Hutchinson-Gilford progeria syndrome (HGPS) is a rare genetic disorder phenotypically characterised by many features of premature aging.¹⁻³ The characteristic features include short stature, prominent eyes, micrognathia, craniofacial disproportion, loss of subcutaneous fat, alopecia, beaked nose, “plucked-bird” appearance, coax valga, pathologic bone fractures, atherosclerosis, and cardiovascular disorders. At birth, the appearance of patients with HGPS is generally normal, but by 1 year of age patients show severe growth retardation, balding, and sclerodermatous skin changes. They average ~1 m in height and usually weigh less than 15 kg even as teenagers. The age at death ranges from 7 to 28 years, with a median age of 13.4 years. Over 80% of deaths are due to heart attacks or congestive heart failure. We previously reported an extraordinarily long-lived patient with HGPS who survived to age 45.³ At birth he appeared normal and his large head was noted at 1 year. Growth retardation was first noticed at the age of 12. He began to lose his hair during childhood and had total alopecia at age 20. When we examined him at the age of 44, he was 132 cm in height and 24.5 kg in weight and had all of the characteristic features listed above. He died of myocardial infarction at age 45. Thus, compared to classical cases of HGPS, the onset of the disease in our patient was late and it took a longer time to develop full-blown HGPS.

Recently, mutations in the lamin A/C gene (LMNA) which cause classical cases of HGPS have been identified.³⁻⁷ The molecular mechanism by which these mutations produce the characteristic manifestations of HGPS remains to be elucidated. Therefore, it is of great interest to investigate the molecular basis of our patient, which allowed him to live about three fold longer than classical HGPS patients.

METHODS

Subjects and sample preparation

This study was approved through the institutional review board at the University of Alabama at Birmingham. A Japanese patient with HGPS was previously described elsewhere.⁴ Lymphoblastoid cell lines (LCLs) were established from peripheral blood samples from the patient and healthy control subjects using Epstein-Barr virus. LCLs were used to prepare protein, RNA, and genomic DNA. LCLs were cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO₂/95% air at 37°C. In order to determine if the same single-base substitution (1868 C>G (T623S)) found in our patient in LMNA presents in a Japanese population, genomic DNA samples extracted from peripheral blood of 115 unrelated Japanese subjects were screened by polymerase chain reaction (PCR) amplification as described below. All of the subjects gave their written consent for genetic analysis to be performed, which was approved by the ethical committees of Osaka University.

Key points

- Hutchinson-Gilford progeria syndrome (HGPS) is a very rare genetic disorder characterised by premature aging phenotype. The median age at death is 13.4 years. Recently, an identical de novo mutation, 1824 C>T (G608G), within exon 11 in the lamin A gene (LMNA) was reported to cause the vast majority of classical HGPS cases. This silent mutation activates a cryptic splice site, resulting in a protein product that deletes 50 amino acids.
- We previously described an exceptionally long-lived patient with HGPS, who survived to age 45. We have sequenced all exons of LMNA using genomic DNA extracted from a cell line established from this patient. mRNA for lamin A expressed in the cells was studied by RT-PCR followed by DNA sequencing. Levels and species of lamin A/C in the cells were compared to those in normal control cells.
- Our patient had a new heterozygous transversion, 1868 C>G (T623S), in LMNA. Approximately 80% of normal-length mRNA for lamin A derived from the mutant allele, suggesting a reduction in normal lamin A. Furthermore, the mutation activated a cryptic splice site to produce a protein product that truncates 35 amino acids. This 35 amino acid truncation entirely overlaps the 50 amino acid truncation caused by the 1824 C>T mutation.
- An amino acid truncation of lamin A involving a C-terminal region encoded by exon 11 causes HGPS. The 50 amino acid truncation may be more harmful than the 35 amino acid truncation that granted a longer lifespan to our patient.

Genotyping

Genomic DNA was extracted from the LCL established from the patient with HGPS. All of the exons of LMNA including exon–intron boundaries in the HGPS patient were amplified through PCR using previously published primers⁵ and then sequenced using an ABI 377 automated DNA sequencer (BigDye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA).

For detection of the single-base substitution (1868 C>G (T623S)) in LMNA in a Japanese population, PCR amplification of genomic DNA samples from 115 unrelated Japanese

Abbreviations: HGPS, Hutchinson-Gilford progeria syndrome; LCL, lymphoblastoid cell line; PCR, polymerase chain reaction; WS, Werner syndrome
subjects was performed using a forward primer, 5'-CGG GAT CCT GGT TGG GCC TGA GTG GTC AGT C-3', a reverse primer, 5'-CGG GAT CCT GGT TGG GCC TGA GTG GTC AGT C-3', and a reverse primer specific for the 1868 C>G mutation, 5'-CAC ACT GGT GTA GGT GCC AC-3'. The PCR was carried out in a 25 µl volume containing 100 ng genomic DNA template, 200 nM each primer, 200 µM each dNTP, 1 x PCR AmpliTaq Gold buffer (Applied Biosystem Japan, Tokyo) and 1.25 U AmpliTaq Gold (Applied Biosystem Japan). The samples were amplified through 35 cycles consisting of 30 s at 94°C, 30 s at 63°C, and 30 s at 72°C. The last DNA synthesis was extended to 5 min. The PCR amplified products were analysed by electrophoresis on 1.7% agarose gel. By this PCR, exon 11 of LMNA was amplified as a 394 base pair (bp) fragment and the 1868 C>G (T623S) mutant allele yielded an additional 261 bp fragment.

For cloning of LMNA exon 11 from the HGPS LCL, exon 11 was amplified by PCR using a forward primer, 5'-CGG GAT CCT GGT TGG GCC TGA GTG GTC AGT C-3', and a reverse primer, 5'-CGG AAT TCA GGG ACC CGC CTG CAG GAT TTG TGG GTG C-3'. A BamHI or EcoRI restriction enzyme site (underlined) was added to the forward or reverse primer, respectively. The PCR was carried out in a 50 µl volume containing 50 ng genomic DNA template, 200 nM each primer, 200 µM each dNTP, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2.5 mM MgCl₂ and 1.25 U Taq polymerase (Roche, Indianapolis, IN). The PCR products were digested with BamHI and EcoRI restriction enzymes and cloned onto the same restriction enzyme sites in pSP72 vector (Promega, Madison, WI). After transforming DH5α competent cells (Invitrogen, Carlsbad, CA), 25 clones with exon 11 insert were randomly selected for DNA sequencing from both directions using a T7 primer and an oligonucleotide primer, 5'-GAG TAC AAC CTG CGC TAC-3'.

Analysis of cDNA for lamin A by RT-PCR
Total RNA from the LCL was extracted using an E.Z.N.A. total RNA kit according to the manufacturer's protocol (Omega Bio-Tek, Lilburn, GA). cDNA was synthesised using an Omniscript reverse transcriptase kit according to the manufacturer's protocol (Qiagen, Valencia, CA). After cDNA synthesis, RNA was digested with E.coli RNase H (0.1 U/µl). Lamin A cDNA encoding part of exon 9 through exon 12 was synthesised by PCR amplification using a forward primer, 5'-CGG GAT CCT GGT TGG GCC TGA GTG GTC AGT C-3', and a reverse primer specific for the 1868 C>G (T623S) mutation, 5'-GAG TAC AAC CTG CGC TAC TGT CAC TG-3'. Twenty-six out of the 33 clones had the transversion at nucleotide 1868 C>G (T623S) in exon 11 of LMNA (fig 1A). This transversion causes a conservative substitution of serine for threonine. This transversion was not found in 115 unrelated healthy Japanese (fig 1B). There was no other alteration in any of the exons of LMNA in this patient. DNA fragments containing exon 11 of LMNA from the patient LCL were amplified by PCR and cloned into a plasmid vector. Twenty five plasmid clones were randomly isolated and the DNA sequence of each clone was determined. Eleven out of the 25 clones (44%) contained the C to G transversion at nucleotide 1868 and the rest had no mutation, indicating that the patient LCL was heterozygous for the transversion but not mosaic.

Results
Identification of a novel heterozygous LMNA mutation
We have determined genomic DNA sequences of all exons of LMNA and their flanking regions in an LCL established from a Japanese patient with HGPS. The patient had a heterozygous transversion at nucleotide 1868 C>G (T623S) in exon 11 of LMNA (fig 1A). This transversion causes a conservative substitution of serine for threonine. This transversion was not found in 115 unrelated healthy Japanese (fig 1B). There was no other alteration in any of the exons of LMNA in this patient. DNA fragments containing exon 11 of LMNA from the patient LCL were amplified by PCR and cloned into a plasmid vector. Twenty five plasmid clones were randomly isolated and the DNA sequence of each clone was determined. Eleven out of the 25 clones (44%) contained the C to G transversion at nucleotide 1868 and the rest had no mutation, indicating that the patient LCL was heterozygous for the transversion but not mosaic.

Activation of a cryptic splice site
Total RNA was extracted from the patient and control LCLs. Part of lamin A cDNA was synthesised by RT-PCR amplification using primers located in exons 9 and 12 (fig 2A). This primer pair should produce a DNA fragment 501 bp in length from normal length lamin A mRNA. As predicted, the 501 bp fragment was observed in both the patient and control samples (fig 2B). A smaller DNA fragment with an approximate length of 400 bp was additionally identified in the patient but not in the control samples. DNA sequencing of the 400 bp fragment revealed a 105 bp deletion in lamin A cDNA (1864–1968del), presumably causing a 35 amino acid truncation (codon 622 through 656) in lamin A (fig 2C). This 105 bp deletion was thought to be caused by activation of a cryptic splice site due to the transversion at nucleotide 1868 C>G because the transversion improves the match to a consensus splice donor sequence (fig 1A). Lamin C should not be affected by the mutation. In order to determine the efficacy of the cryptic splice site, the 501 bp fragment amplified by PCR from the patient sample was cloned into a plasmid and independently isolated 33 clones with the ~500 bp fragment insert were analysed by DNA sequencing. Twenty-six of the 33 clones had the transversion at nucleotide 1868 C>G and the rest showed a wild type by
DNA sequence. These results indicate that normal length lamin A mRNA as well as lamin A mRNA with the 105 deletion was produced from the mutant allele and further suggest that the majority of full length lamin A mRNA might be transcribed from the mutant allele in the patient LCL.

**DISCUSSION**

We have identified a novel mutation, 1868 C>G (T623S), in LMNA, which caused HGPS. Our patient was heterozygous for the mutation. The mutation produced a conservative substitution of serine for threonine and activated a cryptic splice site. The cryptic splice site was partly functional resulting in expression of lamin A with a 35 amino acid truncation (codon 622–656del) as well as normal length lamin A with an amino acid substitution (T623S). By RT-PCR based analysis, approximately 80% of normal length mRNA for lamin A derived from the mutant allele.

Five LMNA mutations causing HGPS have been reported so far (see Table). While patients with mutations at exon 11 in LMNA have the typical phenotype of HGPS, patients with mutations at either exon 2 or 8 and 9 (compound heterozygote) show unusual clinical features for HGPS, such as ample subcutaneous tissues and persistence of coarse hair on the head for exon 2 mutation and absence of coronary artery disease for exon 8 and 9 mutation. Thus, the typical phenotype of HGPS seems to be caused by the mutations in exon 11, all of which activate cryptic splice sites resulting in lamin A truncation. The 1868 C>G (T623S) mutation found in our patient produced lamin A with a 35 amino acid truncation while the 1824 C>T (G608G) or 1822 G>A...
same membrane. Lamin A/C in each lane, an antibody reacting with actin was used on the HGPS sample and not in the controls. To normalise the signal for bands for lamin A/C, a protein band of 572 amino acid residues of human lamin A/C. In addition to the normal protein samples were visualised by JoL2 antibody reacting with the 464–572 amino acid residues of human lamin A/C. In addition to the normal protein samples were visualised by JoL2 antibody reacting with the 464–572 amino acid residues of human lamin A/C.

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This internal proteolytic cleavage is essential for generation of mature lamin A from prelamin A. As suggested by Eriksson et al, incompletely processed prelamin A with an amino acid truncation may be responsible for the typical HGPS phenotype.

Our patient seemed to have a mild form of HGPS in terms of the onset and progression of the disease compared to patients with classical HGPS. The most straightforward explanation for this is that lamin A with the 35 amino acid truncation completely overlaps the 50 amino acid truncation (fig 2A). Both truncated proteins retain a \textit{CAAX}-box motif for farnesylation but lack an endoproteolytic cleavage site. This internal proteolytic cleavage is essential for generation of mature lamin A from prelamin A. As suggested by Eriksson et al, incompletely processed prelamin A with an amino acid truncation may be responsible for the typical HGPS phenotype.

Another progeroid syndrome, Werner syndrome (WS), shares some phenotypic features with HGPS such as loss of hair, atherosclerosis, short stature, scleroderma-like skin, and reduction in proliferative life span of fibroblasts. Most cases of Werner syndrome are caused by mutations at the \textit{WRN} helicase gene. Very recently, Chen et al reported that a small subset of atypical Werner syndrome subjects had mutations in \textit{LMNA}. Considering the proximity of lamin A and \textit{WRN} helicase, lamin A may not only functionally but also physically interact with \textit{WRN} helicase, and certain mutations in lamin A may have a detrimental influence on \textit{WRN} helicase resulting in the shared features. These mutations may provide insight into the connection between lamin A and \textit{WRN} helicase.

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<table>
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<th>Exon</th>
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<td>8, 9</td>
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<td>E145K</td>
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AA del, amino acid deletion.

(G608S) mutation found in classical HGPS cases created lamin A with a 50 amino acid truncation. The 35 amino acid truncation completely overlaps the 50 amino acid truncation (fig 2A). Both truncated proteins retain a \textit{CAAX}-box motif for farnesylation but lack an endoproteolytic cleavage site. This internal proteolytic cleavage is essential for generation of mature lamin A from prelamin A. As suggested by Eriksson et al, incompletely processed prelamin A with an amino acid truncation may be responsible for the typical HGPS phenotype.

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