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FAST TRACK ARTICLE

Dominant inheritance of premature ovarian failure associated with mutant mitochondrial DNA polymerase gamma

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BACKGROUND: Premature ovarian failure (POF) results in menopause before the age of 40. Recently, mutations in the catalytic subunit of mitochondrial DNA polymerase gamma (POLG) were shown to segregate with POF in families with progressive external ophthalmoplegia (PEO) and multiple large-scale rearrangements of mitochondrial DNA (mtDNA). METHODS AND RESULTS: A patient, mother and maternal grandmother are described, all presenting with POF and PEO. The mother developed parkinsonism in her sixth decade. Normal mtDNA sequence excluded mitochondrial inheritance. Sequence analysis of polymerase gamma revealed a dominant Y955C mutation that segregated with disease. Southern blot analysis demonstrated mtDNA depletion in fibroblasts (43% of controls). In contrast, multiple rearrangements of mtDNA were seen in skeletal muscle, consistent with the relative sparing of nuclear-encoded complex II activity compared with other respiratory chain enzymes. Immunoblotting of native gels showed that DNA polymerase gamma stability was not affected, whereas a reverse-transcriptase primer-extension assay suggested a trend towards reduced polymerase activity in fibroblasts. CONCLUSIONS: This study confirms that *POLG* mutations can segregate with POF and parkinsonism and demonstrates for the first time that the Y955C mutation can lead to mtDNA depletion. Future screening projects will determine the frequency with which *POLG* is involved in the aetiology of POF and its impact on reproductive counselling.

Key words: mitochondrial DNA depletion/parkinsonism/PEO/POLG/premature menopause

Introduction

Premature menopause, also known as premature ovarian failure (POF), is the cessation of menstruation before the age of 40 and occurs in approximately 1% of women. POF is aetiologically heterogeneous, and known causes include autoimmunity, environmental toxins, infections, galactosaemia and anticancer treatments (Goswami and Conway, 2005). There is growing evidence for a major genetic component from familial cases, and a recent study demonstrates that age at natural menopause shows a heritability of around 50% (Murabito *et al.*, 2005). Female carriers of a premutation CGG expansion (61–200 copies) in *FMR1* appear to have an increased risk of POF (Allingham-Hawkins *et al.*, 1999), whereas longer expansions (>200) cause fragile X mental retardation syndrome in males and some females. POF is associated with Turner's syndrome (45, X) and can also be caused by balanced X;autosomal translocations most frequently involving two specific regions: POF1 at Xq26–q28 (Tharapel *et al.*, 1993) and POF2 at Xq13.3–Xq21.1 (Powell *et al.*, 1994).

In a recent study, patients with progressive external ophthalmoplegia (PEO) from seven unrelated families were shown to harbour mutations resulting in a defective mitochondrial DNA polymerase gamma. In three of these families, the mutations also segregated with POF, and in five families, segregation with parkinsonism was observed (Luoma *et al.*, 2004). DNA polymerase gamma is the only polymerase responsible for the replication of the 16.6-kb mitochondrial genome. It comprises a 137-kDa catalytic subunit (POLG) and a 52-kDa accessory subunit (POLG2). Mutations in the gene encoding POLG have

previously been associated with a variety of clinical features, including PEO, Alpers syndrome, ataxia and epilepsy (Van Goethem *et al*., 2001; Naviaux and Nguyen, 2004; Winterthun *et al*., 2005). All these conditions arise in combination either with large-scale rearrangements or with a quantitative defect of mtDNA. In addition, polymorphisms in the CAG expansion in exon 2 of *POLG* have been associated with male infertility (Rovio *et al*., 2001), although this association has recently been questioned (Aknin-Seifer *et al*., 2005).

Here, we describe dominantly inherited POF in a three-generation pedigree with PEO. Despite the pedigree showing apparent maternal inheritance, the recent link between *POLG* mutations and premature menopause (Luoma *et al*., 2004) prompted *POLG* sequence analysis. We demonstrate the segregation of the Y955C mutation with POF in this family and provide a detailed biochemical analysis of the phenotypic consequences of Y955C.

Materials and methods

Patient details

The proband (IV-2), her mother (III-2) and maternal grandmother (II-2) all presented with ptosis and PEO with onset in their early twenties and subsequently developed POF at the age of 28, 35 and 32 years, respectively (Figure 1A). At the age of 57, III-2 was noted to have a progressive, parkinsonian-like resting tremor and reduced rapid alternating movements affecting her left arm and leg, and mild bradykinesia. The tremor, which was not observed in II-2 or IV-2 (although the latter is currently only 33), responded to levodopa treatment. IV-2 is of above-average intelligence with a professional career. Additional symptoms experienced by all three women are proximal muscle weakness, exertional dyspnoea and sensory ataxia. II-2 and III-2 developed dysphagia in later life. IV-2 suffers episodes of palpitations and has unusual chest, arm and leg pain thought to be related to peripheral neuropathy. Of note in the family history, the maternal great grandmother I-2 is reported to have had a neuromuscular disorder (labelled myasthenia gravis), although clinical records are incomplete and DNA samples were not available from this individual for analysis. Two sisters of the proband (IV-1 and IV-3) are asymptomatic and have not presented with POF, aged 36 and 30, respectively.

Gonadotrophins were measured in IV-2: FSH levels were raised on two successive occasions 3 months apart (9.0 and 32.4 IU/l aged 28 and 3 months and 28 and 6 months, respectively, adult female premenopausal range 1.0–8.0 IU/l), whereas LH was still normal (4.4 and 7.4 IU/l, adult female non-ovulatory peak 2.0–15.0 and higher postmenopause). Resting plasma lactate was elevated in IV-2 (3.5 mM, normal range 0.5–2.2 mM), whereas in III-2, serum lactate was borderline (1.9 mM) and cerebrospinal fluid lactate was normal (1.1 mM).

Peripheral neuropathy was confirmed by electro-physiological studies in IV-2. Muscle histology in III-2 (biopsied at the age of 52) demonstrated the presence of ragged-red fibres in the modified

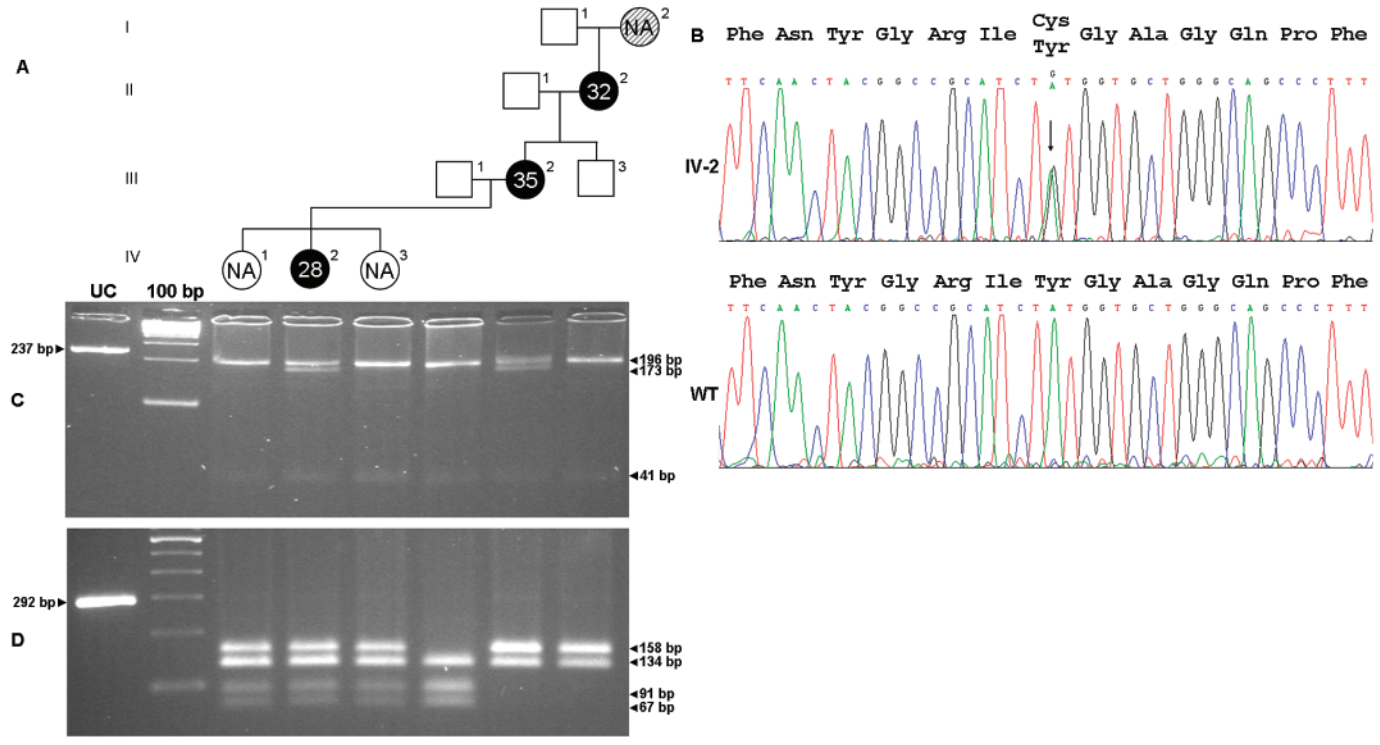

Figure 1. (**A**) Pedigree. Black symbols indicate progressive external ophthalmoplegia (PEO), hatched symbol indicates probable PEO, and numbers indicate age at menopause. NA, not applicable (not yet reached menopause or not known). (**B**) Sequencing electropherograms from 18R primer with amino acid translation. IV-2 harbours a heterozygous Y955C mutation (arrow). (**C**) *Hpy*CH4III digestion of amplification-created restriction site PCR products. The 196- and 173-bp fragments were separated on a 4% agarose gel and correspond to the wild-type and Y955C alleles, respectively. Y955C segregates with the disease. (**D**) *Bst*F5I digestion of the exon 23 amplicon. The fragments were separated on a 2.5% agarose gel. The wild-type 158-bp fragment is cut into 91- and 67-bp fragments in the Q1236H allele. This shows that Q1236H has been paternally inherited. DNA from the maternal grandparents and great grandparents was not available. Gel lanes are aligned with generations III and IV of the pedigree. UC, uncut.

Table I. Comparison of mitochondrial respiratory chain activities in skeletal muscle from III-2 and in fibroblasts from IV-2 with control samples

Assay	Skeletal muscle		Fibroblasts	
	III-2	Observed range in controls (n = 6–17)	IV-2	Observed range in controls (n = 12)
Complex I	0.067[a]	0.100–0.470	Not determined	Not determined
Complex II	0.223	0.110–0.330	0.105	0.095–0.205
Complex II + III	0.084[a]	0.115–0.460	0.129	0.070–0.243
Complex III	0.097[a]	0.100–0.300	Not determined	Not determined
Complex IV	0.008[a]	0.012–0.064	0.011	0.007–0.036

Enzyme activities expressed as ratio to citrate synthase.
[a]The ratios lie outside the control range.

Gomori trichome stain (4% of muscle fibres) and irregular dense NADH dehydrogenase and succinate dehydrogenase staining. Ten percent of muscle fibres were cytochrome *c* oxidase negative. Spectrophotometric analysis of muscle mitochondrial respiratory chain (RC) enzymes indicated normal complex II, whereas the activities of all other complexes were reduced (Table I).

As complex II is the only RC complex that does not include any mitochondrial-encoded subunits, this suggested a defect in either the maintenance of mtDNA or the translation of mitochondrial mRNAs. However, an initial Southern blot of *Bam*HI-digested DNA from III-2's muscle had not shown any large-scale rearrangements of mtDNA, and testing for the common Leu$^{(UUR)}$/Lys tRNA mutations (3243A>G and 8344A>G) in hair follicles from III-2 was also negative.

mtDNA sequencing

The entire sequence of the mitochondrial genome was amplified with AmpliTaq Gold™ DNA polymerase (Applied Biosystems, Warrington, UK) and a panel of 28 M13-tagged primer pairs generating overlapping fragments of between 600 and 700 bp (Taylor *et al*., 2001). Amplified samples were purified (ExoSapIT, Amersham Biosciences, Bucks, UK), sequenced using BigDye Terminator cycle sequencing chemistries (v3.1, Applied Biosystems) on an ABI3100 Genetic Analyser (Applied Biosystems) and directly compared with the revised Cambridge Reference Sequence using SeqScape software (Applied Biosystems).

POLG sequence analysis

Exons 2–23 and all intron–exon boundaries were amplified with primer sequences as previously described (Van Goethem *et al*., 2001). Primers and unincorporated dNTPs were removed using the QIAquick PCR purification kit (Qiagen, Crawley, UK). Sequencing reactions were set up with the BigDye Terminator cycle sequencing kit (v3.1, Applied Biosystems) and then run on the MegaBACE™ automated capillary sequencer (Amersham Biosciences). Sequence analysis was performed using Sequencher™ software (v4.2, Gene Codes, Ann Arbor MI, USA).

Restriction fragment length polymorphism analysis of Y955C (2864A>G)

To confirm the mutation and screen additional family members for the mutation, we designed an amplification-created restriction site (ACRS) primer (5′-AATCTTCAACTACGGCCGCA**A**C-3′, mismatch in bold) and used with the 18R sequencing primer. Digestion of the wild-type PCR product with *Hpy*CH4III (NEB, Hitchin, UK) gave fragments of 196 and 41 bp, whilst the 2864A>G allele resulted in fragments of 173, 41 and 23 bp.

Restriction fragment length polymorphism analysis of Q1236H (3708G>T)

*Bst*F5I (SibEnzyme, Novosibirsk, Russia) digested the 292-bp exon 23 amplicon into 158- and 134-bp fragments in wild type, whereas 3708G>T introduced a further site which cut the 158-bp fragment into 91- and 67-bp fragments.

Biochemical analysis of muscle and fibroblasts

A skin biopsy was taken from IV-2 at the age of 31. Fibroblasts derived from this were cultured in HAMS F10 (Invitrogen, Paisley, UK) supplemented with 12% FBS (Invitrogen) and 50 mg/l of uridine (Sigma, Poole, UK). The analysis of RC enzymes was carried out using standard procedures as described previously for muscle and fibroblasts (Rahman *et al*., 1996).

Southern blot analysis

Genomic DNA was digested with *Bam*HI, *Sna*BI or *Pvu*II, resolved on a 0.6% agarose gel, transferred to a Hybond membrane (XL for fibroblast DNA, N+ for muscle samples, Amersham Biosciences) using standard procedures and then probed with heat-denatured ^{32}P-labelled mtDNA. A probe for the multi-copy nuclear 18S rRNA gene was used to determine the ratio of mtDNA to nuclear DNA (nDNA), essentially as previously described (Blake *et al*., 1999). In addition to the experimental variability, 18S rRNA copy number is polymorphic in mammals (Rowe *et al*., 1996). Therefore, multiple control samples were run on quantitative blots; depletion was indicated by an mtDNA/nDNA ratio outside the control range. *Pvu*II-digested DNA was used for quantitative Southern blots, as the *Bam*HI- and *Sna*BI-digested fragments containing the 18S rRNA gene are not suitably sized.

Quantitative PCR methods

The percentage of deleted mtDNA was calculated by the real-time amplification of fragments within mtDNA regions that are usually deleted (*ND4*) or retained (*ND1*) in deleted mtDNA genomes, as previously described (He *et al*., 2002). To determine the overall abundance of mtDNA, we compared the real-time amplification of *ND1* with a single-copy nuclear reference gene. Sixty-nine base pairs from exon 24 of the *CFTR* gene (chosen on the account of the lack of single-nucleotide polymorphisms) were amplified using forward primer 5′-GAAGAGAACAAAGTGCGGCAG-3′ and reverse primer 5′-TTGCCGGAAGAGGCTCCT-3′. The fluorogenic probe sequence was 5′-ACGATTCCATCCAGAAACTGCTGAACGA-3′. For both experiments, DNA from six adult muscle samples (from needle biopsies, obtained with informed consent) was used as controls, and results are the means of two independent runs, with samples assayed in triplicate in each run.

Blue native gel analysis of DNA polymerase gamma stability

For immunoblot analysis of one-dimensional native gels, mitochondrial fractions were isolated by differential centrifugation in 250 mM sucrose, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 µg/ml of pepstatin A and 1 µg/ml of leupeptin. Proteins were solubilized with *n*-dodecyl-beta-D-maltoside and resolved on blue native 8–18% polyacrylamide gels as devised by Schägger (Schägger, 1995; Williams *et al*., 2004). Proteins were transferred to a 0.45-µm Hybond-P transfer membrane (Amersham Biosciences) and probed with a 1:400 dilution of POLG Ab3 N-terminal antibodies (NeoMarkers, Lab Vision Corporation, Newmarket, UK), a dilution of POLG2 antibodies as previously described (Magnusson *et al*., 2003) or a 1:7500 dilution of MTCO2 antibodies (Molecular Probes™, Invitrogen).

Measurement of DNA polymerase gamma activity

DNA polymerase gamma activity was determined by means of an RNA-dependent DNA polymerase assay in the presence of the nuclear DNA polymerase inhibitor aphidicolin, adapted from Longley *et al*. (1998). Mitochondrial fractions (prepared as described above) were lysed in an equal volume of 200 mM NaCl, 50 mM HEPES·KOH (pH 8.0) and 2% Triton X-100, so that the protein concentration was between 1.5 and 2.5 mg/ml. After lysis on ice for 20 min, samples were centrifuged at 16 000 × *g* for 10 min at 4°C. Five microlitres of supernatant was assayed in a final volume of 50 µl containing 100 mM NaCl, 25 mM HEPES·KOH (pH 8.0), 2.5 mM β-mercaptoethanol, 0.5 mM $MnCl_2$, 0.1 mM aphidicolin, 10 µM dTTP, 60 µCi/ml of [α-^{32}P]dTTP (specific activity: 3000 Ci/mmol; Amersham Biosciences), 100 µg/ml of acetylated bovine serum albumin (Promega, Southampton, UK), 500 U/ml of RNasin (Promega) and 50 µg/ml of poly(rA)·oligo$(dT)_{1–18}$ (Amersham Biosciences). The lysate was added to the assay mixture on ice, followed by a 20-min incubation in a 37°C water bath. The reaction was stopped on ice and 10-µl aliquots were spotted on DE81 anion exchanger chromatography paper (Whatman, Maidstone, Kent, UK). The paper was washed three times for 5 min each in 300 mM NaCl, 30 mM sodium citrate (pH 7.0) and then once in ethanol, followed by air-drying. The quantification of total incorporated dTTP was carried out by counting the radioactivity in Ultima Gold liquid scintillation cocktail (Perkin Elmer, Beaconsfield, Bucks, UK). For each lysate, a control assay without template was used to assess background labelling. All assays were performed in triplicate.

Results

The apparent maternal transmission of disease in this pedigree (Figure 1A) initially led us to sequence mtDNA in skeletal muscle from III-2. The sequence differed from the revised Cambridge Reference Sequence at 35 positions. Of these, only a single base-change (4561T>C) was absent from the MITOMAP database of neutral mtDNA polymorphisms (http://www.mitomap.org). 4561T>C appeared to be homoplasmic on the sequence electropherogram and predicted a V31A amino acid change in the ND2 subunit of RC complex I. However, it was unlikely that a mutation in a complex I subunit could be responsible for multiple RC defects and cytochrome *c* oxidase-negative fibres. 4561T>C was also represented in approximately 1% of sequences in the large Uppsala database of mtDNA sequence variants found in healthy individuals (http://www.genpat.uu.se/mtDB) and was therefore deemed non-pathogenic.

Sequence analysis of *POLG* in IV-2 revealed a heterozygous A→G transition mutation at nucleotide position 2864 in exon 18 (Figure 1B). This predicted a tyrosine-to-cysteine change at residue 955 in the highly conserved polymerase motif B (Y955C). The proband's mother (III-2) was also heterozygous for the mutation, whereas her two unaffected sisters, father and maternal uncle were all wild type, demonstrating segregation of the mutation with disease in this pedigree (Figure 1C). Five additional heterozygous *POLG* polymorphisms were found in IV-2. One of these (rs3087374) predicts a Q1236H substitution near the C terminus. Recent evidence indicates that Q1236H can modify the function of a pathogenic mutation (R627Q) when found in *cis* (Luoma *et al*., 2005). However, the digestion of exon 23 amplicons with *Bst*F5I indicated that Q1236H was inherited from the father (who was homozygous for Q1236H, Figure 1D) and so was in *trans* with Y955C in IV-2.

In some patients with mtDNA depletion syndrome, mtDNA progressively depletes in cultured fibroblasts and myoblasts (Taanman *et al*., 1997). Therefore, mtDNA levels were investigated in six alternate cell passages (from 11 to 21) of a fibroblast culture from IV-2. Although the RC defect was not expressed in these fibroblasts (Table I), Southern blot indicated that the levels of mtDNA in IV-2's fibroblasts were depleted but remained stable at around 43% of three controls (Figure 2).

Repeat Southern analysis of skeletal muscle DNA from III-2 (performed in the light of the molecular findings described above) demonstrated multiple deletions with *Bam*HI-, *Sna*BI- and *Pvu*II-digested DNA (Figure 3 and data not shown). The relatively low levels of these deletions explain why they were missed in the initial screen for rearrangements in muscle mtDNA. Quantitative PCR (qPCR) was used to confirm the presence of deleted mtDNA in the muscle sample. The ratio of *ND4* : *ND1* in the muscle sample from III-2 (0.73 in both analyses) was reduced in comparison with six controls (mean ± SD: 1.007 ± 0.085; observed range: 0.911–1.137). This suggested that approximately 27% of mtDNA molecules harboured deletions that span *ND4*. The presence of these shorter mtDNA molecules caused difficulties in the quantification of the signal from the 18S rRNA gene (which runs at 12 kb) by Southern blot analysis, and so qPCR was again used to determine the amount of mtDNA in relation to nDNA using a nuclear probe for CFTR. The ratio of *ND1* : *CFTR* in the muscle sample from III-2 (1.67 and 1.74 in two separate analyses) was higher than the mean value of six controls (mean ± SD: 1.17 ± 0.67; observed range: 0.498–2.077), thus there was no evidence of mtDNA depletion in III-2's skeletal muscle.

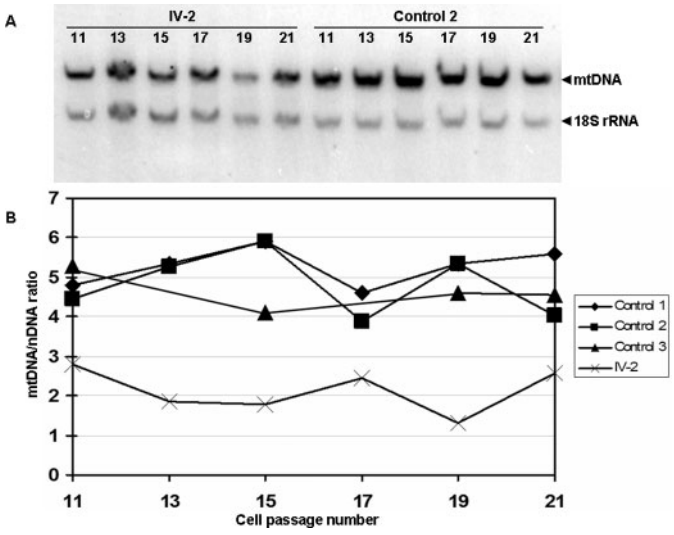

Figure 2. (**A**) Southern blot probed for mitochondrial DNA (mtDNA) and the 18S rRNA gene from alternate passage numbers of IV-2's fibroblasts and a control culture. (**B**) The mtDNA/nDNA ratios, quantified by phosphorimaging, were 2.13 (±0.57, 1.3–2.8) and 4.94 (±0.65, 3.9–5.9) for IV-2 and controls, respectively. The values are expressed as mean (±SD, observed range).

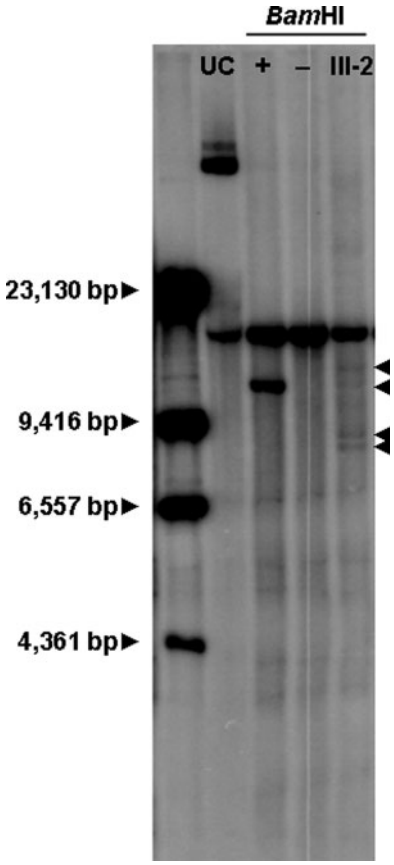

Figure 3. Southern blot of muscle DNA from III-2 reveals low levels of multiple mitochondrial DNA (mtDNA) rearrangements. UC, Uncut; +, DNA from a patient with a single large-scale mtDNA deletion; –, control DNA. Rearrangements are indicated with arrow heads.

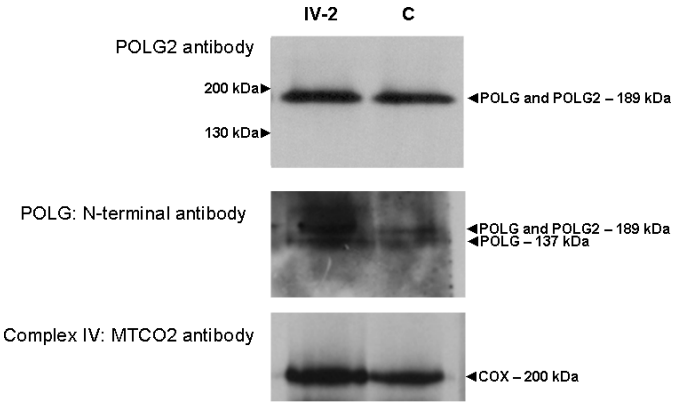

Figure 4. Immunoblots of blue native gels showing the mitochondrial DNA polymerase gamma enzyme and complex IV. Complex IV was probed to control for loading amounts. C, control sample. The migration of complex II (130 kDa) and complex IV (200 kDa) is indicated in the top panel. The sizes of POLG and POLG2 refer to the mature proteins after the cleavage of N-terminal targeting sequences.

Immunoblotting of blue native gels with antibodies against POLG2 or POLG showed a single band migrating just below the ~200-kDa RC complex IV with anti-POLG2 and a double band migrating between complexes IV and II (~130 kDa) with anti-POLG (Figure 4). The upper band recognized by anti-POLG co-migrated with the band recognized by anti-POLG2. These results suggest that the upper band represents the 189-kDa POLG–POLG2 complex, whereas the lower band represents the 137-kDa POLG protein on its own. The intensity of the bands in IV-2 was similar to that in the control, indicating that the stability of holo-DNA polymerase gamma was not affected in IV-2's fibroblasts.

To assess the activity of the enzyme, we determined the reverse-transcriptase activity in mitochondrial fractions of cultured fibroblasts by measuring the dTTP incorporation using poly(rA) as template. The activity of IV-2's sample was 9.38 pmol incorporated dTTP/mg of protein/min, compared with a mean activity (±SD, observed range) of 14.03 (±3.92, 7.30–19.92) in the 12 control samples. Thus, in its heterozygous state, Y955C appears to result in a trend towards a decrease in DNA polymerase gamma activity (67% of controls); however, the trend was not significant.

Discussion

We describe a three-generation pedigree with familial premature menopause associated with PEO, proximal myopathy, sensory ataxia and parkinsonism. In this family, the disease segregates with a dominant Y955C mutation in the highly conserved catalytic polymerase domain (POL B motif) of POLG. Although DNA from the grandmother (II-2) was not available, it can be assumed that she harboured Y955C since she also presented with PEO. To our knowledge, these three women constitute only the second reported family in whom mutations in POLG segregate with POF in multiple generations. Luoma *et al*. (2004) reported *POLG* mutations in seven PEO families. Five women from three of these families exhibited premature menopause (<40 years) or primary amenorrhoea. Two of these families harboured the dominant Y955C mutation found here, whereas a woman from the third family was a compound heterozygote for the N468D and A1105T mutations. In one of the Y955C families described by Luoma *et al*. (family S), POF was also documented in the extended family (Melberg *et al*., 1996), whereas the other two pedigrees each contained only a single case of POF. The data described here support the causal link between mutant POLG and POF and suggests that POF may be a relatively common feature in women with PEO caused by mutations in *POLG* and by Y955C in particular. Women with Y955C and other *POLG* mutations should be counselled about their reproductive options.

In family S described by Luoma *et al*., affected men had testicular atrophy (Lundberg, 1962). We have not been able to confirm an effect of Y955C on male fertility, because all affected family members were female in this pedigree.

In 25% of three-generation pedigrees such as the one described here, the autosomal dominant mutation will have been inherited from the maternal grandmother. When the unknown disease-gene relates to energy metabolism, it is tempting to assume a maternal mode of inheritance and proceed immediately to mtDNA sequencing. This study highlights that nuclear genes must also be considered.

mtDNA levels in cultured fibroblasts from IV-2 were consistently lower than in three control cell lines. This demonstrates

for the first time that the Y955C mutation can result in mtDNA depletion in fibroblasts. The levels of mtDNA depletion did not increase with passage number and were not severe enough to affect RC enzymes (Table I). The expression of cytochrome *c* oxidase subunit 1, as determined by quantitative immunocytochemistry (Taanman *et al*., 2003), was also normal (data not shown). In contrast, multiple mtDNA rearrangements were seen in skeletal muscle, consistent with the relative sparing of nuclear-encoded complex II activity compared with other RC enzymes. Unfortunately, fibroblasts from III-2 and muscle tissue from IV-2 were not available to further investigate this apparent tissue specificity. Further studies are needed to elucidate the pathogenic mechanism of ovarian failure associated with *POLG* mutations, for example qPCR could be used to determine the presence of mtDNA depletion and/or multiple rearrangements in single oocytes.

Y955C, the first mutation described in *POLG*, was initially found in a Belgian pedigree presenting with PEO associated with the accumulation of large-scale mtDNA deletions (Van Goethem *et al*., 2001). More recent studies have failed to detect a common haplotype around Y955C, suggesting that this site might be a hotspot for mutations rather than an ancestral mutation (Lamantea *et al*., 2002). A recent structural model of mitochondrial DNA polymerase gamma, based on the T7 and other DNA polymerases, indicates that this residue is likely to participate in the recognition of the incoming dNTP (Graziewicz *et al*., 2004). Our results support this hypothesis, because polymerase activity fell towards the lower end of the observed control range, despite native gels demonstrating normal stability of the enzyme.

Although the initial Southern blot of skeletal muscle DNA did not show any evidence of mtDNA rearrangements, repeat blots using three different restriction enzymes indicated that low levels of deleted molecules were present. These results demonstrate the experimental variability between different Southern blots and the care needed with their interpretation. As shown here, qPCR-based methods are now able to quantify mtDNA deletions and depletion and can be used to confirm Southern blot data. In cases where mtDNA deletions are present at even lower levels, more sensitive long-range PCR methods have previously been used (Van Goethem *et al*., 2004; Luoma *et al*., 2005). These PCR-based methods are especially important when limited amounts of tissue are available for study. A single Southern blot requires approximately 3 μg of DNA, whereas PCR methods can be used on lysates from single-muscle fibres (He *et al*., 2002). In this case, 27% of mtDNA was found to harbour deletions containing ND4, using qPCR.

The mature oocyte has the highest mtDNA copy number of all cells (Steuerwald *et al*., 2000), so it is likely to be more sensitive to reduced DNA polymerase gamma activity than other tissues. Indeed, a recent study demonstrated a significantly higher mtDNA copy number in human oocytes that were successfully fertilized and developed into embryos than in those that did not survive (Almeida Santos *et al*. 2006). A recent report in this journal highlights the importance of oxidative phosphorylation (and therefore mtDNA) in the normal development of mouse ovarian follicles (Wycherley *et al*., 2005). Reduced fertility has been observed in a mouse model with a proofreading-deficient version of polymerase gamma (Trifunovic *et al*., 2004). In the light of these results, it may be worthwhile screening POLG (including CAG repeat length) in women with POF and with other forms of reduced fertility, even in the absence of PEO. This would help to determine the extent and variability of the *POLG* mutant phenotype. If dominantly acting variations in *POLG* can influence the age of menopause in the absence of PEO, this will raise important reproductive counselling issues for affected women (e.g. timing of families and possibility of cryopreservation of ovarian tissue), especially if the current trend towards having children later in life continues.

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