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A p.R369G *POLG2* mutation associated with adPEO and multiple mtDNA deletions causes decreased affinity between polymerase γ subunits

Kate Craig^{*,1}, Matthew J. Young^{†,1}, Emma L. Blakely^{*}, Matthew J. Longley[†], Douglass M. Turnbull^{*}, William C. Copeland[†], and Robert W. Taylor^{*}

^{*}Mitochondrial Research Group, Institute for Ageing and Health, The Medical School, Framlington Place, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK

[†]Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA

Abstract

Human mitochondrial DNA (mtDNA) polymerase (γ) is the sole enzyme required to replicate and maintain the integrity of the mitochondrial genome. It comprises two subunits, a catalytic p140 subunit and a smaller p55 accessory subunit encoded by the *POLG2* gene. We describe the molecular characterization of a potential dominant *POLG2* mutation (p.R369G) in a patient with adPEO and multiple mtDNA deletions. Biochemical studies of the recombinant mutant p55 protein showed a reduced affinity to the γ p140 subunit, leading to impaired processivity of the holoenzyme complex but did not show sensitivity to N-ethylmaleimide (NEM) inhibition, inferring a novel disease mechanism.

Keywords

mitochondrial DNA polymerase; *POLG2*; adPEO; multiple mtDNA deletions; recombinant enzyme; replication stalling

1. Introduction

The biosynthesis of ATP by mitochondrial oxidative phosphorylation is dependent upon the co-ordinated expression and interaction of both nuclear and mitochondrial-encoded gene products. Mutations in an increasing number of nuclear genes involved in the maintenance and replication of mitochondrial DNA (mtDNA) are being described, associated with an extensive spectrum of recessive and dominantly-inherited clinical phenotypes ranging from severe encephalopathy in infancy and childhood to late-onset, progressive external ophthalmoplegia (PEO), ataxia and myopathy [1, 2]. The secondary mtDNA defect may be expressed in two forms; mtDNA depletion syndromes are characterised by a quantitative loss of mtDNA copy number, leading to isolated organ or multi-systemic paediatric mitochondrial disease. Alternatively, this may manifest as the accumulation of multiple mtDNA deletions in clinically-affected tissues, leading to an associated respiratory chain defect which is often demonstrated as a mosaic pattern of cytochrome *c* oxidase (COX)-

Corresponding Author: Professor R.W. Taylor, Mitochondrial Research Group, Institute for Ageing and Health, The Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, United Kingdom, Telephone: +44-191-2223685, Fax: +44-191-2824375, robert.taylor@ncl.ac.uk.

¹denotes joint first authors

deficient cells [3]. Clinically, patients present with a range of symptoms in which PEO is the predominant feature [4, 5]. The majority of autosomal recessive PEO (arPEO) cases are due to mutations in *POLG*, the gene encoding the catalytic alpha-subunit of the mitochondrial DNA polymerase (pol γ) [6] or *TYMP*, encoding thymidine phosphorylase [7]. Autosomal dominant PEO (adPEO) families have mutations in one of several mtDNA maintenance genes including *POLG* [6], *SLC25A4* encoding ANT-1 [8], *PEO1* encoding Twinkle helicase [9], *RRM2B* [10, 11], *OPA1* [12, 13] or *POLG2* [14] that segregate with disease.

The *POLG2* gene encodes a 55kDa homodimeric accessory subunit of pol γ , conferring high processivity on the enzyme complex formed with the p140 catalytic subunit by increasing its affinity to DNA. Whilst in excess of 150 different *POLG* mutations have been described in paediatric and adult presentations of mtDNA maintenance disorders [15], mutations of the *POLG2* gene have rarely been described. To date, only two patients with multiple mtDNA deletions in muscle, ptosis and late-onset PEO have been described. The first mutation described was associated with adPEO (c.1352G>A; p.G451E), affecting a glutamic acid residue in a region of the p55 protein shown to interact with the p140 subunit, leading to decreased processivity of the enzyme complex via weakened p55-p140 subunit interaction [14]. A further patient with PEO was described with a c.1207-1208ins24 mutation, causing mis-splicing and skipping of exon 7, thus impairing the C-terminal domain predicted to be required for enzyme processivity [16]. More recently several novel *POLG2* variants have been reported in a cohort of patients with suspected *POLG*-related disease who presented with a range of clinical features but no firm family history of mitochondrial disease [17].

Here we describe our investigation of a 55-year-old patient with PEO and a suspected dominant family history due to a heterozygous c.1105A>G *POLG2* variant, predicting a missense mutation in a conserved region of the p55 protein, similar to one of the patients described in [17]. Extensive biochemical studies of the recombinant mutant protein (p.R369G) reveal a reduced affinity to the DNA pol γ p140 catalytic subunit, leading to impaired processivity of the holoenzyme complex which confirm the pathogenicity of the p.R369G mutation, shedding light on the underlying molecular mechanism.

2. Materials and Methods

2.1. Patient details

Our patient is a 55-year-old gentleman, born to non-consanguineous parents. He first presented with symptoms of ptosis at the age of 30, with gradual progression and development of associated ophthalmoplegia. During the last 10 years he has developed other symptoms, in particular slurring of his speech and swallowing problems, which have both been progressive. Within the last 5 years he has developed increasing difficulty with his gait, associated with marked ataxia and cerebellar features, compounded by a peripheral neuropathy.

Examination reveals bilateral ptosis, chronic progressive ophthalmoparesis with eye movements restricted to about 10° in all directions. There was proximal muscle weakness and absent ankle jerks and he was unable to walk heel to toe. ECG showed normal sinus rhythm with no evidence of left ventricular hypertrophy. There was a family history of ptosis and PEO with a paternal uncle and nephew both clinically-affected, although unfortunately they have declined to be investigated further. The patient's father had died at the age of 71 some years earlier, precluding genetic studies. He was not reported to have had PEO, suggesting the *POLG2* mutation identified in his son showed variable penetrance.

All human studies were approved and performed under the ethical guidelines issued by Newcastle University, with written informed consent obtained from the patient.

2.2. Muscle biopsy analysis

Left quadriceps needle muscle biopsy was performed under local anaesthetic. Histological and histochemical analyses of mitochondrial enzyme activities, including the sequential reaction for COX and succinate dehydrogenase (SDH) activities, were performed using 10µm serial cross-sections according to standard procedures [18].

2.3. Molecular genetic analysis of patient samples

Total genomic DNA was extracted from muscle and EDTA-blood by standard procedures. Large-scale mtDNA rearrangements were screened by long-range PCR. Two, separate assays were employed to amplify muscle mtDNA across the major arc, using a pair of primers (L6249 (nucleotides 6249-6265) and H16215 (nucleotides 16225-16196)) to amplify a ~9.9 kb product in wild-type mtDNA, or a second pair (L1157 (nucleotides 1157-1177) and H19 (nucleotides 19-1)) to amplify a ~15.4 kb PCR product (GenBank Accession number NC_012920). The level of deleted mtDNA in individual COX-deficient and COX-positive staining muscle fibres isolated by laser microcapture was determined by quantitative real-time PCR using the ABI PRISM® Step One real-time PCR System (Applied Biosystems) as previously described [19, 20]. Furthermore, the assessment of mtDNA copy number in patient muscle was investigated by real-time PCR [21].

The entire coding regions, including intron-exon boundaries, of the *POLG* (NM_002693), *PEO1* (AF_292005), *RRM2B* (NM_015713), *SLC25A4* (BC_061589) and *POLG2* (NM_007215) genes were amplified using intronic M13-tailed primers by standard PCR. Purified PCR products (ExoSapIT, GE Healthcare) were sequenced with BigDye Terminator cycle sequencing chemistries on an ABI3130xl Genetic Analyzer (Applied Biosystems) and analysed using Mutation Surveyor software (Softgenetics).

One hundred and eighty-one healthy, adult control samples (362 chromosomes) were screened for the c.1105A>G (p.R369G) mutation by direct sequence analysis of exon 5 of the *POLG2* gene.

2.4. Construction of p55 and p.R369G p55 proteins

Mutations were generated separately in the codon optimized pET-p55CHIS plasmid [14] using the QuikChange site-directed mutagenesis kit (Stratagene) and therefore WT p55 and R369G p55 harbour C-terminal hexa-histidiny tags. The oligonucleotides containing the point mutation (lowercase) for introducing the p.R369G mutation in *POLG2* are 5'-CAA GAA AGA AAA ATC TTC ATg GAA AGG TAC TTA AAC TTC ACC C-3' and 5'-GGG TGA AGT TTA AGT ACC TTT CcA TGA AGA TTT TTC TTT CTT G-3.' The mutation encoding the p.R369G substitution was confirmed by DNA sequencing of the *POLG2* insert.

2.5. Expression and purification of p55 and p140

Human p55 and p.R369G p55 were expressed in BL21(DE3) *E. coli* without the 25 amino acid N-terminal mitochondrial targeting sequence as described previously [22]. For this study the exonuclease-deficient (Exo⁻) pol lacking the N-terminal 25 amino acid residue mitochondrial targeting sequence was denoted as wild-type p140 [23, 24]. Overexpression of the N-terminal hexa-histidiny form of this protein, in *Spodoptera frugiperda* (Sf9) insect cells, and purification was carried out as described [21].

2.6. Affinity of p55 to p140

Affinities were calculated as previously described [24]. Briefly, functional assay enzyme dilution buffer (FEDB) consisted of 50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 0.01% NP-40, 75 mM NaCl, 50 µg/ml BSA. Samples (20 µl) of purified p140 (12.5 nM) and

either WT or R369G p55 (varying concentrations) were premixed in FEDB, and 4 μ l was added to a cocktail to make reactions (50 μ l) containing 25 mM HEPES-KOH, pH 7.5, 2.5 mM β -mercaptoethanol, 0.5 mM MnCl₂, 200 μ g/ml BSA, 25 μ M dTTP, 13.32 nM [γ -³²P] dTTP (3000 Ci/mmol, PerkinElmer Life Sciences), 220 mM NaCl, 50 μ g/ml poly(rA)•oligo(dT)_{12–18} (Amersham Biosciences), 1 nM p140, and 0 – 20 nM p55 or variant protein (calculated as dimers). Reactions were incubated at 37°C for 10 minutes. Reactions were stopped by placing in an ice bath followed by the immediate addition of 0.2 ml stop solution (1 mg/ml BSA, 0.1 M sodium pyrophosphate tetrabasic decahydrate) and 1 ml of 10% trichloroacetic acid (TCA). TCA-insoluble radioactivity was determined by liquid scintillation counting and binding isotherms were fit to a quadratic equation as described [25].

2.7. NEM inhibition assay

The standard 50 μ l polymerase reaction with poly(rA)•oligo(dT)_{12–18} (Amersham Biosciences), described in the *Affinity of p55 to p140* section, was performed at either 75 mM NaCl or 220 mM NaCl with varying concentrations of N-ethylmaleimide (NEM) (0 – 0.5 mM) for both wild-type p55 and p.R369G p55 enzymes. Both p140 and p55 or R369G p55 were added at the fixed concentration of 1 nM.

2.8. Stimulation of activity of the catalytic subunit by the p55 variants

In vitro 5'-³²P-end-labeled 35-mer primer extension assays were incubated for 20 min as described previously [22] with the exception that 5 nM p140 and 10.5 nM monomeric p55 or p.R369G p55 variant was utilized and reactions were adjusted to 100 mM NaCl unless otherwise indicated. Reactions were resolved by 12% denaturing polyacrylamide gel electrophoresis as described [22].

3. Results

3.1. Mitochondrial histochemistry and mtDNA analysis

Routine histology and histochemistry of the patients' skeletal muscle biopsy revealed some mild inflammatory changes and evidence of mitochondrial respiratory chain deficiency, with sequential COX-SDH histochemistry identifying ~10% COX-deficient fibres. In addition, ragged-red fibres indicating subsarcolemmal mitochondrial accumulation were present. Long-range PCR amplification of skeletal muscle DNA clearly showed the presence of multiple mtDNA deletions in both the 9.9 kb and 15.4 kb amplifications (Fig. 1A), indicative of a disorder of mtDNA maintenance. Real-time of individual, lasercaptured COX-deficient fibres showed that the majority, but not all, fibres have high levels of clonally-expanded mtDNA deletion involving the *MTND4* gene (Fig. 1B), a consistent observation in patients with multiple mtDNA deletion disorders [12, 14, 19]. No major abnormality of mtDNA copy number was detected in the patient's muscle DNA sample (data not shown).

3.2. Molecular genetic analysis

Screening of the *POLG*, *PEO1*, *RRM2B* and *SLC25A4* genes revealed no pathogenic mutations. Direct sequencing of the 8 coding exons of the *POLG2* gene identified a single, heterozygous change (c.1105A>G) in exon 5 of our patient (Fig. 2A) which was not detected in the analysis of 362 control chromosomes. The transition is predicted to alter a highly conserved arginine to glycine at codon 369 (p.R369G, Fig. 2B).

3.3. The R369G p55 holoenzyme has impaired processivity

To address the consequence of this mutation on the function of the protein we overproduced the human recombinant p55 harbouring the R369G change and compared it to the wild type p55. The affinity of the p.R369G p55 to the catalytic subunit as compared to the wild-type p55 was previously compared by analysis of the activity on poly(rA)•oligo(dT)_{12–18} at 220 mM NaCl [17]. At this salt concentration the activity of the isolated p140 catalytic subunit is almost entirely inhibited but is significantly stimulated by the presence of the p55 accessory subunit. This difference in activities between the holoenzyme and isolated p140 at 220 mM NaCl allows for the titration of p55 into the reaction for determination of binding isotherms. This analysis showed a 4-fold weaker affinity for the p140 catalytic subunit by the p.R369G p55 as compared to the wild type ($K_d = 0.19 \pm 0.05$ nM for the WT and 0.85 ± 0.01 nM for the p.R369G p55) [17]. This weakened interaction suggests that the processivity of DNA replication by the holoenzyme may be affected.

Processivity was examined using an end-labelled primer extension assay to test the ability of p.R369G p55 to stimulate p140 under a range of 0 to 250 mM supplemental NaCl (Fig. 3). While still on ice a ~900-fold excess of unlabeled, sonicated, heat-denatured calf-thymus “trap” DNA harbouring ~18 pmol of random 3'-ends was added last to the reaction to restrict the polymerase to a single DNA binding event (Fig. 3A). Processivity reactions were initiated by incubation at 37°C for 20 min. The p140 catalytic subunit alone is a salt sensitive polymerase, which becomes inhibited with increasing concentration of NaCl (lanes 2–7, Fig. 3A). When WT p55 is added to the reaction, salt tolerance is restored to the holoenzyme (compare lanes 8–13 with lanes 2–7 in Fig. 3A). As shown in Fig. 3B, the p.R369G-pol complex produces products 10 nucleotides shorter at the lower salt concentrations (0 – 200 mM) and ~40 nucleotides shorter at the high salt concentration (250 mM) as compared to the wild type complex.

The decreased length of primer extension products indicated reduced processivity at all salt concentrations tested for p.R369G relative to WT p55 (Fig. 3A, compare lanes 14 to 19 and 8 to 13). The reduced processivity displayed by the p.R369G p55 holoenzyme prompted us to examine the overall intensity of the products formed in Fig. 3A. To estimate the relative mtDNA polymerase activities, densitometric analysis of the radioactively labelled primer extension products at each salt concentration revealed that the total amount of products synthesized by the isolated catalytic subunit was maximal in the absence of additional salt and declined dramatically upon salt addition (Fig. 3C, open circles). However, addition of the wild type p55 to the pol catalytic subunit showed maximal activity at 100–150 mM NaCl (open triangles). At 250 mM NaCl only trace amount of isolated catalytic activity could be detected and quantified while the wild type holoenzyme had as much activity at 250 mM NaCl as it did at no salt. When the processivity of the p.R369G-p55 holoenzyme was tested, the shape of the salt profile was similar to the wild type holoenzyme (Fig. 3A) but the total extended products was less, about 80% at 100 – 150 mM NaCl which decreased to 50% at the higher salt concentrations tested (Fig. 3C, closed circles).

Compared to the wild type holoenzyme, quantitation demonstrated a reduced activity in the p.R369G p55 holoenzyme, albeit, quantitation of end-labelled products under-estimates the specific activity of the enzyme. Therefore, we also determined the specific activity of the wild type and p.R369G p55 holoenzyme as well as the isolated catalytic subunit using poly(rA)•oligo(dT)_{12–18} at 220 mM salt. This substrate allows the direct quantitation of radiolabel incorporated into DNA and demonstrated that the isolated catalytic subunit could only incorporate 0.4 ± 0.2 ($\pm$ SDM) pmol of total dTTP into acid insoluble material in 1 hour at 37°C. However, the wild type p55-holoenzyme complex was able to incorporate 13.1 ± 0.3 pmol/hr of dTTP at this salt concentration. This assay reinforces the salt tolerance

imparted by the accessory subunit on overall activity. In comparison, the p.R369G-p55 holoenzyme could only incorporate 7.9 ± 0.3 pmol/hr of dTTP under these conditions.

3.5. NEM inhibition assay

The ability of the accessory subunit to physically associate with the catalytic subunit can be estimated by the degree of inhibition of the catalytic subunit by NEM with and without the accessory subunit. In the absence of p55 the p140 catalytic subunit is irreversibly inhibited by the action of NEM on cysteine sulphydryl groups while in the presence of wild type p55 the catalytic subunit is protected 100-fold from NEM inhibition [22]. This assay was instrumental in determining the disrupted association between the p.G451E p55 disease variant and p140 [14]. Using the standard polymerase reaction to test the two accessory subunits at 75 mM NaCl, there was no noticeable difference between the percent maximal activity of the wild-type p55 and p.R369G p55 (data not shown). Next we carried out the NEM inhibition assay at 220 mM NaCl which is slightly higher than the physiological concentration of ~150 mM (Fig. 4). On average a 1.6-fold decrease in the specific activity of the holoenzyme harbouring p.R369G p55 relative to the wild-type was observed across a range of 0 to 0.5 mM NEM. By EMSAs we previously observed that p.R369G p55 binds to a 47 bp double-stranded oligonucleotide identically to wild-type p55 [17]. These results corroborate our activity analysis and suggest that, although the p.R369G p55 was effective in protecting the catalytic subunit from NEM inhibition, the overall stimulation of activity is compromised at higher salt. This is the first *POLG2* variant that has shown a salt dependent defect in stimulation of activity and processivity while maintaining NEM protection and DNA binding.

4. Discussion

We report here on the investigation of a family with PEO and multiple mtDNA deletions in muscle due to a heterozygous p.R369G mutation in the *POLG2* gene, encoding the p55 accessory subunit of mitochondrial pol . Several lines of evidence support the pathogenic role of the p.R369G *POLG2* mutation; our patient presents with the typical clinical and histochemical features associated with adult-onset presentations of mtDNA maintenance disorders, namely a suggestive dominant family history of PEO associated with focal respiratory chain deficits in muscle due to clonally-expanded, multiple mtDNA deletions (Fig. 1). In the absence of pathogenic mutations in several other mtDNA maintenance genes known to cause adPEO, we identified a heterozygous mutation in *POLG2* which affects a highly conserved amino acid residue (p.R369G) and which has recently been identified in another patient with the clinical features of mitochondrial disease and confirmed biochemical abnormalities in pol function [17]. We were unable to demonstrate that other clinically-affected family members harbour the p.R369G mutation since they declined further investigation, and the lack of observable PEO in the patient's father suggests that the mutation was associated with variable penetrance within the family. In further support of the p.R369G variant being associated with clinical disease, we were not able to detect this mutation in 362 control chromosomes.

To complement the human studies and establish the c.1105A>G mutation (encoding p.R369G) as disease-causing, we performed a biochemical characterisation of the purified, recombinant p.R369G protein *in vitro*. In processivity reactions, p.R369G p55 synthesized fewer products over a 0 – 250 mM range of NaCl in comparison to wild-type p55. In these reactions the p.R369G variant had reduced activity which declined even further at the higher salt concentrations, 80% the activity of the wild type holoenzyme at low salt, declining to 50% activity at the higher salt concentrations (Fig. 3). Processivity is defined as the number of nucleotides incorporated per binding event and is measured by primer extension in the presence of a DNA-trap that prevents rebinding of the polymerase to the end-radiolabeled

substrate. In general, the processivity of the isolated catalytic subunit is ~50 nucleotides and is inhibited by addition of salt. In contrast, the wild type p55 imparts a salt tolerance to the complex, enhances processivity, and causes tighter DNA binding, which allows for longer products to be synthesized at higher salt concentrations [24]. Both the wild type and the p.R369G-p55 holoenzymes displayed maximal activity between 100–150 mM NaCl but the length of the products synthesized by p.R369G were shorter at the lower salt concentrations (~10 nucleotides) and even less at the higher salt concentrations (~40 nucleotides). The results were similar when the reactions were performed without trap DNA (data not shown). For the p.R369G containing holoenzyme at 250 mM NaCl, no significant DNA products were extended past 50 nucleotides (the pause site halfway up the gel which occurs at a stable stem-loop structure in the M13 substrate) in the presence or absence of trap (Fig. 3A, lane 19 and data not shown) while the wild type, although showing salt inhibition at this high NaCl concentration, still extended at least 50 nucleotides past this pause site (Fig. 3A, lane 13 and data not shown).

Analysis of the specific activities of the mtDNA polymerases revealed that the p.R369G-p55 holoenzyme only had 60% activity as compared to the wild type complex at 220 mM NaCl. These analyses demonstrate two important defects by the p.R369G holoenzyme, a decline in processivity and a reduction in total polymerase activity. Both of these reductions would cause the pol_γ complex to stall more often in difficult sequence (stem-loops). Stalling by the pol_γ complex may allow the opportunity for deletions to occur in mtDNA [25].

Wild type p55 has been shown to impart high processivity to the holoenzyme through a tighter interaction with DNA [24]. This enhanced stimulation and processivity is dependent on the tight association of the accessory subunit with the catalytic subunit. The G451E p55 mutation provides an example of an extreme defect in the interaction between the accessory subunit and the catalytic subunit [14]. The p.G451E p55 protein showed no stimulation or enhancement of processivity due to an inability to interact with the catalytic pol_γ [14]. As a potential kinetic explanation for the reduced processivity and activity by the R369G-p55 we also determined the K_d of interaction between the p.R369G-p55 and the p140 catalytic subunit. We found a ~4.5-fold reduced affinity for p140 when compared to wild-type p55 ($K_{d(p140)}$ of 0.85 nM versus 0.19 nM). Normally, higher salt is thought to stimulate the interaction between the p55 accessory subunit and the p140 catalytic subunit [24]. However, the p.R369G p55 interaction appears to become more compromised at higher salt as indicated by the weaker K_d . This weaker interaction mostly likely provides a molecular explanation for the decreased salt tolerance of the p.R369G holoenzyme as compared to the wild type holoenzyme.

The first reported *POLG2* pathogenic mutation (p.G451E) demonstrated a defect in the protection from NEM inhibition due to the loss of interaction with the catalytic subunit / modification [14]. Although the total synthesis of activity in the NEM assay is reduced by the p.R369G variant due to the weakened interaction with the catalytic subunit, the p.R369G variant did protect against NEM inhibition. This is the first time that a *POLG2* variant has not shown sensitivity to NEM while affecting the interaction with the catalytic subunit. Furthermore, these findings demonstrate that the cysteine residue on the p140 catalytic subunit, which is modified by NEM is not affected by the Arg369 to Gly substitution and therefore is probably not located in the region of the protein which interacts with the Arg369 area of p55. Presently the NEM modified cysteine residue is unknown. As shown in the crystal structure of the holoenzyme the catalytic subunit binds to p55 over a large area encompassing ~3500 Å² and helps to explain the tight binding between subunits [26]. Our results suggest that the p.R369G modification weakens part of this subunit interaction but appears not to be in proximity to the NEM sensitive cysteine residue, consistent with the weakened but not eliminated interaction. Thus, in comparison to the p.G451E variant, the

p.R369G mutation causes a novel separation-of-function mutation regarding NEM protection and interaction.

A 3-dimensional structure of the human pol γ holoenzyme has been solved at 3.2 Å, providing insight into the consequences of various disease mutations on a structural level [26]. Fig. 5 depicts the location of the Arg369 p55 residue in the p140-p55 complex. The 3-dimensional crystal structure [26] shows that the thumb subdomain of p140 (grey) containing the Leu473 residue (yellow colour) appears to make a van der Waals interaction with the Arg369 residue (yellow and blue) in the proximal p55 monomer (purple). Elimination of the Arg side chain in the mutant p.R369G protein would alter this interaction and helps to explain the apparent weakened interaction between these two proteins, and hence gives further confirmation to the mechanism by which the p.R369G mutation leads to disease.

In conclusion, we have described a detailed characterization of a p.R369G mutation in the *POLG2* gene, confirming this to be a pathogenic mutation and the cause of adPEO in this family. Disrupting the interaction of the p55 accessory subunit with the p140 catalytic subunit could promote stalling of the replication fork and induce multiple mtDNA deletions. We predict that these mtDNA deletions would subsequently clonally-expand over time, causing a focal COX deficiency in skeletal muscle, leading to the clinical phenotype. Although less frequent in comparison to mutations in the *POLG*, *PEO1* and *RRM2B* genes [11, 14, 16], our findings confirm that *POLG2* should be included as part of the regular diagnostic work-up of similar patients.

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References

1. DiMauro S, Schon EA. Mitochondrial disorders in the nervous system. *Ann. Rev. Neurosci.* 2008; 31:91–123. [PubMed: 18333761]
2. McFarland R, Taylor RW, Turnbull DM. A neurological perspective on mitochondrial disease. *Lancet Neurol.* 2010; 9:829–840. [PubMed: 20650404]
3. Fratter C, Gorman G, Stewart JD, et al. The clinical, histochemical and molecular spectrum of *PEO1* (Twinkle)-linked adPEO. *Neurology.* 2010; 74:1619–1626. [PubMed: 20479361]
4. Zeviani M, Servidei S, Gellera C, et al. An autosomal dominant disorder with multiple deletions of mitochondrial DNA starting at the D-loop region. *Nature.* 1989; 339:309–311. [PubMed: 2725645]
5. Suomalainen A, Majander A, Haltia M, et al. Multiple deletions of mitochondrial DNA in several tissues of a patient with severe retarded depression and familial progressive external ophthalmoplegia. *J. Clin. Invest.* 1992; 90:61–66. [PubMed: 1634620]
6. Van Goethem G, Dermaut B, Löfgren A, et al. Mutation of *POLG* is associated with progressive external ophthalmoplegia characterized by mtDNA deletions. *Nat. Genet.* 2001; 28:211–212. [PubMed: 11431686]
7. Nishino I, Spinazzola A, Hirano M. Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. *Science.* 1999; 283:689–692. [PubMed: 9924029]
8. Kaukonen J, Juselius JK, Tiranti V, et al. Role of adenine nucleotide translocator 1 in mtDNA maintenance. *Science.* 2000; 289:782–785. [PubMed: 10926541]
9. Spelbrink JN, Li FY, Tiranti V, et al. Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat. Genet.* 2001; 28:223–231. [PubMed: 11431692]

10. Tyynismaa H, Ylikallio E, Patel M, et al. A heterozygous truncating mutation in *RRM2B* causes autosomal-dominant progressive external ophthalmoplegia with multiple mtDNA deletions. *Am. J. Hum. Genet.* 2009; 85:290–295. [PubMed: 19664747]
11. Fratter C, Raman P, Alston CL, et al. *RRM2B* mutations are frequent in familial PEO with multiple mtDNA deletions. *Neurology.* 2011; 76:2032–2034. [PubMed: 21646632]
12. Hudson G, Amati-Bonneau P, Blakely EL, et al. Mutation of *OPA1* causes dominant optic atrophy with external ophthalmoplegia, ataxia, deafness and multiple mitochondrial DNA deletions: a novel disorder of mtDNA maintenance. *Brain.* 2008; 131:329–337. [PubMed: 18065439]
13. Amati-Bonneau P, Valentino ML, Reynier P, et al. *OPA1* mutations induce mitochondrial DNA instability and optic atrophy 'plus' phenotypes. *Brain.* 2008; 131:338–351. [PubMed: 18158317]
14. Longley MJ, Clark S, Yu Wai Man C, et al. Mutant *POLG2* disrupts DNA polymerase gamma subunits and causes Progressive External Ophthalmoplegia. *Am. J. Hum. Genet.* 2006; 78:1026–1034. [PubMed: 16685652]
15. Stumpf JD, Copeland WC. Mitochondrial DNA replication and disease: insights from DNA polymerase mutations. *Cell Mol. Life Sci.* 2011; 68:219–233. [PubMed: 20927567]
16. Walter MC, Czermin B, Muller-Ziermann S, et al. Late-onset ptosis and myopathy in a patient with a heterozygous insertion in *POLG2*. *J. Neurol.* 2010; 257:1517–1523. [PubMed: 20405137]
17. Young MJ, Longley MJ, Li F-Y, et al. Biochemical analysis of human *POLG2* variants associated with mitochondrial disease. *Hum. Mol. Genet.* 2011; 20:3052–3066. [PubMed: 21555342]
18. Taylor RW, Barron MJ, Borthwick GM, et al. Mitochondrial DNA mutations in human colonic crypt stem cells. *J. Clin. Invest.* 2003; 112:1351–1360. [PubMed: 14597761]
19. He L, Chinnery PF, Durham SE, et al. Detection and quantification of mitochondrial DNA deletions in individual cells by real-time PCR. *Nucleic Acids Res.* 2002; 30:e68. [PubMed: 12136116]
20. Krishnan KJ, Bender A, Taylor RW, et al. A multiplex real-time PCR method to detect and quantify mitochondrial DNA deletions in individual cells. *Anal. Biochem.* 2007; 370:127–129. [PubMed: 17662684]
21. Blakely EL, He L, Gardner JL, et al. Novel mutations in the *TK2* gene associated with fatal mitochondrial DNA depletion myopathy. *Neuromusc. Disord.* 2008; 18:557–560. [PubMed: 18508266]
22. Kasiviswanathan R, Longley MJ, Young MJ, et al. Purification and functional characterization of human mitochondrial DNA polymerase gamma harboring disease mutations. *Methods.* 2010; 51:379–384. [PubMed: 20176107]
23. Longley MJ, Ropp PA, Lim SE, et al. Characterization of the native and recombinant catalytic subunit of human DNA polymerase gamma: identification of residues critical for exonuclease activity and dideoxynucleotide sensitivity. *Biochemistry.* 1998; 37:10529–10539. [PubMed: 9671525]
24. Lim SE, Longley MJ, Copeland WC. The mitochondrial p55 accessory subunit of human DNA polymerase gamma enhances DNA binding, promotes processive DNA synthesis, and confers N-ethylmaleimide resistance. *J. Biol. Chem.* 1999; 274:38197–38203. [PubMed: 10608893]
25. Krishnan KJ, Reeve AK, Samuels DC, et al. What causes mitochondrial DNA deletions in human cells? *Nat. Genet.* 2008; 40:275–279. [PubMed: 18305478]
26. Lee YS, Kennedy WD, Yin YW. Structural insight into processive human mitochondrial DNA synthesis and disease-related polymerase mutations. *Cell.* 2009; 139:312–324. [PubMed: 19837034]

Figure 1. Identification of multiple mtDNA deletions in patient muscle

A, Long range PCR of muscle DNA (9.9 kb and 15.4 kb amplifications) shows significant evidence of multiple mtDNA deletions. C = control; P = patient. B, Quantitative, single fibre real-time-PCR reveals the majority – but not all – of COX-deficient fibres contain high levels of a clonally-expanded mtDNA deletion involving the *MTND4* gene, an observation which is entirely consistent with a diagnosis of multiple mtDNA deletions.

Figure 2. Identification of a p.R369G *POLG2* mutation

A, Sequence chromatogram highlighting the heterozygous c.1105A>G (p.R369G) *POLG2* mutation in our patient, compared to a control sequence of this region. B, Amino acid alignments of p55 orthologs, showing the region in which the p.R369G substitution is located. The Arg369 residue is identified in bold and the p.R369G mutation is shown in red. Invariant amino acid residues are indicated by an asterisk.

Figure 3. Pol γ processivity with the WT and p.R369G p53

Reactions were analyzed by denaturing PAGE and contained 2 nM singly primed M13mp18 DNA, 5 nM p140, and 10.5 nM monomeric p53 or p.R369G p53. Reactions contained 0, 50, 100, 150, 200, or 250 mM supplemental NaCl and the gradient from low (L, none added) to high salt (H, 250 mM NaCl) is indicated by black triangles. The hyphen (–) indicates the absence of p53 in these reactions (p140 only). Primer, no protein added to the reaction. A, Processivity reactions containing unlabeled, sonicated, heat-denatured calf-thymus DNA harbouring ~18 pmol of random 3'-ends to "trap" the polymerase following its first disassociation from the labelled substrate. B, Densitometric tracing of the length of the products in panel A. Closed circles are the length of products with the holoenzyme containing wildtype p53 and open triangles are the length of products with the p.R369G holoenzyme. C, The total radioactivity extended past the primer in the processivity reaction

in the presence of trap was quantitated by image analysis and graphed as percent maximal activity. Open circles denote p140 subunit only, open triangles denote holoenzyme containing WT p55, closed circles denote holoenzyme containing p.R369G p55. The quantitation of a representative gel is shown.

Figure 4. Treatment of pol γ harbouring either WT or p.R369G accessory subunits with *N*-ethylmaleimide (NEM)

p55 and p.R369G p55 protect DNA pol γ from inactivation by NEM at 220 mM NaCl. The specific activity of the holoenzyme harboring p.R369G p55 is reduced 1.6-fold relative to the WT p55 in the absence of NEM. Specific activity is defined as U/ng p140 where 1 U = 1 pmol of total dTTP incorporated into acid insoluble material in 1 hour at 37°C. Open circles denote p140 subunit, open triangles denote holoenzyme containing WT p55 subunit, closed circles denote holoenzyme containing p.R369G p55 subunit.

Figure 5. Location of the R369 p55 residue in the p140-p55 complex

The thumb subdomain of p140 (grey) contains the L473 residue (yellow colour) while the proximal p55 monomer (purple) harbours the R369 residue (yellow and blue). The figure was generated using the program PyMOL and the published crystal structure PDB ID 3IKM [26].