LMNA mutations in atypical Werner’s syndrome

Summary

Background Werner’s syndrome is a progeroid syndrome caused by mutations at the WRN helicase locus. Some features of this disorder are also present in laminopathies caused by mutant LMNA encoding nuclear lamin A/C. Because of this similarity, we sequenced LMNA in individuals with atypical Werner’s syndrome (wild-type WRN).

Methods Of 129 index patients referred to our international registry for molecular diagnosis of Werner’s syndrome, 26 (20%) had a WRN coding region and were categorised as having atypical Werner’s syndrome on the basis of molecular criteria. We sequenced all exons of LMNA in these individuals. Mutations were confirmed at the mRNA level by RT-PCR sequencing. In one patient in whom an LMNA mutation was detected and fibroblasts were available, we established nuclear morphology and subnuclear localisation.

Findings In four (15%) of 26 patients with atypical Werner’s syndrome, we noted heterozygosity for novel missense mutations in LMNA, specifically A57P, R133L (in two people), and L140R. The mutations altered relatively conserved residues within lamin A/C. Fibroblasts from the patient with the L140R mutation had a substantially enhanced proportion of nuclei with altered morphology and mislocalised lamins. Individuals with atypical Werner’s syndrome with mutations in LMNA had a more severe phenotype than did those with the disorder due to mutant WRN.

Interpretation Our findings indicate that Werner’s syndrome is molecularly heterogeneous, and a subset of the disorder can be judged a laminopathy.

Lancet 2003; 362: 440–45
See Commentary page 416

Introduction Werner’s syndrome is an autosomal, recessively inherited, segmental progeroid syndrome, in which multiple aspects (or segments) of ageing phenotypes seem to be entailed. The disorder is caused by mutations in WRN, which is a member of the RECQ family of DNA helicases.1 We used a set of clinical criteria to prospectively classify patients enrolled in a positional cloning study that defined the locus of this gene.2 When we did not find any mutations in WRN, we tentatively designated these patients as having atypical Werner’s syndrome (or non-WRN).

Several diseases that overlap partly with the phenotype of Werner’s syndrome share mutations at the LMNA (lamin A/C) gene; they have therefore been referred to as laminopathies.3 These diseases include Emery-Dreifuss muscular dystrophy,4–6 dilated cardiomyopathy type 1A,4 limb-girdle muscular dystrophy type 1B,5 familial partial lipodystrophy,6–8 Charcot-Marie-Tooth disease type 2,9 mandibuloacral dysplasia,10 and a rare childhood syndrome of premature ageing, Hutchinson-Gilford syndrome.11–13 LMNA mutations causing familial partial lipodystrophy, for example, are associated with insulin resistance, type 2 diabetes, and atherosclerosis; these features are similar to those seen in people with Werner’s syndrome. These observations prompted us to investigate the LMNA gene in our subset of individuals with atypical (non-WRN) Werner’s syndrome.

Methods Between January, 1987, and November, 2003, we enrolled all patients diagnosed with Werner’s syndrome via the international registry of Werner syndrome.14 Criteria for diagnosis of Werner’s syndrome are summarised in table 1. We enrolled controls from the national long-term care survey (Department of Pathology, University of Washington, Seattle, WA, USA), a population-based sampling of US residents. Written informed consent was sought at the time of enrolment from both patients and controls. We obtained blood samples from all participants. We established lymphoblastoid cell lines from patients’ blood samples with Epstein-Barr virus, and primary fibroblast cultures were made from punch skin biopsy samples.2 This protocol was approved by the internal review board at the University of Washington, Seattle, WA, USA.

When we received the samples, we did initial mutational analysis by amplification of LMNA exons from genomic DNA with published primers,3 followed by cycle sequencing of the PCR product with Thermo Sequenase (Bioline, Canton, MA, USA) with phosphor-33 dideoxynucleotide triphosphate terminators (Amersham Pharmacia, Piscataway, NY, USA). For confirmation, poly(A) RNA was reverse transcribed, and we amplified LMNA cDNA with primers shown in the panel, and sequenced the product with internal primers.
Table 1: Characteristics of individuals with LMNA mutations

<table>
<thead>
<tr>
<th>Mutation in LMNA protein (gene)</th>
<th>PORTU8010</th>
<th>ATLANT010</th>
<th>NORWAY1010</th>
<th>IRAN1010</th>
</tr>
</thead>
<tbody>
<tr>
<td>R133L (813G→T)</td>
<td>R133L</td>
<td>L140R</td>
<td>L140R</td>
<td>L140R</td>
</tr>
<tr>
<td>R133L (813G→T)</td>
<td>L140R</td>
<td>L140R</td>
<td>L140R</td>
<td>L140R</td>
</tr>
<tr>
<td>L140R (584G→C)</td>
<td>L140R</td>
<td>L140R</td>
<td>L140R</td>
<td>L140R</td>
</tr>
<tr>
<td>L140R (584G→C)</td>
<td>L140R</td>
<td>L140R</td>
<td>L140R</td>
<td>L140R</td>
</tr>
</tbody>
</table>

**Key signs**

- Cataracts: Yes/No
- Scleroderma: Yes/No
- Skin short stature: Yes/No
- Gyring/thinning of hair: Yes/No
- Increased urinary hyaluronic acid: Yes/No
- Other signs of Werner’s syndrome:
  - Diabetes mellitus, type 2: Yes/No
  - Hypogonadism: Yes/No
  - Osteoporosis: Yes/No
  - Bone atherosclerosis: Yes/No
  - Soft-tissue calcification: Yes/No
  - Premature atherosclerosis: Yes/No
  - Mesenchymal neoplasms: Yes/No
  - Voice changes: Yes/No
  - Other manifestations: Yes/No

**Werner’s syndrome Possible diagnosis**

NA: not available.

**Diagnosis of Werner’s syndrome is based on previously described criteria.**

**Diabetes mellitus**, **Yes, at age 23**, **Yes, at age 18**, **No**, **No**

**Hypogonadism**, **Yes**, **Yes**, **Yes**, **Yes**

**Osteoporosis**, **Yes**, **Yes**, **Yes**, **Yes**

**Bone atherosclerosis**, **Yes**, **Yes**, **Yes**, **Yes**

**Soft-tissue calcification**, **Yes**, **Yes**, **Yes**, **Yes**

**Premature atherosclerosis**, **Yes**, **Yes**, **Yes**, **Yes**

**Mesenchymal neoplasms**, **Yes**, **Yes**, **Yes**, **Yes**

**Voice changes**, **Yes**, **Paternal inheritance**, **Yes**, **Aortic stenosis/insufficiency/died at age 36 years**, **Yes**, **No**, **Dilated cardiomyopathy/pathy/sloping shoulders**, **Possible**

**Other manifestations**, **Possible**

**Werner’s syndrome Possible diagnosis**

**Table 1**: Characteristics of individuals with LMNA mutations

**Figure 1**: Pedigrees of families with atypical Werner’s syndrome

Filled-in symbols indicate affected individuals.
A base substitution in exon 2 (813G→T), which altered aminoacid 133 by changing Arg (CGG) to Leu (CTG), was seen in two affected individuals—ATLAN1010 and PORTU8010. We did not record this mutation in either parent or in a younger sister in the PORTU pedigree, indicating that the R133L mutation was a de novo mutation. A different mutation (834T→G)—a Leu (CTG) to Arg (CGG) substitution at aminoacid 140—was seen in the NORWAY1010 individual. A third new mutation (584G→C), which altered Ala (GCA) to Pro (CCA) at aminoacid 57, was recorded in the IRAN1010 patient. All these heterozygous mutations were detected by genomic PCR sequencing and confirmed by sequencing RT-PCR products (figure 2).

The dominant presenting features of the four patients were their appearances (looking older than their age) and short stature, which was especially striking for the IRAN1010 patient. Cardiovascular pathological findings, osteoporosis, lipodystrophy, and muscular atrophy were also typical. Doctors reported atrophic skin, loss of subcutaneous tissues, and various degrees of muscle atrophy in these patients. Grey or sparse hair was also reported in all patients. Insulin-resistant diabetes mellitus was seen in both patients with the R133L mutation (ATLAN1010 and PORTU8010). The deceased father, paternal aunt, and paternal grandmother of ATLAN1010 were also diagnosed with severe insulin deficiency syndrome, suggesting that the R133L mutation might have been paternally inherited (figure 1).

**Figure 2: LMNA mutations in patients with atypical Werner’s syndrome**

(A) Genomic PCR sequencing result. Arrows show the presence of 813G→T substitution in ATLAN1010 and PORTU8010 (causing R133L mutation), 834T→G in NORWAY1010 (causing L140R mutation), and 584G→C in IRAN1010 (causing A57P mutation). *Individuals with wildtype LMNA.

(B) RT-PCR sequencing of index cases. *Western-blot analysis of lamin A/C, WRN protein, and β-actin in lymphoblastoid cell lines from IRAN, ATLAN, and PORTU pedigrees, and primary fibroblasts from the NORWAY patient. 82-6 is a line of control primary skin fibroblasts. The autoradiograph with the highest exposure is shown here.

**Figure 3: Structure of lamin A and disease mutants**

(A) Domain organisation of lamin A and locations of A57P, R133L, and L140R mutations. (B) Multiple sequence alignment of selected nuclear lamins in the vicinity of these mutation sites (yellow). Columns in grey depict invariant positions. Boxes correspond to the α-helical, coiled-coil segments. Red marks the first and fourth positions of a coiled-coil heptad repeat; in dimeric coiled-coils, they are usually apolar or hydrophobic and are internalised to stabilise the structure. The selected lamin sequences shown are HsLaminA, Homo sapiens lamin A/C (databank code LAMA_HUMAN); GgLaminA, Gallus gallus lamin A (LAMA_GALLUS); XlLaminA, Xenopus laevis lamin A (LAMA_XENLA); HsVim, Homo sapiens vimentin (VIME_HUMAN).
cell types—eg, lymphoblastoid cell lines and primary fibroblasts—irrespective of the presence of heterozygous mutations. In lymphoblastoid cell lines, lamin C was expressed, on average, at concentrations 47% higher than lamin A. In fibroblasts, lamin C was expressed at amounts 2.6-fold higher than lamin A (figure 2). We detected normal WRN expression in LMNA mutant cells (figure 2).

To elucidate the pathological importance of the three LMNA mutations, we investigated sequence conservation in homologous proteins (figure 3). Multiple sequence alignment indicated that invariant portions are confined largely to α-helical coiled-coil segment 1A and the C-terminus of segment 2B2 (data not shown; figure 3). The A57P site is usually occupied by hydrophilic aminoacids, R133L is generally polar, and L140R can tolerate a range of substitutions (figure 3). The R133L and L140R mutations are located in a heptad repeat region of 1B (figure 3) that seems to be unique to nuclear lamins.

Many laminopathy-associated LMNA mutations lead to perturbed nuclear structure. We therefore did DAPI (diaminophenylindole) staining on primary fibroblasts from the NORWAY1010 patient to examine whether LMNA mutations leading to atypical Werner’s syndrome caused a similar phenotype. Labelling indices of control primary fibroblasts and NORWAY1010 fibroblasts were comparable (65% and 72%, respectively). To determine the correlation between misshapen nuclei and absence of intranuclear lamin A/C, cells were first scored for nuclear shape and then examined for intranuclear lamin A/C foci. We recorded that most of the NORWAY1010 cells showed altered nuclear structure compared with the control primary fibroblasts (61% vs 25%, p=0.0037; table 2). Many nuclei were irregularly shaped and some displayed apparent leakage of DAPI into the cytoplasm (figure 4). We also examined lamin A/C localisation. Normally in fibroblasts, lamin A/C localises to both the nuclear envelope and internal foci of undefined function. In the subset of NORWAY1010 cells with nuclei that seemed normal, lamin A/C cells were first scored for nuclear shape and then examined for intranuclear lamin A/C foci. We recorded that most of the NORWAY1010 cells showed altered nuclear structure compared with the control primary fibroblasts (61% vs 25%, p=0.0037; table 2). Many nuclei were irregularly shaped and some displayed apparent leakage of DAPI into the cytoplasm (figure 4).

We sequenced LMNA in 116 controls, who were mainly of white ethnic origin. None of the alterations described was seen in these individuals (p<0.0001).

The LMNA gene encodes gene products produced by alternative splicing, lamin A and lamin C. Results of the three independent Western-blot analyses showed that lamins A/C are expressed at similar amounts in the same

<table>
<thead>
<tr>
<th></th>
<th>Mean percentage of misshapen nuclei (SD)</th>
<th>Mean percentage of cells lacking intranuclear lamin A/C foci (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control primary fibroblasts (82-6)</td>
<td>24.7 (0-4)</td>
<td>-</td>
</tr>
<tr>
<td>NORWAY1010</td>
<td>60.7 (2-2)</td>
<td>-</td>
</tr>
<tr>
<td>Cells with normal nuclei</td>
<td>-</td>
<td>4.3 (1-6)</td>
</tr>
<tr>
<td>Cells with misshapen nuclei</td>
<td>-</td>
<td>9.0 (2-0)</td>
</tr>
<tr>
<td>NORWAY1010</td>
<td>-</td>
<td>9.3 (1-1)</td>
</tr>
<tr>
<td>Cells with normal nuclei</td>
<td>-</td>
<td>46.7 (6-9)</td>
</tr>
</tbody>
</table>

Data represent the mean (SD) from three independent experiments. In every experiment, 100 cells were counted for every phenotype in three independent experiments.

Table 2: Nuclear morphology and lamin localisation

Figure 4: Nuclear structure and subcellular localisation of lamin A/C. DAPI staining was used to establish nuclear shape in control (A) and NORWAY1010 primary fibroblasts (B, C). Indirect immunofluorescence in control (D–I) and NORWAY1010 (J–L) primary fibroblasts (green). DAPI staining marks DNA (blue). Volume views are shown, which are composites of several images taken at distances of 0.2 μm apart through most of the nucleus.
MECHANISMS OF DISEASE

Discussion

We have shown that a subset of patients with Werner’s syndrome without mutations at the WRN locus have mutations at the LMNA locus that cause the disorder. These observations, however, do not preclude the possibility that a WRN function or functions could be altered.

Imura and colleagues14 grouped patients with Werner’s syndrome according to similar clusters of clinical features. They inferred that there were at least three distinct clinical types of the disease, with type 2 Werner’s syndrome being the least typical and having the earliest age of onset—resembling the LMNA-type Werner’s syndrome we report here.

The age at initial signs in patients with atypical Werner’s syndrome was 6 years earlier than the mean age for the appearance of grey hair, which is usually the earliest sign of classic Werner’s syndrome, aside from the short stature, which is recognised only retrospectively.28

The rates of progression of disease in this group of patients with atypical Werner’s syndrome could be greater than is typically the case for those with classic disease.

At least six polymorphisms have been identified in LMNA, which do not change the encoded aminocides. These polymorphisms include 1276T→C (A287A), 1753T→C (D446D), and 2113C→T (H566H) in the coding region, and IVS4–13T→A and 2393C→T (398 bases downstream from stop codon) in the 3′ untranslated region.38 These polymorphisms were noted in some of the patients with atypical Werner’s syndrome and controls.

Segment 1A of LMNA protein is believed to form one AMPHIPATHIC α-helix in the monomeric (open) form and a coiled-coil in the dimeric (closed) form.26 Thus, any disruption of the helix induced by the proline in the A57P mutation might not be important for the lamin A/C segment 1A monomer. A57P could affect the assembly, dynamics, or both of a filamentous network because this (subtle) conformational change could affect how 1A segments interact in the dimer. Segments 1B, 2A, 2B1, and 2B2 constitute the central rod domain and are the primary building blocks of the coiled-coil dimer.25 An locations of R133L and L140R at the surface position of a heptad repeat suggests the mutations might not affect the structure of the lamin A/C dimer itself, but rather perturb intermolecular interactions.

Most of the mutations reported in patients with autosomal dominant Emery-Dreiffuss muscular dystrophy, dilated cardiomyopathy type 1A, limb-girdle muscular dystrophy type 1B, and familial partial lipodystrophy are heterozygous missense mutations. They map throughout the LMNA exons.1 Many of these are predicted to cause failure of nuclear lamina assembly. The observation of an unaffected father bearing one of the compound heterozygous mutations in a patient with Emery-Dreiffuss muscular dystrophy suggests that a threshold of lamin abnormality might exist for lamin assembly.1 Alternatively, a patient with Emery-Dreiffuss muscular dystrophy with a heterogeneous stop codon mutation at aminoacid 6 raises the possibility that HAPLOINSUFFICIENCY of lamins may partly account for the nuclear fragility.4 An R133P substitution—similar to the R133L mutation reported in atypical Werner’s syndrome—has been reported in a 40-year-old patient with Emery-Dreiffuss muscular dystrophy who had disease onset at age 7 years and atrial fibrillation at age 32 years.44 Neither R133L nor R133P mutations were not detected in the parents and siblings of two of our patients, indicating that they most probably arose de novo.3

The overlapping syndromes of Emery-Dreiffuss muscular dystrophy, dilated cardiomyopathy type 1A, and limb-girdle muscular dystrophy type 1B have been widely noted,1,3,17 which could be due to alterations in lamina structure. For example, the homozygous A298C mutation reported in autosomal recessive Charcot-Marie-Tooth disease type 2, resides at the α-helical rod domain, which probably perturbs the lateral interactions of lamin A.27

The NORWAY1010 individual, who died of ureaemia at age 36 years, had extensive arterial calcifications consistent with premature atherosclerosis. Coronary atherosclerosis is the major cause of death in patients with either Werner’s syndrome or Hutchinson-Gilford syndrome, but happens at a much earlier age in those with Hutchinson-Gilford syndrome. Sloping shoulders and osteosclerosis of finger phalanges—characteristics of mandibuloacral dysplasia—were noted in the IRAN1010 patient. Osteosclerosis of the distal phalanges has also been described in patients with typical Werner’s syndrome.39 Dilated cardiomyopathy is not a feature of mandibuloacral dysplasia, caused by homozygous mutation at R327H. The location of the mutation in the IRAN1010 patient, A57P, is close to mutations seen in dilated cardiomyopathy type 1A and Emery-Dreiffuss muscular dystrophy, which might also present with cardiomyopathies.

Clinical features of our patients are distinct from classic Hutchinson-Gilford syndrome. Onset of this disorder is during the first year of life, and mean age of death is 13 years. Five different mutations have been reported in Hutchinson-Gilford syndrome—G608G, G608S, E145K, R471C, and R527C.11–14 The most typical mutation, G608S, results in an in-frame deletion of 50 aminocids in the C-terminal region of LMNA, including the endoproteolytic cleavage site.12–14 The E145K mutation is within the heptad repeat of LMNA, and is close to the R133L and L140R mutations. The clinical phenotype of E145K was unusual for Hutchinson-Gilford syndrome in that scalp hair and ample subcutaneous tissues over arms and legs were present.11

Why do LMNA missense mutations lead to so many diseases that in some cases show little phenotypic overlap? Two non-exclusive models have been suggested.1 In one, general alterations in nuclear structure resulting from mutations in LMNA lead to cell instability and ultimately tissue atrophy. This model does not clearly account for tissue specificity. Moreover, since most mutations are associated with similar proportions of nuclear shape anomalies when expressed in fibroblasts, the reason why the range of affected tissues would differ so widely from one mutation to the next is not evident. The second model is that lamins A and C act to regulate several important nuclear components whose activities are important to prevent disease onset.30 The range of phenotypes with respect to each missense mutation would then depend on which interactions were affected. Our structural analysis suggests that phenotypic differences in our patients may result from the specific tissue or cell-type nature of regulatory or cross-bridging partners. Despite the appeal of this model, only a few of these possible interacting factors—such as emerin, lamin-associated protein, and nesprin α—have been identified to date.

Why might mutation of LMNA promote progeroid phenotypes? One obvious possibility is that WRN function is impaired. Although we find that LMNA mutant fibroblasts have reduced amounts of protein, to establish whether WRN protein localisation and activity is normal will be important before we can rule out this hypothesis. A second possibility is that retinoblastoma...
protein function is altered in patients with atypical Werner’s syndrome that contain LMNA mutations. We have discovered that lamin A/C, a known retinoblastoma protein-binding-protein, is needed to maintain retinoblastoma protein stability (Johnson B, Kennedy B K, unpublished). Also, interference with function of this retinoblastoma protein is known to delay the onset of senescence in cell culture. These LMNA mutations might enhance retinoblastoma activity leading to an accelerated senescence programme. Whatever the cause, our findings suggest that proper nuclear organisation is important to prevent or delay the progeroid features noted in patients with LMNA mutations.

Most (82-9%) patients with Werner’s syndrome have mutations in either WRN or LMNA. Molecular diagnosis can stratify and confirm a clinical diagnosis only when the phenotype is unclear. Candidate genes that may account for these remaining cases would include those coding for domains of the WRN protein, including DNA-PK complex, TRF2, and TP53. The LMNA or WRN mutations could also be secondary to a mutation in another mutator gene, since several pedigrees of the LMNA mutations were not inherited from the parents and arose only in the germiline of parents.

Contributors
L Chen did sequencing and protein analysis. L Lee did sequencing and established cell lines. B A Kudlow did immunofluorescence studies. G Dos Santos provided the PORTU pedigree. O Stroffolini provided the NORWAY case. Y Shafaghati provided the IRAN case. E G Botha provided the ATLAN pedigree. A Gargin initiated immunofluorescence studies and provided necessary scientific and technical information. N B Hanson organised shipment of samples and provided necessary technical information. G M Martin checked every patient for the clinical diagnosis of Werner’s syndrome. S I Mian did structural studies. B K Kennedy did analyses of immunofluorescence studies and contributed substantially to the Discussion section of the report. J Oshima analysed sequencing results and designed the experimental plans.

Conflict of interest statement
None declared.

Acknowledgments
This work was supported by grants from the National Institute of Health and the Progeria Research Foundation.

References