A LMNA SPLICING MUTATION IN TWO SISTERS WITH SEVERE DUNNIGAN-TYPE FAMILIAL PARTIAL LIPODYSTROPHY (FPLD2)

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SHORT TITLE: FPLD2 LMNA SPLICING MUTATION

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ABSTRACT

Context: To date, all cases of familial partial lipodystrophy type 2 (FPLD2, MIM 151660) result from missense mutations in LMNA, which encodes for nuclear lamin A/C (MIM 150330).

Objective: To carry out mutational analysis of LMNA in two sisters with a particularly severe FPLD2 phenotype.

Design: Descriptive case report with molecular studies.

Setting: Referral Center.

Patients: We report two sisters of South Asian origin. The first presented with acanthosis nigricans at age 5 years, diabetes with insulin resistance, hypertension and hypertriglyceridemia at age 13 years and partial lipodystrophy starting at puberty. Her sister and their mother had a similar metabolic profile and physical features, and their mother died of vascular disease at age 32.

Interventions: None.

Main outcome measures and results: LMNA sequencing showed that the sisters were each heterozygous for a novel G>C mutation at the intron 8 consensus splice donor site, which was absent from the genomes of 300 healthy individuals. The retention of intron 8 in mRNA predicted a prematurely truncated lamin A isoform (516 instead of 664 amino acids) with 20 nonsense 3'-terminal residues. The mutant lamin A isoform failed to interact normally with emerin and failed to localize to the nuclear envelope.

Conclusions: This is the first LMNA splicing mutation to be associated with FPLD2, and it causes a severe clinical and metabolic phenotype.
Lipodystrophies are characterized by loss of fat in specific anatomical sites (1,2). There are two types of Dunnigan-type familial partial lipodystrophy (FPLD): FPLD2 (MIM 151660) results from heterozygous mutations in \( LMNA \) (MIM 150330), encoding nuclear lamin A/C, while FPLD3 (MIM 604367) results from heterozygous mutations in \( PPARG \) (MIM 601487), encoding peroxisomal proliferator-activated receptor (PPAR)\( \gamma \) (2). FPLD2 is a laminopathy and is one of 16 distinct disease phenotypes that have been shown to result from more than 100 different \( LMNA \) mutations, including 12 autosomal dominant (AD) and four autosomal recessive (AR) phenotypes (3). The mutation position in the primary genomic DNA sequence of \( LMNA \) is associated with tissue and organ pathology (3). To date, more than 90% of \( LMNA \) mutations are missense mutations (3). All reported mutations in FPLD2 are \( LMNA \) missense mutations, usually occurring within the 3’-half of the protein (3). A few heterozygous nonsense mutations underlie some cases of dilated cardiomyopathy (3) and Emery-Dreifuss muscular dystrophy (3). Heterozygous \( LMNA \) splicing mutations have been reported in some cases of progeria (4), restrictive dermopathy (5) and limb girdle muscular dystrophy (6). We report the first individuals with FPLD2 who are heterozygous for a novel \( LMNA \) mutation that affects RNA splicing and who initially presented with strikingly severe clinical and metabolic features of FPLD2.

**SUBJECTS AND METHODS**

Study subjects
Proband 1. The index patient, a female of South Asian ancestry, was referred to the Medical Genetics clinic at age 20. Her medical history revealed the presence of acanthosis nigricans starting at age 5. Hypertension, hypertriglyceridemia and hyperinsulinemia were diagnosed at age 13, and she developed type 2 diabetes (T2DM) soon thereafter. At age 13, significant facial and body hirsutism, a male body habitus and moderately severe acne were noted, and hidradenitis suppurativa was diagnosed after removal of cysts from pubic and axillary regions. She had menarche at age 9, with secondary amenorrhea since age 10. At age 10 she was diagnosed with depression. A renal ultrasound at age 13 and head CT scan at age 14 were both normal. At age 14, an electrocardiogram (EKG) showed prolongation of the QT interval with diffuse repolarization abnormalities, while an echocardiogram was normal. Acute pancreatitis secondary to hypertriglyceridemia resulted in one hospital admission at age 17, at which time no hepatomegaly was seen on abdominal CT scanning. At age 21, she had a vocal cord polyp removed; an EKG revealed normal sinus rhythm and a persantine myocardial perfusion study was normal.

On physical examination at age 20 she was noted to have a round face and a deep and hoarse voice. She had generalized acanthosis nigricans, skin tags and cysts in axillary and pubic regions, severe lipodystrophy with muscle and labia majora pseudohypertrophy, prominent veins, absence of gluteal fat, hirsutism and Cushingoid habitus, but without catabolic or other somatic manifestations.
Proband 2. The sister of proband 1 was first assessed in the Medical Genetics clinic at age 26. She was diagnosed with T2DM, hypertriglyceridemia and hypertension at age 17. EKG at age 17 revealed a left ventricular strain pattern, although an echocardiogram was normal. At age 18, she was noted to have numerous skin findings, including velvety light-brown papillomatous hypertrophic plaques over the nape and sides of her neck, axillae and groin, verrucous hyperkeratosis of the flexural regions of the elbows, dorsum of hands and ankles and numerous skin tags over neck and axillae. At age 19, she was hospitalized with acute pancreatitis, secondary to hypertriglyceridemia, which was complicated by a pseudocyst requiring subtotal pancreatectomy. An abdominal CT scan showed diffuse fatty liver and hepatomegaly. Upon starting insulin treatment, the dermatologic changes regressed, leaving only mild acanthosis nigricans. Menses were normal. Bilateral sixth cranial nerve palsies occurred on two occasions. She was also diagnosed with depression during her adolescence. At age 24, she noted lower extremity weakness with decreased endurance. A neurological evaluation demonstrated proximal muscle weakness of both upper and lower extremities, decreased temperature and pain sensation of the lower limbs and decreased deep tendon reflexes. An electromyogram (EMG) study revealed non-specific abnormalities consistent with a myopathic process. Serum creatine kinase was not elevated.

On physical examination at age 26, she was noted to have a round face, mild nuchal acanthosis nigricans, skin tags, severe lipodystrophy with muscle pseudohypertrophy, prominent veins, absence of gluteal fat and hirsutism. Her
lipodystrophy phenotype was less severe than that of proband 1. Like her sister, she reported a voracious appetite and heat intolerance.

*Probands’ mother.* The history of the probands’ mother was obtained from medical records. At age 23, atrial fibrillation was diagnosed and treated with digoxin. She did not receive anticoagulation therapy. At age 25, she complained of recurrent abdominal pain and was diagnosed with ‘paroxysmal tachycardia’. Hepatomegaly and elevated serum transaminases were reported on several occasions. Investigations at age 27 for suspected muscle atrophy revealed a normal neurological examination, EMG and serum creatine kinase concentration. A muscle biopsy showed small type 1 fibers and increased connective tissue, consistent with a benign fiber type disproportion syndrome. T2DM was diagnosed at age 28, however, the lipid profile result was unavailable. She had persistent severe acne. At age 28, she presented with right hemiparesis caused by a left cerebrovascular accident of cardiogenic origin. Echocardiography revealed a bicuspid aortic valve, mitral stenosis and cardiomyopathy that was considered secondary to valvular disease. Anticoagulation was started and a cardiac pacemaker was placed the next year. Compliance with medication was inconsistent. At age 32, she presented with a massive right hemisphere infarct. At the time, she was noted to be hypertensive, and to have multiple papulo-pustular lesions over her back, chest and extremities, hirsutism, and significant atrophy of muscles on extremities with preservation of girdle musculature. She died of respiratory failure shortly thereafter.
Review of childhood photographs of the sisters and their mother suggested that they had normal fat distribution as children, with lipodystrophy beginning at the onset of puberty.

*Other family history.* The probands’ non-consanguineous parents were of Punjabi origin. A maternal aunt had a cardiac problem but no diabetes or lipodystrophy. A maternal uncle had T2DM since age 60 and his son had diabetes since age 3. The maternal grandfather had longstanding hypertension. The girls’ father had hypertension and two paternal aunts and the paternal grandmother had diabetes.

**Anthropometric measurements**

Height and body weight were measured by standard procedures. Skinfold thickness was measured with a Lange caliper (Cambridge Scientific Industries, Cambridge, MD) at five truncal (chest, mid-axillary, abdomen, subscapular and suprailliac), and four peripheral (biceps, triceps, mid-thigh and calf) sites on the right side of the body. The mean of three repeat measurements at each site was calculated. Circumferences were also obtained of the chest, waist, hip, mid-arm, mid-thigh, and calf. Skinfold thickness and circumference measurement results were compared with the U.S. population reference values for the same age and sex-group, in percentile (7). The percentage of total body fat was determined by bioelectrical impedance analysis (BIA) under fasting conditions, as described (8), and from the sum of skinfold thicknesses measured at four sites (9). Skeletal muscle mass was estimated from BIA (10).
Genomic DNA analysis

The study was approved by the ethics review panel of the University of Western Ontario (protocol #07920E). Both patients provided informed consent to participate in the studies and for publication of their clinical, biochemical and molecular genetic information. The coding regions and intron-exon boundaries of the LMNA gene were amplified, purified and genomic DNA sequence was read on a 3730 Automated DNA Sequencer (PE-Applied Biosystems, Mississauga, ON) using established protocols (11). Screening for the mutation employed direct sequencing of exon 8; genomic DNA from 150 healthy individuals each of Caucasian and South Asian ethnicity was examined.

Gene expression analysis

Total RNA was isolated from patient whole blood using the PAXgene blood RNA kit (Qiagen, Mississauga, ON). First strand cDNA was synthesized from total RNA with an oligoT primer (Superscript First-strand Synthesis System, Invitrogen, Burlington, ON). Two µl of the first strand was amplified in a total of volume of 30 µl containing specific LMNA primers: 5′-GTC AGT CTG CTG AGA GGA AC and 5′-GAC ACT GGA GGC AGA AGA GC, which span from exon 5 to exon 11, inclusive of LMNA cDNA. Thirty amplification cycles were performed at an annealing temperature of 60°C. The PCR products were gel purified (QIAquick gel extraction kit, Qiagen, Mississauga, ON) and directly
sequenced on an ABI 3730 Automated DNA Sequencer (PE-Applied Biosystems, Mississauga, ON).

Recombinant vector constructions

A 2.1 kb fragment containing human LMNA full length cDNA was obtained by amplification using the primer pairs: 5'-TCC GAG CAG TCT CTG TCC TT and 5' CTG GCA GGT GGC AGA TTA CAT and first strand cDNA from patients as a template. The product was ligated into the TA Cloning 2.1 vector (Invitrogen, Burlington, ON). The insert was subcloned to pcDNA3.1/myc-His vector (Invitrogen, Burlington, ON) after EcoRI digestion. For immunofluorescence microscopy, the amplified product of LMNA cDNA was subcloned into the pcDNA3.1/His vector with N-terminal tag encoding the Xpress epitope (Invitrogen, Burlington, ON) after EcoRI digestion using primers: 5'-CGG AAT TCA TGG AGA CCC CGT CCC AG and 5'- CGG AAT TCT TAC ATG ATG CTG CAG TTC TG. The fidelity of DNA amplification and satisfactory insertion of the respective control and mutant cDNAs were confirmed by DNA sequencing.

Cell culture transient transfection assay

Human HepG2 cells were grown in alpha-minimum essential medium (MEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% [v/v] heated-inactivated fetal calf serum (Invitrogen, Burlington, ON). COS-7 cells were maintained in Dulbecco’s MEM (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were seeded on culture
plates to achieve >90% confluence and were transfected, respectively, with normal and mutant *LMNA* constructs and empty control vector using Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen, Burlington, ON). Cells were extracted after addition of Mammalian Protein Extraction reagent (Pierce, Rockford IL) 48 h post-transfection and precleared cell lysates were used for subsequent Western blot analysis.

**Gel electrophoresis and immunoblotting**

Cell lysates were diluted in SDS-PAGE sample buffer and resolved on 10% SDS-PAGE (Novex pre-cast gel, Invitrogen, Burlington, ON). Proteins were transferred to nylon membranes following manufacturer’s protocol (Invitrogen, Burlington, ON). Membranes were incubated with blocking buffer (5% milk powder [w/v] and 0.05% Tween-20 in TBS) for 1 h at room temperature. Rabbit anti-lamin A/C (Cell Signaling Technology, Beverley, MA) was used at 1:1000 dilution and incubated with membrane for overnight at 4°C. Membranes were rinsed three times with washing buffer (0.05% Tween-20 in TBS) and then incubated with 1:2000 dilution of horse radish peroxidase-conjugated second antibodies (donkey anti-rabbit, Amersham, Oakville, ON) for 1 h at room temperature. After rinsing, proteins on membranes were detected using SuperSignal West Pico Chemiluminescent substrates (Pierce, Rockford IL).

**Co-immunoprecipitation analysis with emerin**
The ProFound Mammalian Co-immunoprecipitation Kit (Pierce, Rockord IL) was used according to the manufacturer’s instructions. Briefly, precleared cell lysates from transfected Hep-G2 cells were incubated for 2 h with anti-emerin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) coupling gel at room temperature. Immunoprecipitated proteins and whole lysates obtained in RIPA buffer were subjected to Western blotting. The membrane was probed with specific anti-emerin and anti-lamin A/C antibodies as described (12).

Immunofluorescence microscopy

*LMNA* cDNA was amplified using primers 5’-CGG AAT TCA TGG AGA CCC CGT CCC AG and 5’-CGG AAT TCT TAC ATG ATG CTG CAG TTC TG. The amplified fragment was digested and then in-frame cloned into pcDNA3.1/His vector with N-terminal tag encoding the Xpress epitope (Invitrogen, Burlington, ON). The fidelity of amplification and insertion of respective control and mutant cDNAs were confirmed by DNA sequence analysis. COS-7 cells were cultured as described above. Transfected cells were grown on glass coverslips and were fixed in methanol for 6 min at 10°C. Following washes with PBS, the fixed cells were incubated with anti-Express-FITC antibody at 1:500 dilution (Invitrogen, Burlington, ON), for 30 minutes at 37°C, washed with PBS and mounted on slides. Transfection efficiency was approximately 30%. Immunofluorescence microscopy was performed as described (13).
Statistical analysis

All statistical analyses were performed using SAS v8.2 (Cary, NC), with nominal $P<0.05$.

RESULTS

Anthropometry and fat distribution

The height, weight and body mass index of proband 1 were 165 cm, 60.2 kg, and 22.1 kg/m$^2$, respectively and those of proband 2 were 160.3 cm, 49.2 kg and 19.1 kg/m$^2$, respectively. Proband 1 had the following skinfold thickness measurements: chest 8 mm, mid-axillary 9 mm, subscapular 16 mm, suprailliac 11 mm, abdomen 14 mm, biceps 5 mm, triceps 6 mm, thigh 7 mm, and calf 5 mm. Circumference measurements were as follows: chest 93 cm, waist 73.5 cm (5-10$^{th}$ %ile for age and sex), hip 87 cm, mid-arm 26.2 cm (15-25$^{th}$ %ile), mid-thigh 41.2 cm (<5$^{th}$ %ile) and calf 34 cm (15-25$^{th}$ %ile). Proband 2 had the following the skinfold thickness measurements: chest 6 mm, mid-axillary 6 mm, subscapular 12 mm, suprailliac 6 mm, abdomen 9 mm, biceps 3 mm, triceps 5 mm, thigh 5 mm, calf 4 mm. Circumference measurements were chest 84 cm, waist 68.5 cm (<5%ile), hip 78.5 cm mid-arm 22.2 cm (<5%ile), thigh 36.2 cm (<5%ile) and calf 30.5 cm (<5%ile). The skinfold measurements are similar to those previously reported in individuals with FPLD2 (11), with all measurements falling in the low range (usually <5%ile) compared to the normal population, with the exception of the subscapular skinfold measurement, which was ~50$^{th}$ %ile of normal for Proband 1 and ~25$^{th}$ %ile for Proband 2, respectively. Proband 1 also
had a suprailiac skinfold measurement that fell between the 25th and 50th %ile. Circumference measurements demonstrated low-normal values. Bioelectrical impedance analysis indicated 23.1% body fat, 76.9% fat-free mass and 25.1 kg of skeletal muscle mass for Proband 1 and 17.8% body fat, 82.2% fat-free mass and 23.7 kg of skeletal muscle mass for Proband 2. It is notable that these sisters did not demonstrate any obvious abnormality in muscle mass. The clinical appearance of Proband 1 is shown in Figure 1.

DNA sequence analysis

The proband and her sister were each heterozygous for a single nucleotide change in their genomic DNA, specifically a novel G>C mutation at the intron 8 consensus splice donor site (Figure 2A). The mutation was absent from the genomes of 300 healthy individuals, including 150 individuals of South Asian ethnicity.

Reverse transcription of leukocyte mRNA from both patients followed by gel electrophoresis showed that each had two distinct cDNA species with fragment sizes 1121 and 1037 bp, compared to only a single 1037 bp fragment reverse-transcribed from leukocyte mRNA from a healthy control subject (Figure 2B). The sequence of the mutant 1121 bp band showed retention of intron 8 within the mRNA sequence: the aberrant transcript encoded 496 normal lamin A/C residues, followed by 20 new amino acids and a premature stop codon (Figure 2C).
Western analysis and co-immunoprecipitation with emerin

Results of western analysis of lysates of HepG2 cells transfected with WT and mutant *LMNA* cDNA from proband P1 and probed with anti-lamin A antibody are shown in Figure 3A. Normal lamin A/C was detected in all transfected cell lysates, but the truncated lamin A isoform was only detected in the lysate of the cells that were transfected with mutant *LMNA*. Thus, the truncation mutation could be translated and transcribed *in vitro*.

Results of western analysis of HepG2 cell lysates that were co-immunoprecipitated with emerin antibodies are shown in Figure 3B. Probing of these whole cell lysates with anti-lamin A antibody showed strong bands at the position of normal lamin A for all transfected cells, and a strong band at the position of the truncated mutant lamin A for cells transfected with mutant *LMNA*. Probing of whole cell lysates with anti-emerin antibody showed strong bands at the position of normal emerin for all transfected cells. After co-immunoprecipitation with anti-emerin and blotting, probing of whole cell lysates with anti-lamin A antibody showed bands at the position of normal lamin A for all transfected cells, and loss of the band at the position of mutant lamin A for cells transfected with mutant *LMNA*, suggesting that the mutant lamin A had lost the ability to interact with emerin. After co-immunoprecipitation with anti-emerin and blotting, probing of whole cell lysates with anti-emerin antibody showed bands at the position of emerin for all transfected cells. Thus, emerin co-precipitated with normal lamin A but with not the truncated isoform.
Immunofluorescence microscopy

Immunostained nuclei from cells transfected with wild-type and mutant LMNA cDNA are shown in Figure 4. Lamin localized specifically around the nuclear envelope in wild-type transfected cells, but was diffusely localized throughout the cytoplasm in mutant transfected cells. From 70 cells that were transfected with WT LMNA, 19 had a visible lamin A signal: in 18 cells (95%) lamin A co-localized with the nuclear rim (Figure 4A), with the one remaining cell showing presence of lamin A signal in the cytoplasm. From 89 cells that were transfected with mutant LMNA, 16 had a visible lamin A signal: in all 16 (100%), lamin A signal was seen in the cytoplasm, with no co-localization along the nuclear rim (Figure 4B). This difference in proportion of cells transfected with wild-type versus mutant LMNA showing nuclear rim versus cytoplasmic localization of the anti-lamin antibody was highly significant (2X2 chi-square=31.2, P<0.00001).

DISCUSSION

We report two sisters with a severe FPLD2 phenotype associated with heterozygosity for a novel LMNA splice variant. A clinical description of their mother suggested that she had the same disease. This is the first splicing mutation reported in FPLD2 patients. The mutation encoded a lamin A isoform that was truncated by ~30%. The mutant protein was expressed in substantial quantities in vitro, suggesting that it is not subject to nonsense-mediated decay or degradation. Co-immunoprecipitation studies indicated that the mutant protein
did not interact with emerin. Unlike normal lamin A, the mutant protein failed to localize to the nuclear membrane and, instead, was present diffusely throughout the cytoplasm of transiently transfected cells. The in vitro studies indicate that the mutant isoform is expressed and has impaired function, although it is not clear whether the pathogenic mechanism in vivo in heterozygous patients is a deficiency of normal function or the result of interference with the product of the normal allele by the product of the mutant allele.

The most striking features in the family we are reporting are the early age of onset and the severity of the typical features of FPLD2. The onset of clinical features in FPLD2 typically occurs at puberty (10). Based on clinical observations in FPLD2 patients with LMNA missense mutations R482Q and R482W, it has been assumed that the precipitating event is the loss of fat tissue occurring late in childhood or early in puberty. It was presumed that insulin resistance and the intermediate biochemical phenotypes followed the loss of fat. However, it is of interest that proband 1 in this report had stigmata of insulin resistance, specifically acanthosis nigricans, at age 5, with early menarche followed by a diagnosis of T2DM years before the fat loss was noted clinically. This anecdotal observation suggests that insulin resistance might precede the clinically apparent fat loss making it, temporally the primary disturbance. Fat loss has traditionally been considered to be the inciting event that precedes the development of metabolic complications in FPLD2, such as insulin resistance (1,2). The ability to genotype children in FPLD2 families, combined with more sensitive biochemical markers and non-invasive tools to detect fat loss may help
to sort out the precise order of evolution of the characteristic disturbances in FPLD2.

Other clinical features in our patients that emphasize the severity of the FPLD2 phenotype associated with this LMNA splicing mutation include the early age of onset of hypertension, T2DM and severe hypertriglyceridemia with secondary pancreatitis, occurring in the teenage years for both probands. In addition, our patients have several features not previously reported in FPLD2. Proband 1 had hidradenitis suppurativa, an acneiform infection of the cutaneous apocrine glands that also can involve adjacent subcutaneous tissue and fascia. Proband 2 had two occurrences of sixth nerve palsy. Although depression has been reported in at least one other patient (11), our patients were diagnosed during childhood and proband 1 required numerous hospitalizations during her teenage years. They continue to be followed and treated.

There is some suggestion of overlapping involvement of other organ systems, such as cardiac and neurological systems, associated with this mutation in the probands and their mother. However, the occurrences of sixth cranial nerve palsies in proband 2 have not been reported in FPLD2 due to other LMNA mutations. Similarly, the possibility of a cardiomyopathy in the probands’ mother was suggested by atrial fibrillation diagnosed at age 25 and a diagnosis of non-specific cardiomyopathy necessitating placement of a cardiac pacemaker. Another FPLD2 patient with the LMNA R62G mutation had cardiomyopathy and conduction defects in addition to the typical features of FPLD (11). Other patients had cardiac and lipodystrophic signs associated with LMNA R527P and
R60G mutations (12). A patient with the LMNA R133L mutation had hypertrophic cardiomyopathy with valvular involvement (13). Muscle weakness previously reported in patients with FPLD2 tends to be proximal and several have significant limb girdle weakness. Proband 2 had muscle weakness, but had no elevation in serum creatine kinase which has been reported previously (14,15). A patient with the LMNA R482W missense mutation had severe limb girdle muscle dystrophy with loss of ambulation, in addition to FPLD2 (14). Recently, a patient with arthropathy, tendinous calcinosis and progeria was found to have a homozygous LMNA S573L missense mutation (16).

In summary, we report the first LMNA splicing mutation in FPLD2, which was associated with a severe phenotype and altered in vitro function, including inability to interact with emerin and failure to localize at the nuclear envelope. In general, the position of the mutation within the LMNA gene determines the type and extent of tissue involvement (3), particularly for missense mutations. However, this unique mutation might serve as a natural “probe” in future in vitro and in vivo studies designed to understand pathogenesis and phenotypic consequences of disrupted nuclear envelope function in human disease.
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REFERENCES


2. **Hegele RA** 2003 Monogenic forms of insulin resistance: apertures that expose the common metabolic syndrome. Trends Endocrinol Metab 14:371-377


FIGURE LEGENDS

Figure 1. Clinical photographs of proband P1 (see detailed clinical description in Methods and Results sections).

Figure 2. DNA sequence analysis. A) The upper panel shows genomic DNA sequence near the exon 8-intron 8 border from a healthy control subject and from the proband P1, as indicated. The proband was heterozygous for a single nucleotide change in her genomic DNA, specifically a novel G>C mutation at the intron 8 consensus splice donor site. The mutation was also found in patient 2 (homozygote), but was absent from the genomes of 300 healthy individuals. B) The middle panel shows an agarose gel with RT-PCR products from mRNA of a healthy subject (N) and from the two sisters A1 and A2 with the additional mutant 1121 bp PCR fragment that contains intron 8 (84 bp in length). C) The lower panel shows cDNA sequence surrounding the exon 8-exon 9 junction from a normal individual (above) and from the proband P1 (below). The long mutant cDNA sequence shows retention of intron 8 within the mRNA sequence. This aberrant transcript encoded 496 normal lamin A/C residues, followed by 20 new amino acids and a premature stop codon, indicated by capitalized and underlined TAA in the nucleotide sequence line. Six nucleotides corresponding to the first two non-translated codons of exon 9 are shown at the 3’ end of the mutant cDNA sequence.
Figure 3. Western analyses.  A) The results of western analysis are shown in the upper panel. HepG2 cells were transfected with normal LMNA (N), mutant LMNA (P1) constructs and vector only (B). Anti-lamin A antibody was used to probe the Western blot. Normal lamin A/C was detected in all three transfected cell lysates but the truncated lamin A isoform was only detected when mutant LMNA was transfected. B) Results of western analysis of cell lysates that were co-immunoprecipitated with emerin antibodies are shown: whole cell lysates are shown the left side and lysates after co-immunoprecipitation with emerin are shown on the right side. The top and bottom sections represent probing with anti-lamin A and anti-emerin antibody, respectively. Cells transfected with wild-type, mutant and empty vector are designated N, M and B, respectively. For the whole lysates, strong bands were seen at 70 and 64 kD position of normal lamin A for all transfected cells. In addition, a strong band was present at the 50 kD position of the truncated mutant lamin A for cells transfected with mutant LMNA. Finally, probing of whole cell lysates with anti-emerin antibody showed strong bands at the 34 kD position of normal emerin for all transfected cells. After coimmunoprecipitation with anti-emerin and blotting, probing of cell lysates with anti-lamin A antibody showed bands at the position of normal lamin A for all transfected cells (right half). In addition, there was a loss of the band at the 50 kD position of mutant lamin A for cells transfected with mutant LMNA, suggesting that the mutant lamin A had lost the ability to interact with emerin. After coimmunoprecipitation with anti-emerin and blotting, probing of whole cell lysates with anti-emerin antibody showed bands at the position of emerin for all
transfected cells. Therefore, emerin co-precipitated with normal lamin A but with not the truncated isoform.

**Figure 4. Immunofluorescence microscopy.** Immunostained nuclei from cells transfected with wild-type lamin A cDNA (panel A) and mutant *LMNA* cDNA (panel B). The nuclei are stained blue, and the lamin antibody is yellow. The lamin antibody localized specifically around the nuclear envelope in wild-type transfected cells (sharp nuclear border in panel A) in 95% of cells visualized (N=19). In contrast, signal from the anti-lamin A antibody did not localize around the nuclear rim in any of the mutant transfected cells visualized (N=16) and instead was diffusely localized throughout the cytoplasm in 100% of mutant transfected cells (diffuse staining in panel B).
A normal gDNA

proband gDNA

IVS8 +5G>C

B M N P1 P2

1121 bp
1037 bp

C exon 8 exon 9

5' GTG ACG ATC TGG 3'

normal cDNA

5'GTGACGgtgacgtgagcgcttggaagagctgtggcttgctgatggagctggAgggTAAgtgtcc ttc tct ctcagATCTGG3'

IVS8 +5G>C

Stop
**A**

- **N**
- **M**
- **B**

64 kD → lamin A/C

50 kD → mutant lamin A

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**B**

**Whole lysate**

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**Emerin IP**

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