Published in final edited form as:
Mitochondrion. 2009 September ; 9(5): 340–345. doi:10.1016/j.mito.2009.05.002.

De Novo Mutation in *POLG* Leads to Haplotype Insufficiency and Alpers Syndrome

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Abstract

Mutations in *POLG* are a major contributor to pediatric and adult mitochondrial diseases. However, the consequences of many *POLG* mutations are not well understood. We investigated the molecular cause of Alpers syndome in a patient harboring the *POLG* mutations A467T *in trans* with c.2157 +5_+6 gc→ag in intron 12. Analysis of transcripts arising from the c.2157+5_+6 gc→ag allele revealed alternative splicing with an insertion of 30 intronic nucleotides leading to a premature termination codon. These transcripts were subsequently removed through nonsense-mediated decay, leading to haplotype insufficiency due to expression of the A467T allele and decreased expression of the c.2157+5_+6 gc→ag allele, which is likely responsible for the Alpers syndrome phenotype.

Keywords

Mitochondrial disease; mitochondrial DNA replication; Alpers syndrome; nonsense-mediated decay; *de novo* mutation; haplotype insufficiency

1. INTRODUCTION

Mitochondrial diseases arise because of disturbances within the mitochondria (Dimauro and Davidzon, 2005; Wallace, 1999). One subset of these diseases encompasses are due to defects in mitochondrial DNA (mtDNA) stability (Copeland, 2008). *POLG* encodes the mtDNA polymerase (pol γ) and is a major locus of mitochondrial disease (Chan and Copeland, 2008).

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Patients with *POLG* mitochondrial disease show mtDNA deletions or depletion in symptomatic tissues as pol γ is essential for mtDNA replication and repair. To date, approximately 150 mutations in *POLG* have been identified (http://tools.niehs.nih.gov/polg/), with three main disease phenotypes, that is Alpers syndrome and related mtDNA depletion disorders, ataxia-neuropathy syndromes, and progressive external ophthalmoplegia.

Alpers syndrome is a rare heritable autosomal recessive disorder affecting young children (Alpers, 1931; Harding et al., 1995; Naviaux et al., 1999). The disease is characterized by refractory seizures, psychomotor regression, hepatic dysfunction and depletion of mtDNA (Harding et al., 1995; Naviaux et al., 1999). It generally manifests during the first few weeks to years of life and symptoms develop in a stepwise manner leading eventually to early death. *POLG* mutations were first linked to Alpers syndrome in 2004 (Naviaux and Nguyen, 2004) when two unrelated probands were found to have compound heterozygous mutations in *POLG*, namely the A467T mutation on one allele and a premature termination codon (PTC) mutation, E873X, on the other allele. A467T is the most common *POLG* mutation, and is particularly prevalent in Alpers syndrome where ~65% of patients carry at least one A467T allele (Nguyen et al., 2006). Previous biochemical studies in our laboratory using purified recombinant pol γ containing the A467T mutation showed that this enzyme had very low DNA polymerase activity. Furthermore, pol γ with the A467T mutation had an impaired ability to functionally associate with the pol γ accessory subunit (Chan et al., 2005a), which is needed for highly processive DNA synthesis and enhanced DNA binding (Lim et al., 1999).

We also studied the skin fibroblasts of one patient from the aforementioned Alpers *POLG* study (Naviaux and Nguyen, 2004) in order to understand the contribution of the E873X allele to disease (Chan et al., 2005b). The allele containing the E873X mutation in exon 17 was expected to produce a truncated protein. However, only full-length p140 protein was detected. Sequence analysis of the cDNA from the pre-spliced message showed that both alleles were represented equally. Sequence analysis of cDNA derived from mature message revealed only transcripts containing the A467T mutation indicating that full-length pol γ arose from the allele containing the A467T mutation. Further analyses revealed that transcripts arising from the E873X allele were degraded by the nonsense-mediated mRNA decay (NMD) pathway. The NMD pathway degrades transcripts containing PTCs that are at least 50–55 nucleotides upstream from at least one intron. Additionally, sequencing of a shadow band showed that exon 17 had been skipped by the nonsense-associated alternative splicing pathway (NAS), which produces a frameshift leading to another PTC (Chan et al., 2005b). NMD and NAS destroyed virtually all mRNAs produced from the allele containing the PTC. Thus, the severity of disease for this patient was most likely due to mono-allelic expression of A467T pol γ. Extrapolating to other mutations of this nature, NMD and NAS are expected to remove those *POLG* mRNAs containing PTCs.

There are currently 18 *POLG* mutations that may produce a PTC, frameshift or an alternatively spliced transcript (Table 1). The mechanisms of pathogenesis via intronic *POLG* mutations have not yet been described until now. We report an Alpers syndrome patient with a *POLG* genotype of A467T/c.2157+5_+6 gc→ag *in trans*. We investigated the consequence of the double intronic mutation on splicing and degradation of the *POLG* message. We observed alternative splicing of this allele leading to a PTC and degradation of the message by NMD. This results in a much decreased expression of the c.2157+5_+6 gc→ag allele, with normal expression of the allele containing the A467T mutation, which explains the Alpers syndrome phenotype for this patient.

2. MATERIALS AND METHODS

2.1. Patient history

This patient was previously described as 'Patient 2' or 'Case 2' (Lutz et al., 2009; Wong et al., 2008). The patient was an 18-month-old Caucasian boy born to healthy, unrelated parents. He was the second son of three siblings. Motor development was delayed, with crawling but not walking achieved. He was noted to have lower extremity weakness. His speech development was also delayed. MRI at 17 months showed normal brain findings, but he had a spinal cord syrinx at T11-L1. At 18 months, he experienced an acute episode of emesis while eating, followed by irritability and lethargy. The episode was not preceded by fasting, fever, or illness. During this episode, he progressed to have unresponsiveness, eye deviation, jaw clenching, and hypotonia of the trunk and extremities. During transport to the emergency department, he developed repetitive generalized tonic-clonic seizures that evolved into refractory status epilepticus. He was transferred to a tertiary care facility and placed in a pentobarbital coma for seizure control after failing standard treatment with phenobarbital, fosphenytoin, midazolam, lorazepam, and diazepam. Valproic acid was avoided. After 30 days on this treatment, his seizures resolved and pentobarbital was discontinued. He was left with brain atrophy and a severe encephalopathy characterized by choreo-athetoid movements, cortico-visual impairment, diffuse hypotonia, and severe swallowing dysfunction. Transaminases were mildly elevated during his hospitalization but normalized as his seizures resolved.

The patient became more alert and regained the ability to babble at 20 months. However, by 21 months, his seizures recurred despite treatment with topiramate, levetiracetam, and phenobarbital. At 24 months, he was noted to have non-dependent edema, ascites, and abnormal coagulation studies indicating liver biosynthetic failure. Parents elected to place the child in hospice care, and he died at 25 months. Autopsy confirmed liver fibrosis, with nests of cells with severe vacuolation, areas of bile duct proliferation, and small numbers of lymphocytes scattered throughout. The spleen showed erythrocyte sequestration and lymphoid aggregates with germinal centers. The pancreas appeared normal. Kidneys appeared normal except for hypercellularity. The heart showed diffuse hypercellularity and thin myocytes with frequent dark nuclei. There was anasarca, atelectasis of the lungs, and a large amount of cerebrospinal fluid surrounding a relatively small-sized brain.

The initial laboratory work-up included AST 85 U/L (range 16–46 U/L), ALT 69 U/L (range 29–46), plasma lactate 3.2 mM (range 0.5–2.0), CSF lactate was 3.4 mM (range 0.6–2.2) and protein was 46 mg/dL (range 15–40). Liver transaminases remained mildly elevated throughout his hospitalization. AST peaked at 195 U/L and ALT at 200 U/L. MRI of the brain showed abnormal restricted diffusion involving the subcortical white matter in the left posterior parietal and occipital lobes consistent with multi-regional stroke-like episodes or ischemia. T2 signal was increased bilaterally in the thalami.

2.2. Molecular genetics

The patient had a compound heterozygous *POLG* genotype, with A467T on one allele, and two intronic changes in the other *POLG* allele (2157+5_+6 gc>ag). The proband's mother was heterozygous for the A467T mutation (A467T/WT), as well as his siblings, however the father was wild-type for both *POLG* alleles (Figure 1). Neither the A467T mutation, nor 2157+5_+6 gc>ag were seen in the father's DNA extracted from blood. Analysis of 15 unlinked microsatellite markers was consistent with stated paternity (Wong et al., 2008). This suggests a *de novo* mutation originating on the paternal allele.

2.3.Cell culture

A muscle biopsy was performed on the patient at 19 months. At this time, skin fibroblasts were obtained and cultured. Cells were grown at 37ºC in 5% CO2 in D-MEM/F-12 medium (Invitrogen) supplemented with 10% fetal calf serum, 1 mM pyruvate, non-essential amino acids, penicillin and streptomycin.

2.4. RNA extraction

Total RNA was isolated using the RNeasy kit (Qiagen). To remove any genomic DNA contamination, RNA samples were treated twice with DNaseI, first with Qiagen DNaseI for 15 min at room temperature as stated in the RNeasy protocol, and then with 6 units Ambion RNase-free DNaseI for 1 hr at 37ºC. DNaseI was then removed using the supplied DNase inactivation reagent. DNA-free status was confirmed by performing PCR on purified RNA using the same primers, without the addition of reverse transcriptase.

2.5. cDNA synthesis, PCR, sequencing and cloning

First strand cDNA synthesis was carried out on DNase1-treated RNA with the Superscript III First-Strand Synthesis system (Invitrogen), using a gene specific primer for *POLG* (2995R (Chan et al., 2005b)). Primer sequences for cDNA were as follows:

Exonic primers	
Spanning exons 5 to 8 of *POLG*	
Forward	5'-TTG TGA AGG GCA CCA TGA-3'
Reverse	5'-CTG TGG CTG GTT CCT TCT T-3'
Spanning exons 11 to 14 of *POLG*	
Forward	5'-AGT TCC TGC TCA CTG ACA ATA G-3'
Reverse	5'-GCG TTC CTC CAG AAA GAA ATC-3'
Intronic primers	
Spanning exons 7 to 8 of *POLG*	
Forward	5'-GTC TTG CCT CCT GTG GTC AT-3'
Reverse	5'-CTG TGG CTG GTT CCT TCT T-3'
Spanning exons 11 to 14 of *POLG*	
Forward	5'-TGT CAA TCA ATC CCT GTC TAA AAC C-3'
Reverse	5'-CGG CTC CAG CAG TTA CAC CAA GAA G-3'

PCR amplifications of cDNA were performed in a Perkin Elmer GeneAmp PCR Cycler 9700 using the following conditions: the thermal cycling program consisted of 10 min at 95ºC, followed by 20 cycles of 94ºC 30 s, 60ºC - 50ºC 30s, 72ºC 60 s (touchdown PCR, annealing temperature was decreased 0.5ºC after each cycle). This touchdown step was followed by 10 cycles of 94ºC 30 s, 50ºC 30 s, 72ºC 60 s. The samples were then cooled to 20ºC for 4 min and then kept at 4ºC. PCR products were resolved by agarose gel electrophoresis. Products were gel purified and directly sequenced using a BigDye terminator sequencing kit (Applied Biosystems) on an ABI PRISM 377 DNA sequencer. Alignments of the sequences were performed using SeqWeb Version 2 (Accelrys).

PCR products were also cloned into pCR4Blunt-TOPO plasmid using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen). Plasmids containing inserts from individual colonies were purified using the Miniprep plasmid purification kit (Qiagen) and were

sequenced using BigDye terminator sequencing and one of the primers used for the original PCR.

3. RESULTS

We investigated the molecular cause of Alpers syndrome in a patient with the A467T mutation in one *POLG* allele and a c.2157+5_+6gc→ag insertion mutation in intron 12 in the other *POLG* allele (Figure 2). While the pathogenesis of the A467T allele is well established as a recessive mutation in Alpers syndrome and other *POLG* mitochondrial diseases, the consequence of the c.2157+5_+6 gc>ag mutation is unclear. As this change is close to the canonical splice site, we wanted to determine whether this mutation did indeed affect splicing, which could lead to a mutant pol γ with severe defects in mtDNA replication and repair.

3.1. Alternative splicing of POLG transcripts containing the c.2157+5_+6 gc>ag mutation

The c.2157+5_+6 gc→ag mutation alters two nucleotides at the 5^{th} and 6^{th} base position of *POLG* intron 12. To investigate whether this two-nucleotide change could alter splicing, we first used a splice site prediction program, NetGene2, which uses neural network predictions of splice sites in human DNA (http://www.cbs.dtu.dk/services/NetGene2/; (Brunak et al., 1991; Hebsgaard et al., 1996)). Analysis of the wild-type sequence in this region accurately predicts wild-type exon 11 - intron 11, exon 12 - intron 12, and exon 13 - intron 13 donor splice sites (Figure 3A). However, *POLG* sequence containing the c.2157+5_+6 gc→ag mutation predicts a different exon 12 - intron 12 donor splice site (Figure 3B). This predicted splice site would lead to an alternatively spliced transcript containing an extra 30 nucleotides with a UAG stop codon in the 28^{th} to 30^{th} position.

To assess whether the predicted scenario occurs *in vivo*, we developed a fibroblast cell line from this patient using cells from a muscle biopsy taken at 19 months. Whole cell RNA was isolated from cultured fibroblasts. First-strand *POLG* cDNA was synthesized and used for all experiments described. PCR was performed in order to amplify mature message using exonic primers spanning exons 11 to 14. PCR products were ligated into pCR4Blunt-TOPO plasmids and individual clones isolated. Sequence analysis of 21 individual clones revealed that 81% (95% CI = 58–95%) of the transcripts were correctly spliced, while 19% (95% CI = 5–42%) were alternatively spliced. No exon skipping was observed. The sequence of the alternatively spliced cDNAs indicated bypass of the normal exon 12 - intron 12 splice site, with mature message containing an extra 30 nucleotides of intron 12 sequence (Figure 4). The new splice site corresponds with the predicted splice site by NetGene2 analysis (Figure 3B). All alternatively spliced transcripts, representing 19% of the total transcripts, contained the two intronic c.2157+5_+6 gc→ag mutations.

3.2. Both alleles are represented equally in pre-spliced transcripts

We determined the proportion of both *POLG* alleles prior to splicing. Amplification of prespliced message was performed using intronic primers spanning codon 467 (exon 7). Clones containing these PCR products were constructed as described above and individual clones were sequenced. Sequence analysis of these pre-spliced clones revealed an equal distribution (1:1) of each allele (Ala467:Thr467).

3.3. The bulk of mature POLG transcripts contain the A467T mutation

As we saw an equal representation of transcripts arising from each *POLG* allele prior to splicing, we wanted to determine the proportion of mature transcripts containing the A467T mutation. Direct sequencing of this mature, fully-spliced message showed that the bulk of this message contained Thr467, indicative of the A467T mutation (Figure 5A). To gauge a quantitative level of steady-state A467T message we isolated individual cDNA clones and

subjected them to sequencing. Using the exonic primers to exons 5 and 8, PCR products were ligated into the pCR4Blunt-TOPO plasmids. Forty-six individual clones were picked for sequencing analysis (Figure 5B). Sequence analysis of individual cDNA clones confirm that 89% (95% CI = 76–96%) of the mature message contained the A467T mutation, while only 11% (95% CI = 4–24%) of this population arises from the allele containing the c.2157+5_+6 gc>ag mutation. Thus, the bulk of the transcripts arise from the allele containing the A467T mutation. By comparing the results from 3.1. and by determining the binomial 95% confidence intervals, we conclude that the two populations (that is, those that contain the A467T mutation and those that are spliced correctly) are one and the same ($p = 0.44$). This suggests that the mature *POLG* message containing the c.2157+5_+6 gc>ag are spliced differently and are degraded.

4. DISCUSSION

Here we show that the c.2157+5_+6 gc>ag *POLG* allele is indeed pathogenic by affecting splicing of the *POLG* mRNA. This mutation causes the exon 12 - intron 12 splice junction to be bypassed, allowing the cell to utilize the next likely splice junction at c.2157+30. Within this 30 nucleotide insertion is an in-frame UAG PTC at c.2157+28–30. Transcripts with PTCs that are at least 50–55 nucleotides upstream of an intron are subjected to NMD. The message arising from the c.2157+5_+6 gc>ag *POLG* allele fits this criterion as *POLG* contains 23 exons, and the double mutation occurs in intron 12.

The degradation of the c.2157+5_+6 gc>ag *POLG* message was confirmed by sequencing individual mature cDNA clones. Analysis revealed that 19% of the fully spliced transcripts were alternatively spliced. These alternatively spliced transcripts all contained the two intronic mutations. Sequence analysis of prespliced *POLG* transcripts in the exon 7 region showed an equal proportion of wild-type message and message containing A467T. The discrepancy between the prespliced transcripts and the relatively low amount of c.2157+5_+6 gc>ag *POLG* message indicates that the c.2157+5_+6 gc>ag message was degraded, due to the PTC which triggered the NMD pathway. This caused an effective haplotype insufficiency, where the bulk of the surviving transcripts contain the A467T mutation. Our previous biochemical analysis showed that the A467T mutation results in a pol γ with low intrinsic polymerase activity and a defect in functional association with the pol γ accessory subunit leading to poor mtDNA replication (Chan et al., 2005a). However, this does not address why patients who are homozygous for the A467T mutation can develop one of the three major *POLG* disease phenotypes, and have a longer survival time as compared with compound A467T/W748S heterozygotes (Tzoulis et al., 2006). Our previous study of the first genotyped Alpers patient (A467T/E873X) (Chan et al., 2005b), and this current study take us closer to answering this question. In the previous study, we showed that for a patient with A467T/E873X *POLG*, most transcripts arise from the allele containing the A467T mutation, leading to mono-allelic expression of A467T pol γ, and is the most likely cause of early onset Alpers syndome in this patient (Chan et al., 2005b). Table 1 reveals a striking correlation of PTC mutations *in trans* with either A467T or W748S and early onset Alpers or myocerebrohepatopathy syndrome, while other mutations *in trans* with PTCs are associated with later onset PEO disease. This suggests that functional gene dose of the A467T allele could dictate age of onset.

We present the first analysis of the consequence of an intronic mutation that leads to alternative splicing in a *POLG* disease patient. Our analysis represents the second *POLG* case that causes one of the *POLG* alleles to be removed by NMD. Transcripts derived *from POLG* alleles containing any of the 18 known PTCs or splice-site mutations are also expected to undergo NMD. Thus, transcripts arising from alleles containing *POLG* PTC, frame-shift, or intronic mutations that are predicted to affect splicing should be regarded as possible targets for NMD.

Consequently for patients and physicians, the mutation found in the other *POLG* allele may be a better predictor of disease state.

ACKNOWLEDGMENTS

We would like to thank Drs. Janssen Daly and Raghuvar Dronamraju for critically reviewing this manuscript. Funding was provided from a National Institutes of Health Career Development Award to SSLC (1K99-ES015555-01), and Intramural Research funds (Z01-ES065078) from the National Institute of Environmental Health Sciences, NIH to WCC.

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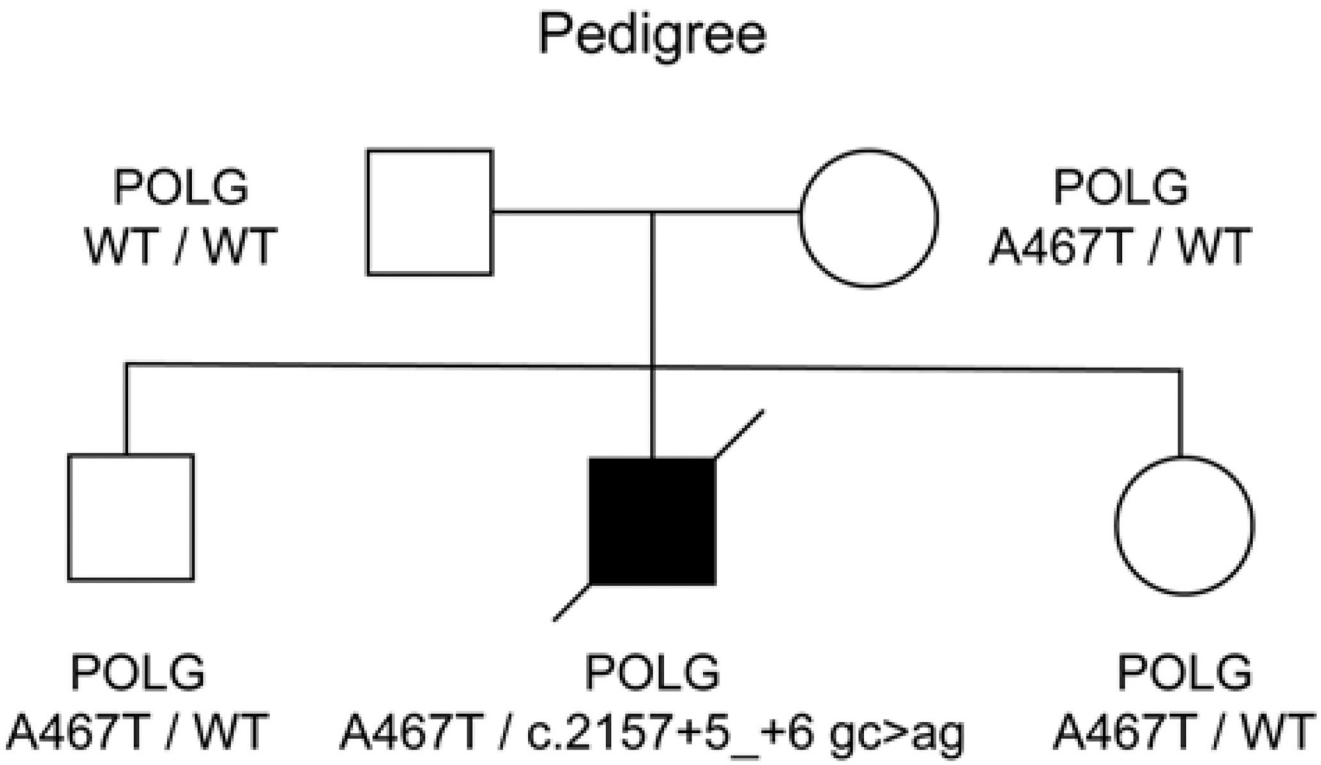

Figure 1.
Pedigree of proband. Black symbol indicates Alpers syndrome. WT = wild-type.

Figure 2.
Genomic DNA sequence of exon 12 - exon 14 from the *POLG* allele containing the c.2157+5_+6 gc>ag mutation. This alters a ‘gc’ to ‘ag’ at the 5th and 6th position 3' of the exon 12 - intron 12 splice junction.

A.

WILD-TYPE *POLG* SEQUENCE
Donor splice sites, direct strand

Confidence	5' exon intron 3'	
1.00	ATGGCAAACG^GTGAGGGCAG	Exon 11 - Intron 11
0.31	GCAGTGCCAG^GTCAACCCCT	
0.91	CCTAGCTCTG^GTGAGCAGTG_	Exon 12 - Intron 12
0.75	GGTTCTCTAG^GTGGGTGCTG	
0.46	GAAAGCCTGG^GTGAGACCTC	
0.95	GCCTCACAAG^GTGTGTCCTG	Exon 13 - Intron 13

B.

MUTANT *POLG* SEQUENCE
Donor splice sites, direct strand

Confidence	5' exon intron 3'	
1.00	ATGGCAAACG^GTGAGGGCAG	Exon 11 - Intron 11
0.31	GCAGTGCCAG^GTCAACCCCT	
0.81	GGTTCTCTAG^GTGGGTGCTG	Alternate Exon 12 - Intron 12
0.46	GAAAGCCTGG^GTGAGACCTC	
0.95	GCCTCACAAG^GTGTGTCCTG	Exon 13 - Intron 13

Figure 3.
Splice sites as predicted by NetGene2 server, which produces neural network prediction of splice sites in human DNA (http://www.cbs.dtu.dk/services/NetGene2/) (Brunak et al., 1991; Hebsgaard et al., 1996). **(A)** Wild-type *POLG* sequence. **(B)***POLG* sequence containing the c2157+5_+6 gc>ag mutation.

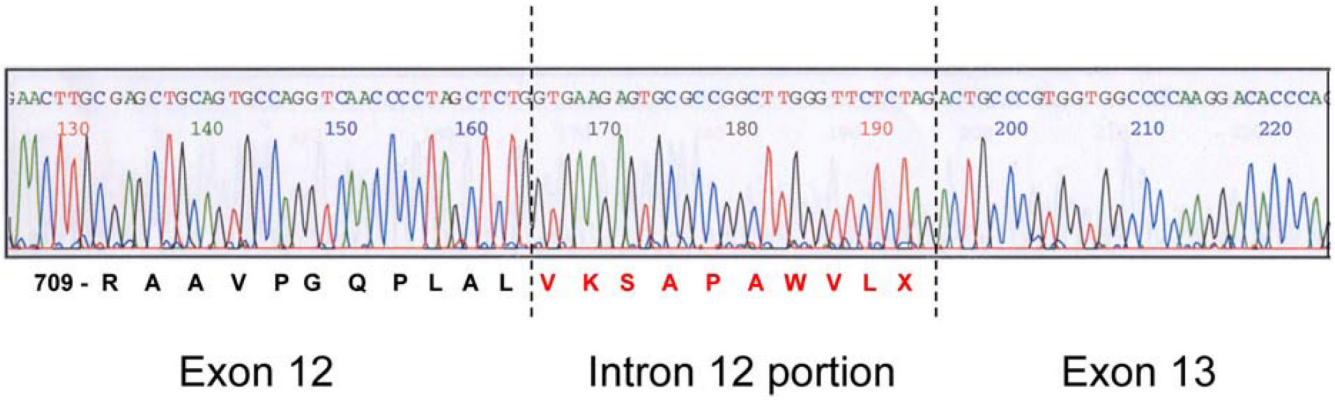

Figure 4.
Sequence analysis of individual clones from cDNA spanning exons 11 – 14 reveals an inclusion of part of intron 12 in the final fully-spliced transcript. The extra 30 nucleotide insert contains an in-frame PTC at nucleotides 28–30 just before the alternative splice site.

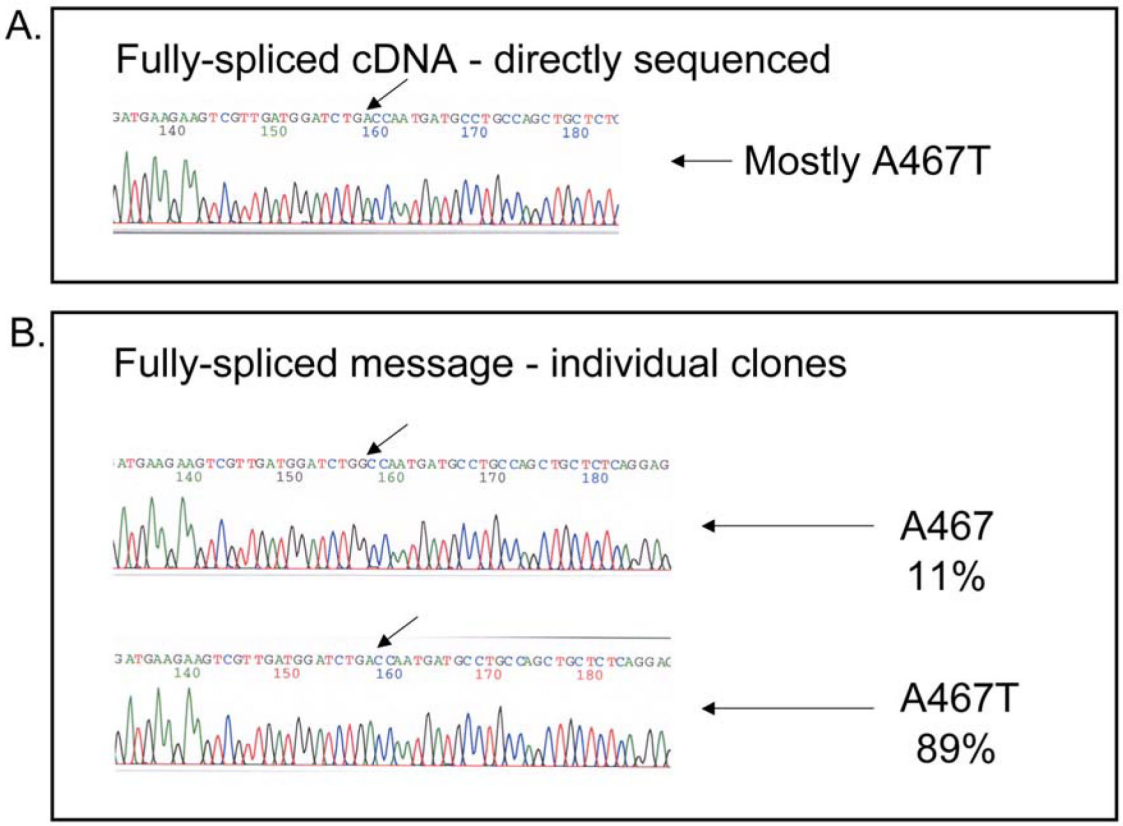

Figure 5.
Sequence analysis of genomic DNA and fully-spliced mRNA fragments. **(A)** Fully-spliced message from fibroblasts (direct sequencing after amplification from cDNA) showed that most transcripts contain the A467T mutation. **(B)** Sequencing of individual clones revealed both populations, with the A467T allele predominating.

Table 1

Premature termination mutations in the *POLG* gene and associated disease.

Amino Acid Substitution	DNA mutation	Disease	Genetics	Reference
Q68X	202 c→t (exon 2)	Alpers	Found *in trans* with A467T	(Wong et al., 2008)
W235X	705 g→a (exon 3)	Myocerebrohepato-pathy syndrome	Found *in trans* with A467T	(de Vries et al., 2006)
T326fsX387	975–976 ins c (exon 4)	Alpers	Found *in trans* with A467T	(Naimi et al., 2006)
R374X	1120 c→t (exon 5)	Alpers	Found *in trans* with A467T.	(Ashley et al., 2008)
L424GfsX28 (CT)deletion-452X	1270–1271 del ct (exon 7)	Alpers	Found *in trans* with A467T	(Wong et al., 2008)
		PEO	Sporadic Found *in trans* with G431V	(Agostino et al., 2003)
T452X	1356 t→g (exon 7)	PEO	Sporadic	(Hudson et al., 2006)
R709X	2125 c→t (exon 12)	PEO	Sporadic, Found *in trans* with T251I–P587L	(Del Bo et al., 2003; Di Fonzo et al., 2003)
Q715X	2143 c→t (exon 12)	Alpers	Found *in trans* with A467T	(Wong et al., 2008)
c. 2157+5_+6	2157 gc→ ag (exon 12)	Alpers	Found *in trans* with A467T	(Wong et al., 2008)
2354Gins at G785	2354 ins G STOP @ codon 806 (exon 14)	arPEO	Found *in trans* with T251I	(Lamantea et al., 2002)
c. 2480+1	2480+1 g→a splice (exon 15)	Alpers	Found *in trans* with W748S–E1143G	(Wong et al., 2008)
IVS15-9-c.2485del 12bp	2485 del 12bp (exon 16)	Alpers	Found *in trans* with A467T. Splice site mutation 3'exon15/intron junction,	(Horvath et al., 2006)
E873X	2617 g→t (exon 17)	Alpers	Found *in trans* with A467T	(Ashley et al., 2008; Chan et al., 2005b; Naviaux and Nguyen, 2004; Naviaux and Nguyen, 2005)
L965X	2894 t→g (exon 18)	arPEO	Found in cis with E1143G and *in trans* with R627Q–Q1236H	(Horvath et al., 2006)
W1020X	3057 g→a (exon 19)	Alpers	Found *in trans* with A467T	(Nguyen et al., 2005)
3482 +2T to C	3482 +2t's splice at intron 21 (exon 21)	Alpers	Exon21/Intron21 splice site mutation at a.a. 1161. Found *in trans* with A467T	(Ferrari et al., 2005; Horvath et al., 2006)
L1173fsX	3518 ins gact, fs in exon 22 (exon 22)	Alpers	Found *in trans* with A467T	(Nguyen et al., 2006)
Y1210fs1216X	3630 ins c (exon 22)	Alpers	Found *in trans* with W748S–E1143G, Mutation causes frameshift to stop at a.a. 1225	(Ferrari et al., 2005; Spinazzola et al., 2009)