of Helsinki (WMA World Medical Association Declaration of Helsinki) and was approved by the Institutional Ethics Committee (IEC) of both the participating institutes. In addition, 156 healthy volunteers from the same ethnical background who had no family history or symptoms of cardiovascular disease and were not related to the patients were recruited as controls.

DNA sequence analysis

DNA was isolated from peripheral blood lymphocytes. The exons and exon-intron boundaries of the lamin A gene were amplified by PCR using seven sets of primers given in Table 1. The amplified PCR products were isolated from gels using a Qiax-II gel extraction kit (Qiagen, Limburg, Netherlands), sequenced using the Big Dye Terminator kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI3730 DNA Analyzer (Perkin Elmer, Applied Biosystems). The sequences obtained were compared with the reference *LMNA* sequence (NG 008692.2) using NCBI BLAST. Coding DNA sequences were compared to *LMNA* transcript 1 encoding lamin A (NM 170707.2) to detect amino acid alterations.

Protein expression studies

The c.1873A>T mutation was introduced into an available lamin A cDNA construct by PCR-based mutagenesis. The lamin A wild-type and mutated construct were cloned into the pEGFP mammalian expression vector. The human embryonic kidney cell line HEK293T was maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Plasmid constructs were transiently transfected into cells for 24 h by lipid-mediated transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) by standard protocols, which yielded ~ 80% transfected cells. After 24 h of transfection, cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature. Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI). Confocal laser-scanning fluorescence microscopy of fixed cells was performed on a Zeiss LSM510 META/NLO confocal microscope (Carl Zeiss, Oberkochen, Germany). DAPI staining of nuclei was viewed in the transmission mode. Images were analyzed with LSM510 META software and assembled using Photoshop CS3. Lysates of transfected cells were analyzed by western blot assays using antibodies to lamin A/C and lamin B1 (Abcam, Cambridge, MA, USA), retinoblastoma protein (pRb, Abcam), phosphorylated pRb-ser807/811 and pRb-ser780 (Cell Signaling Technology, Beverly, MA, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Abcam) and p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RESULTS

LMNA mutation analysis

Molecular screening for mutations in *LMNA* was performed on an Indian cohort of 239 patients who were

recruited with a clinical diagnosis of DCM, as well as 156 healthy, unrelated and ethnically matched controls. The *LMNA* gene was sequenced using primers that amplified all the 12 exons and smaller introns of the gene, as well as the exon-intron boundaries of the larger introns (introns 1 and 2). The sequences were aligned using the *LMNA* gene sequence available in NCBI as the reference sequence. The sequence variants obtained were queried in the NCBI SNP database and lamin mutation databases http://www.umd.be/LMNA and http://www.dmd.nl/lmna_seqvar.html (Leiden Open Variation database) to determine whether they had been reported earlier and shown to be pathogenic (disease-causing) or not. Our analysis revealed 20 synonymous SNPs in 28 patients (Table 2). The SNPs c.1698C>T (exon 10), c.*79G>C (exon 12, in 3'UTR) and c.1157+16G>A (intron 6) were more prevalent than the other SNPs, and these SNPs have been observed earlier in global populations of both patients and healthy subjects at a high frequency. Both of our patients showing the c.*79G>C SNP also harboured the SNP c.1157+16G>A in intron 6. Other SNPs were not associated with each other in the same patient. The exon 12 SNP was also found in four controls in our analysis but the other SNPs were not detected in our controls. Polymorphisms at c.597 and c.1146 have been reported in the databases, while the remaining SNPs have not been reported in the databases. All the variants were heterozygous, except for two cases with homozygous variant c.1698C>T.

A single putative disease-causing heterozygous mutation c.1873A>T in exon 11 of *LMNA* was identified exclusively in one patient (Fig. 1A). This is a rare mutation that has not been reported in the databases, though a change at the same base c.1873A>C has been reported in one case but its significance was not clear. The c.1873A>T mutation predicts a change in amino acid serine to cysteine at position 625. Serine 625 has been reported to be a mitotic phosphorylation site in lamin A [17]. This residue is conserved in lamin A from several mammalian species as well as Xenopus and zebrafish lamin A (Fig. 1B). The replacement of serine by cysteine which cannot be phosphorylated by mitotic protein kinases is expected to have deleterious consequences. No synonymous SNPs were identified in this patient. No pathogenic mutations in *LMNA* were found in the remaining patients or controls.

The patient harbouring the c.1873A>T mutation was a 48-year old female with symptoms of DCM. In the patient, echocardiography revealed mild left ventricular dysfunction (LVEF 50-55%) and left ventricular dilation (LVED 53 mm). The patient was diagnosed with moderate mitral regurgitation and mild aortic regurgitation. Clinical evaluation of her family indicated that the proband had a brother who also showed symptoms of DCM. However his blood sample was not available for mutation analysis.

Expression analysis of mutant lamin S625C

Further experiments were carried out in cultured cell lines with a mutant lamin S625C expression construct to investigate the cellular effects of expressing this mutation. The cellular localization of GFP-tagged wild-type lamin A and mutant lamin S625C was first determined since several mutations in lamin A disrupt lamina assembly and lead to aberrant lamina localization and nuclear abnormalities. In this instance, both wild-type lamin A and lamin S625C assembled normally at the nuclear periphery as a smooth nuclear rim (Fig. 2A). There was also no discernible increase in levels of the mutant lamin in the nucleoplasm, as observed with lamin A mutated at certain sites that are normally phosphorylated during interphase [18].Serine 625 has been earlier identified as a conserved mitotic phosphorylation site in the tail region of lamin A [17]. Since protein phosphorylation is an important regulator of cell cycle progression and defects in mitotic phosphorylation of lamin A may lead to cell cycle defects, we studied expression of the important cell cycle-dependent proteins pRb and Cdk inhibitor p21 by western blot analysis of cells expressing GFP-tagged wild-type lamin A or mutant lamin S625C (Fig. 2B). Total pRb as well as phosphorylated pRb-ser807/811 and pRb-ser780 that are differentially phosphorylated during the cell cycle were analysed. There was a 60% decrease in p21 levels in cells expressing lamin S625C but no alteration in levels of pRb or its phosphorylated forms. These data suggest a compromise in cell cycle regulation in cells expressing lamin S625C.

Table 1. List of PCR primers

Exon	Forward primer 5' to 3'	Reverse primer 5' to 3'
Exon 1	GTCTCTGTCCTTCGACCCGAGCCCCGC	CCCCAACTTGTCCCTGATACCCCCACC
Exon 2	CCCAGAGGCAAGCAGATGCA	GGTAGATCCCATTGGCAGCC
Exon 345	CCTTCCCTGGACCTGTTTCCACATGTG	GCCATCTGACTCCACATC CTGCGACCC
Exon 67	GGCTCTGGGGAAGCTCTGAT	GGGCAAGGATGTTCCTCTCTC
Exon 8910	GGGGTGTGTGTAGATGGAAGGAGAGGC	GTATAGGGAGGAGAGAGAAGAAAGGCC
Exon 11	GGGCACAGAACCACACCTTC	CAGACAAGAGGGGCAGGATG
Exon 12	AGGGCTGGAGTGTGAGGGAT	GAGCAGGAGGATGCAGTGAC

Table 2. Synonymous SNPs identified in DCM patients

Location	Genome position	Nucleotide (aa)	No of Patients
Exon 1	g.5315	c.66G>C (S22S)	One
Exon 1	g.5552	c.303C>G (R101R)	One

Exon 2	g.20984	c.393G>A (Q131Q)	One
Exon 2	g.21101	c.510C>G (A170A)	One
Exon 3	g.24736	c.516T>A (L172L)	One
Exon 3	g.24817	c.597C>A (T199T)	One
Exon 3	g.24850	c.630C>A (I210I)	One
Exon 4	g.25171	c.675A>T (R225R)	One
Exon 4	g.25240	c.744C>A (L248L)	One
Exon 5	g.25535	c.828G>A (Q276Q)	One
Exon 5	g.25607	c.900C>T (D300D)	One
Exon 6	g.26441	c.1146G>C (G382G)	One
Exon 6	g.26468	c.1157+16G>A	Three
Exon 7	g.26638	c.1251A>G (K417K)	One
Exon 8	g.27260	c.1389C>T (S463S)	One
Exon 8	g.27332	c.1461C>T (F487F)	One
Exon 9	g.27464	c.1509G>C (G503G)	One
Exon 10	g.28009	c.1632G>T (V544V)	One
Exon 10	g.28074	c.1698C>T (H566H)	Eight
Exon 12	g.29516	c.*79G>C	Two

Fig 1. *LMNA* mutation analysis. (a) Chromatograms of partial *LMNA* DNA sequence from normal and patient (mutant); arrow indicates A>T heterozygous mutation at position 1873 bases in exon 11. (b) ClustalW2 multiple sequence alignment of lamin A from eight species; arrow indicates conserved serine at position 625 in human lamin A protein

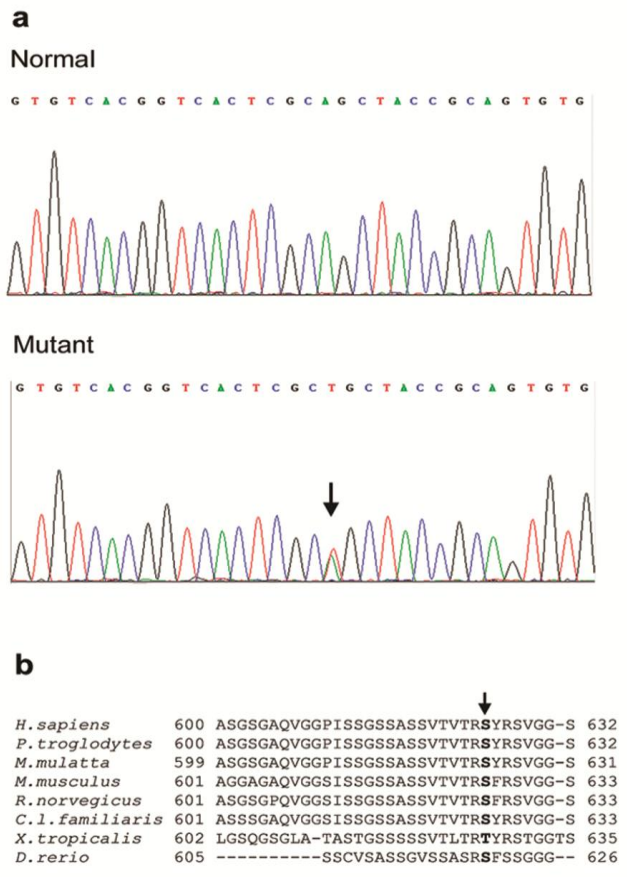
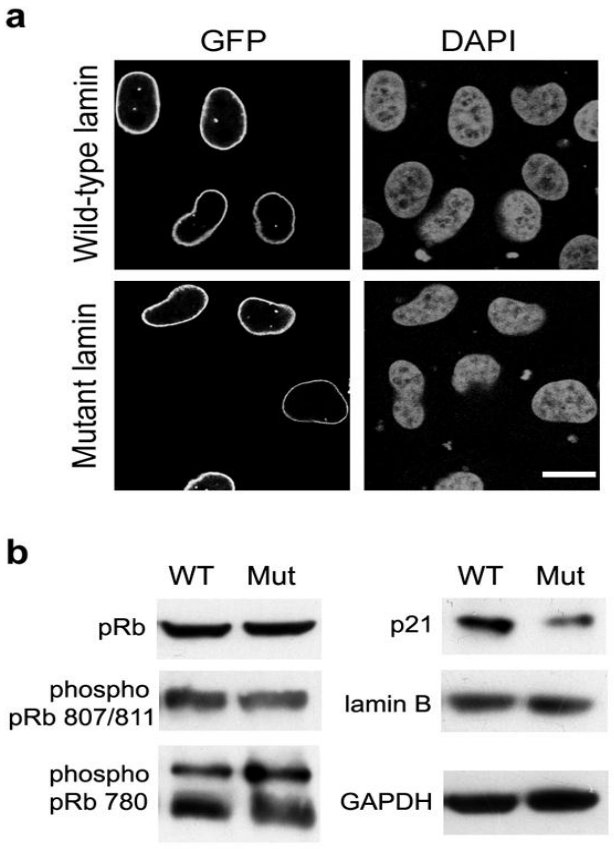


Fig 2. Expression analysis of wild-type and mutant lamin S625C. (a) Localization of GFP-tagged wild-type and mutant lamin S625C. Bar, 10 µm. (b) Western blot analysis of lysates from cells expressing wild-type or mutant lamin S625C, using antibodies to the indicated proteins



DISCUSSION

In this study, we report a rare *LMNA* mutation c.1873A>T in a patient with DCM, which is predicted to cause a S625C mutation at a conserved residue in lamin A that is phosphorylated in mitotic cells. Expression of the mutant lamin S625C in cultured cells led to reduction in the levels of the Cdk inhibitor p21.

LMNA mutations associated with DCM have been identified in almost all exons of the gene. Most are missense heterozygous mutations, while a few double-heterozygous mutations, deletions and nonsense mutations have also been identified. Patients with certain mutations at the C-terminus show overlapping phenotypes of lipodystrophy, progeria and cardiomyopathy, as well as muscle weakness [12]. DCM due to *LMNA* mutations may accompany EMD or LGMD or show minimal skeletal muscle involvement. Though most *LMNA* mutations in DCM patients are associated with a severe clinical phenotype, subtle effects of certain mutations also need to be monitored carefully due to the risk of sudden cardiac death [3]. Our patient with a c.1873A>T mutation displayed mild to moderate symptoms of DCM with LV systolic dysfunction; however no skeletal muscle involvement and no detectable conduction system disease was identified.

We also identified 20 synonymous SNPs in the patients which were non-pathogenic by themselves. However these SNPs might modulate the risk associated with mutations in the gene. Of special interest is the SNP c.1698C>T that occurs adjacent to the lamin A/C splice junction of exon 10. This SNP has been associated with increased weight and body mass index and shown to regulate the ratio of lamin A to lamin C under certain conditions [19]. It is a highly prevalent SNP in global populations and has been found in patients with mandibuloacral dysplasia harbouring a R527H mutation in *LMNA* [20]. The variants c.1157+16G>A and c.1698C>T are frequently present in Caucasian subjects and have also been reported in a separate group of ten Indian DCM patients [15]. Since the remaining SNPs we have identified have not been reported in the databases, these SNPs might be less frequent in Caucasians. A more exhaustive study would need to be carried out in order to infer that these SNPs are exclusively present in Indian populations.

In Caucasian patients, approximately 5-8% of familial DCM cases have been attributed to mutations in *LMNA* [11-13]. In these populations the three most prevalent genes involved in DCM are *LMNA*, cardiac β-myosin heavy chain (*MYH7*) and cardiac troponin T (*TNNT2*) [8]. However, we observed only one case with a putative pathogenic mutation in *LMNA* out of 239 patients (0.4%) in an Indian cohort. There is also a single report of an *LMNA* mutation in a Japanese family with DCM [21]. On the other hand, DCM patients from South Asia have a high prevalence of mutations in cardiac myosin binding protein C, *MYBPC3* [22] and *RAF1* [23] as well as *MYH7* (Rani *et al.* unpublished data).

The lamin protein undergoes a number of post-translational modifications such as phosphorylation, ubiquitylation, acetylation, glycosylation and SUMOylation [24]. Lamin A is extensively phosphorylated during mitosis as well as interphase. Approximately 50 Ser/Thr residues have been identified in lamin A as sites for mitotic phosphorylation by protein kinases by quantitative phosphor-proteomics of nocadazole-treated mitotic HeLa cells [17]. These sites are located in the N-terminal and C-terminal regions flanking the central rod domain and in the tail domain of the protein. Mitotic phosphorylation of lamins leads to depolymerization of the lamina and is induced by cyclin dependent kinase 1 [25-27], while interphase phosphorylation has been attributed to various kinases including protein kinase C and Akt kinase [18,28,29]. The role of critical serine and threonine residues flanking the central rod domain in lamina depolymerization and lamina assembly has been established by mutational analysis [18,25]. However, the role of mitotic phosphorylation of sites in the tail domain is not well understood. Our data suggests that mutation of Ser625 decreases expression levels of the Cdk inhibitor p21 and is likely to affect cell cycle regulation. Mice lacking p21 develop normally but are defective in normal G1 checkpoint control in response to DNA damage [30] while accumulation of Cdk inhibitors like p21 leads to cell cycle arrest primarily at the G1/S transition [31].

There is considerable interest in understanding how mutations in *LMNA* cause laminopathic diseases. An emerging concept in lamin pathogenesis is that laminopathic mutations hinder interactions of lamins with key regulatory factors and alter signaling pathways involved in several cellular functions such as cell proliferation, cell differentiation and response to DNA damage and stress [1-7]. *LMNA* mutations in the central rod domain of the protein cause substantial changes in nuclear morphology and lamin-chromatin interactions due to their effects on lamina assembly, as most of these mutations hinder lamin dimer association. Mutations that permit normal assembly of the lamina are likely to disrupt other functions of the lamina, by affecting binding to key regulatory proteins or altering functionality of a site that normally harbours a post-translational modification, as we have observed with the S625C mutation.

CONCLUSIONS

In conclusion, we report for the first time a rare mutation in *LMNA* (c.1873A>T) in one patient out of a cohort of 239 Indian DCM patients. Expression of a mutant lamin A protein harbouring the predicted S625C mutation in a mitotic phosphorylation site leads to reduction in levels of the key cell cycle inhibitor p21. It would be informative to study the effects of this mutation on tissue development in an *in vivo* model.

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CONFLICT OF INTEREST: Nil

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