

ONLINE MUTATION REPORT

LMNA mutation in a 45 year old Japanese subject with Hutchinson-Gilford progeria syndrome

K Fukuchi, T Katsuya, K Sugimoto, M Kuremura, H D Kim, L Li, T Ogihara

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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, coxa 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 45.⁴ 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.⁵⁻⁷ 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 primers⁸ and then sequenced using a 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 AAT TCA GGG ACC CGC CTG CAG GAT TTG G-3', and a reverse primer specific for the 1868 C>G mutation, 5'-CAC ACT GCG GTA GCT GCG 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× 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 G-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 500 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 T-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 CCG TAC GGC TCT CAT CAA CTC CAC TG-3' and a reverse primer, 5'-CGG AAT TCA GGG GGT GGG CAT GAG GTG AGG AG-3'. A BamHI or EcoRI restriction enzyme site (underlined) was added to the forward or reverse primers, respectively. The PCR was carried out in a 50 µl volume containing 1.5 µl of cDNA synthesis reaction, 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. The PCR products were analysed through 1.5% agarose electrophoresis. This PCR yielded 501 and 396 bp fragments in the HGPS sample but only the 501 bp fragment in controls. The 501 bp fragment corresponding to a normal length cDNA of lamin A in the HGPS sample was isolated, digested with BamHI and EcoRI restriction enzymes, and cloned onto the same restriction enzyme sites in pSP72 vector. After transforming DH5α competent cells, 33 clones with ~500 bp insert were randomly selected for DNA sequencing using an oligonucleotide, 5'-GAG TAC AAC CTG CGC T-3', as a primer. The 396 bp fragment was also sequenced using the same primer.

Western blotting

Cultured lymphoblastoid cells were washed with PBS once and harvested in 1× vol of 2× Laemmli buffer (1× = 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) with protease

inhibitors (1× final concentration of Complete Mini, Roche, Mannheim, Germany). After boiling for 5 min, cell lysates were sheared with 26-gauge needles. Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Three independent culture samples from each of three different LCLs (an HGPS and two normal controls) were similarly prepared and analysed by Western blotting. A 30 µg amount of protein for each sample was applied to 8% Tris-glycine SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were blocked with phosphate-buffered saline (PBS) containing 5% non-fat dried milk (w/v), 0.02% sodium azide, and 0.02% Tween 20, incubated at 4°C for 16 h with JoL2 antibody (Chemicon, Temecula, CA) reacting with the 464–572 amino acid residues of human lamin A/C. Lamin A and C were visualised by Western blot chemiluminescence reagent plus (Perkin Elmer, Boston, MA) according to the manufacturer's protocol. The membranes were reprobated with anti-actin antibody (Chemicon) for normalisation. The relative concentration of lamin A was determined by densitometric scanning of the membrane using a Fluor-S MultiImager and PDQuest software (Bio-Rad, Hercules, CA) and normalised by the actin signal.

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 out of the 33 clones had the transversion at nucleotide 1868 C>G and the rest showed a wild type by

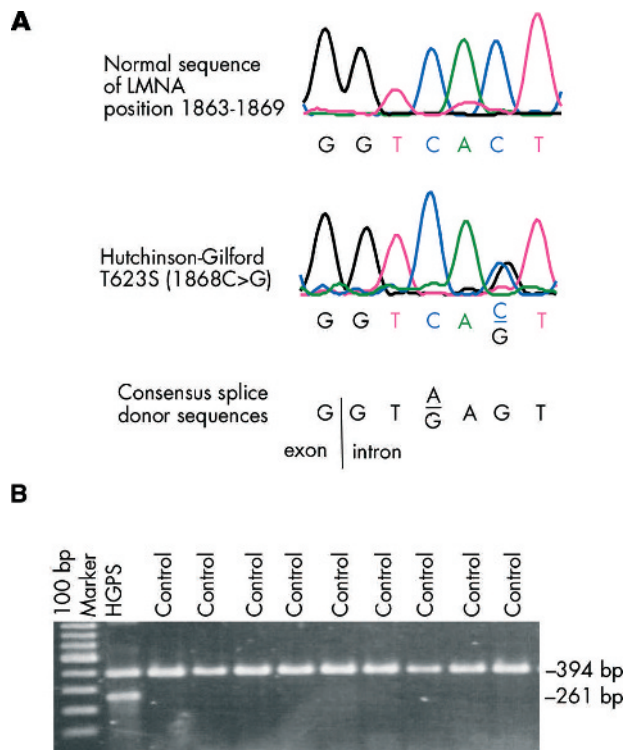


Figure 1 Point mutation and cryptic splice site found in exon 11 of *LMNA* in a patient with HGPS. (A) Genomic DNA sequence traces (position 1863–1869) from a normal control and an HGPS patient. Consensus splice donor sequences are shown for comparison. (B) Screening of healthy control subjects for the 1868 C>G (T623S) mutation by PCR. We established a PCR screening method using a specific primer to detect the 1868 C>G mutation. By this method, a mutant allele with the 1868 C>G mutation produces two DNA fragments 261 and 394 bp in length while a wild type allele produces only the 394 bp fragment. The figure shows typical results of the PCR screening: the 261 bp fragment separated by agarose gel electrophoresis was found in our HGPS patient but not in healthy controls.

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.

Confirmation of a truncated lamin A

Expression levels and species of lamin A and C in the patient and control samples were determined by Western blot analysis using a monoclonal antibody, JoL2, reacting with the 464–572 amino acid residues of human lamin A and C. The levels of lamin A and C were normalised by actin expressed in the same samples. Lamin A and C were identified as approximately 70 and 60 kDa bands, respectively, on the Western blots (fig 3). The levels of lamin A and C in the patient samples were comparable to those in the control samples. Between lamin A and C on the Western blots, an additional band of about 65 kDa in molecular weight, a presumptive lamin A with the 35 amino acid truncation, was identified in the patient samples but not in controls.

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

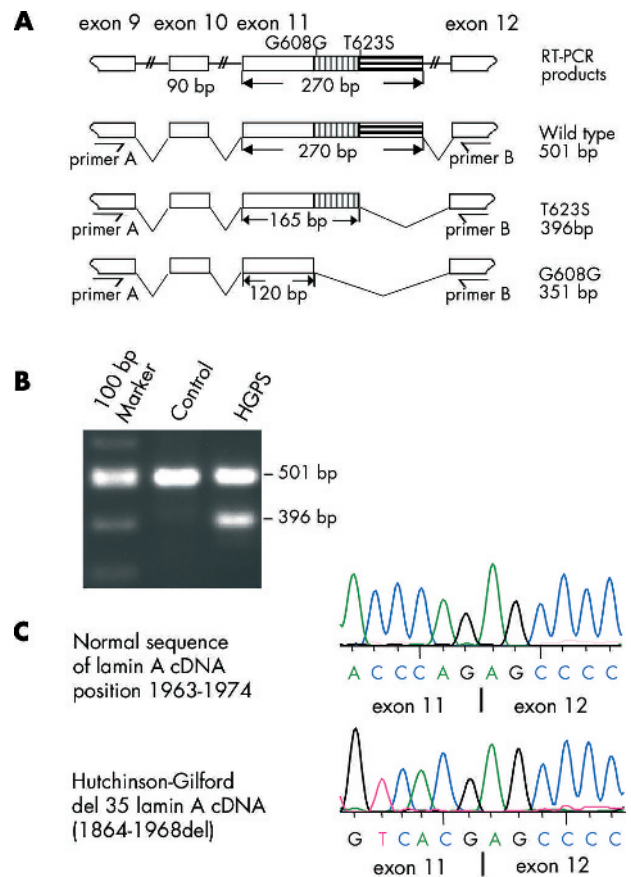


Figure 2 Analysis of lamin A cDNA in an HGPS subject. (A) Structure of three different mRNAs produced from a wild type allele or two mutant alleles with an 1868 C>G (T623S) or 1824 C>T (G608G) mutation. (B) Agarose gel electrophoretic analysis of RT-PCR. RNA was extracted from HGPS and control cell lines and RT-PCR was performed using primers A and B indicated in A. Two DNA fragments with approximate sizes of 500 and 400 bp are seen in the HGPS sample while the control sample produced only the ~500 bp fragment, demonstrating the cryptic splice site activated by the 1868 C>G mutation. (C) DNA sequences of the junction of exons 11 and 12. The ~500 and 400 bp fragments from the control and HGPS samples, respectively, in B were isolated and subjected to DNA sequencing. DNA sequence traces at the junction of exons 11 and 12 in the ~500 bp fragment from a control (position 1963–1974) and the ~400 bp fragment from the patient sample (1864–1968del) are shown.

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.^{3,7} 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

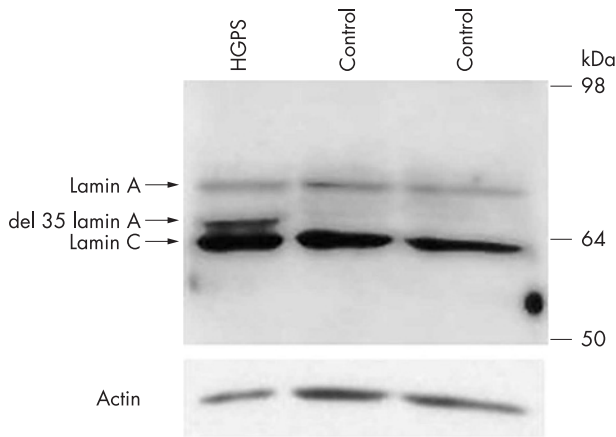


Figure 3 Western blot analyses for lamin A/C expression in HGPS and control cells. Protein samples from HGPS and controls were subjected to 8% Tris-glycine SDS-PAGE. A 30 μ g amount of protein for each sample was loaded on each lane. After electrotransfer to a PVDF membrane, the protein samples were visualised by JoL2 antibody reacting with the 464–572 amino acid residues of human lamin A/C. In addition to the normal bands for lamin A/C, a protein band of ~65 kDa in size is seen only in the HGPS sample and not in the controls. To normalise the signal for lamin A/C in each lane, an antibody reacting with actin was used on the same membrane.

(G608S) mutation found in classical HGPS cases created lamin A with a 50 amino acid truncation.^{5, 6} The 35 amino acid truncation completely overlaps the 50 amino acid truncation (fig 2A). Both truncated proteins retain a CAAX-box motif for farnesylation but lack an endoproteolytic cleavage site.^{9–11} This internal proteolytic cleavage is essential for generation of mature lamin A from prelamin A. As suggested by Eriksson *et al.*,⁵ 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 is less harmful to its function than that with the 50 amino acid truncation. In this scenario, the truncated proteins of lamin A might have dominant negative effects on the nuclear lamina whose architectural main constituent is lamin A. Thus, our results may reflect genotype–phenotype correlation. Because normal length lamin A with the 1868 C>G mutation is expressed in our patient, it is possible that the T623S substitution is responsible for the mild form of HGPS rather than the truncated protein. The latter explanation, however, is less plausible than the former because the 1824 C>T mutation is a silent substitution (G608G). It is also

possible that an amino acid truncation of lamin A involving a C-terminal region encoded by 11 results in a gain-of-function mutation and that lamin A with a longer truncation is functionally more active than that with a shorter one.

De Sandre-Giovannoli *et al.*⁶ reported that cells from HGPS patients with the 1824 C>T mutation had 75% reduction in expression levels of lamin A and lacked expression of the truncated lamin A. They attributed HGPS to a major loss of lamin A expression. Eriksson *et al.*⁵ identified by Western blotting the 50 amino acid truncated lamin A in cell samples from patients with the 1824 C>T mutation, except for one sample, and did not find a major loss of lamin A expression except in the same exceptional sample. The latter authors suggested that expression of the truncated lamin A might be a cause of HGPS. In our patient, the 35 amino acid truncated lamin A was readily detectable and levels of normal length lamin A were comparable to those in control subjects based upon three independent Western blot analyses. Our results are consistent with those by the latter authors. Such a varying degree of truncated lamin A expression among patients could be due to heterogeneity in modifier genes. A mutation in the splice donor site in the sodium channel gene *Scn8a* causes inherited movement disorders in mice, which range in severity from tremor to ataxia, dystonia, and juvenile lethality.^{12, 13} The severity of these disorders is modified by genetic variation in a putative RNA splicing factor, sodium channel modifier 1 (SCNM1), which determines the proportion of correctly spliced transcripts.^{13, 14} Because the age at death even among classical HGPS cases ranges from 7 to 27.5 years, it seems important for a better understanding of the molecular pathogenesis of HGPS to investigate a possible association between the age at death and the proportion of correctly spliced or truncated lamin A transcripts in patients with the 1824 C>T (G608G) or 1822 G>A (G608S) mutation. Such investigation may lead to identification of modifier genes involved in RNA splicing.

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 *WRN* helicase gene.¹⁵ Very recently, Chen *et al.*¹⁶ reported that a small subset of atypical Werner syndrome subjects had mutations in *LMNA*. Considering the proximity of lamin A and *WRN* helicase, lamin A may not only functionally but also physically interact with *WRN* helicase, and certain mutations in lamin A may have a detrimental influence on *WRN* helicase resulting in the shared features. These mutations may provide insight into the connection between lamin A and *WRN* helicase.

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Summary of reported *LMNA* mutations in patients with HGPS

Exon	Mutation	Effect	No of subjects	Reference
11	1824 C>T	G608G Cryptic splice site (50-AA del)	20	5, 6, 7
11	1822 G>A	G608S Cryptic splice site (50-AA del)	1	5, 7
11	1868 C>G	T623S Cryptic splice site (35-AA del)	1	This study
8, 9	1623 C>T, 1791 C>T	R471C, R527C	1 (compound heterozygote)	7
2	433 G>A	E145K	1	5

AA del, amino acid deletion.

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Authors' affiliations

K Fukuchi, H D Kim, Department of Genetics, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, AL 35294-0024, USA

T Katsuya, K Sugimoto, M Kuremura, T Ogihara, Department of Geriatric Medicine, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan

L Li, Department of Medicine, School of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294-0024, USA

Conflicts of interest: none declared.

Correspondence to: K Fukuchi, MD, PhD, UAB Department of Genetics, KHGB 640B, 720 South 20th Street, Birmingham, AL 35294-0024, USA; fukuchi@uab.edu

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