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ORIGINAL ARTICLE

Mutations in mitochondrial DNA polymerase-γ promote breast tumorigenesis

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Decreased mitochondrial oxidative phosphorylation (OXPHOS) is one of the hallmarks of cancer. To date, the identity of nuclear gene(s) responsible for decreased OXPHOS in tumors remains unknown. It is also unclear whether mutations in nuclear gene(s) responsible for decreased OXPHOS affect tumorigenesis. Polymerase-γ (POLG) is the only DNA polymerase known to function in human mitochondria. Mutations in POLG are known to cause mitochondrial DNA (mtDNA) depletion and decreased OXPHOS, resulting in mtDNA depletion syndrome in humans. We therefore sequenced all coding exons (2–23) and flanking intron/splice junctions of POLG in breast tumors. We found that the POLG gene was mutated in 63% of breast tumors. We identified a total of 17 mutations across the POLG gene. Mutations were found in all three domains of the POLG protein, including T251I (the exonuclease domain), P587L (the linker region) and E1143G (the polymerase domain). We identified two novel mutations that include one silent (A703A) and one missense (R628Q) mutation in the evolutionarily conserved POLG linker region. In addition, we identified three novel mutations in the intronic region. Our study also revealed that mtDNA was depleted in breast tumors. Consistently, mutant POLG, when expressed in breast cancer cells, induced a depletion of mtDNA, decreased mitochondrial activity, decreased mitochondrial membrane potential, increased levels of reactive oxygen species and increased Matrigel invasion. Together, our study provides the first comprehensive analysis of the POLG gene mutation in human cancer and suggests a function for POLG (1) in decreased OXPHOS in cancers and (2) in promoting tumorigenicity.
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INTRODUCTION

Decreased mitochondrial oxidative phosphorylation (OXPHOS) is one of the most common and profound phenotypes of cancer cells.[1–10] As early as 1930, the German biochemist, Otto Warburg, described OXPHOS differences in the mitochondria of tumor versus normal cells.[1,2] He proposed that cancer initiates from irreversible injury to OXPHOS.[2] He further proposed that decreased OXPHOS led to an increased rate of aerobic glycolysis in most cancers. This phenomenon is described as the Warburg effect.

In human cells, the OXPHOS system is assembled from 13 mtDNA (mitochondrial DNA) genes and from over 85 nDNA (nuclear DNA) genes. The entire mitochondrial genome is devoted to the production of 13 protein subunits of OXPHOS complexes (I, III, IV and V) involved in respiration and adenosine triphosphate synthesis. We investigated the underlying reason for decreased OXPHOS in breast cancer and discovered that more than 40% of primary breast tumors lack a detectable expression of cytochrome *c*-oxidase subunit II (OXPHOS complex IV) encoded by mtDNA.[11] Other laboratories have measured mtDNA content directly in tumors and report a decrease in mtDNA content in breast, renal, hepatocellular, gastric and prostate tumors.[12–17] Depletion of mtDNA is also supported by a decrease in OXPHOS levels in renal tumors.[18] It is also noteworthy that drugs used for treating human immunodeficiency virus inhibit POLG, which in turn induces mtDNA depletion.[19] Tamoxifen, a commonly used drug for the treatment of breast cancer, also depletes mtDNA.[20] A recent study also demonstrates that depletion of mtDNA correlates with tumor progression and prognosis in breast cancer patients.[21] To date, the identity of nuclear gene(s) responsible for mtDNA depletion and decreased OXPHOS in tumors remains unknown. It is also unclear whether mutations in nuclear gene(s) involved in mtDNA depletion and decreased OXPHOS affect tumorigenesis.

The first mtDNA depletion syndrome (MDS) was described more than 15 years ago.[22] MDS results from mutation(s) in nuclear-encoded genes that participate in mtDNA replication, in mitochondrial nucleotide metabolism and in the nucleotide salvage pathway. So far, only six MDS genes have been identified. These nuclear genes include mtDNA polymerase-γ (POLG), mtDNA helicase twinkle,[23] thymidine kinase 2,[24,25] deoxyguanosine kinase,[26,27] SUCLA2, the gene-encoding β-subunit of the adenosine diphosphate-forming succinyl coenzyme A synthetase ligase[28,29] and MPV17, a mitochondrial inner membrane protein.[28,30] Of these nuclear genes, POLG is the most frequent target

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of mutation and is involved in a variety of mitochondrial diseases. To date, more than 150 mutations in POLG have been identified.[31]

Polymerase-γ is the only DNA polymerase that is currently known in human mitochondria. POLG is essential for the development of an embryo.[32] It contains a large catalytic subunit, POLG (140-kDa), and two identical accessory subunits encoded by POLG2 (55-kDa).[33] POLG belongs to the family of A-type DNA polymerases,[34] consisting of an exonuclease domain with three exo motifs, I, II and III, and a polymerase domain with three pol motifs, A, B and C, along with an intervening linker region.[35] As with any other polymerase, POLG has been involved in DNA polymerase, 3′–5′ exonuclease and in the 5′dRP lyase activities of mtDNA replication.[36]

The POLG gene maps to 15q25 is 21 kb in size and consists of 23 exons. POLG contains CAG trinucleotide repeats that code for polyglutamine in the second exon, which is not present in any of the polymerases or orthologs.[37] As the first identification of POLG mutations in progressive external ophthalmoplegia (PEO), it has become evident that mutations in POLG are a major cause of many human diseases, ranging from Alpers' syndrome to male infertility, Parkinsonism and other mitochondrial diseases.[36,38–41] Most disease phenotypes associated with mutations in POLG are due to mutations and/or depletions in mtDNA.

In this study, we analyzed POLG gene mutations and the associated reduction in mtDNA content in breast tumors. We performed mutational analyses of all coding exons and flanking intron/splice junctions of POLG. This study reports novel somatic mutations in POLG that are frequently found in breast cancer. In addition, we provide evidence that mutations in POLG gene promote tumorigenesis.

MATERIALS AND METHODS

Tumor samples

Tissue samples were collected from patients with breast tumors undergoing surgery for treatment at the Roswell Park cancer institute and from Cooperative Human Tissue Network (CHTN), with their informed consent.

Cell culture

Breast cell lines MCF7 and MDAMB231 were grown in Dulbecco's modified Eagle's media and control cell lines MCF12A and MCF12ARho0 were grown in a 1:1 mixture of DMEM and Ham's F12 media (Cellgro, Herndon, VA, USA), supplemented with 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Invitrogen, Carlsbad, CA, USA). MCF7 Tet-On Advanced cells (Clontech, Mountain View, CA, USA) were grown in DMEM supplemented with 10% Tet System Approved fetal bovine serum (FBS) (Clontech), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Invitrogen), 100 μg ml^{-1} G418 (Cellgro) and 50 μg ml^{-1} uridine (Sigma, St Louis, MO, USA). Cells were maintained in a 37 °C, 5% CO_2 environment.

Plasmid construction and site-directed mutagenesis

Full-length POLG cDNA was subcloned into the inducible mammalian expression vector, pTRE-Tight-BI-AcGFP1 (Clontech). Site-directed mutants were created for the mutations T251I (exonuclease domain); P587L (linker domain); T251I and P587L (double mutant); D1135A and E1143G (polymerase domain), using the site-directed mutagenesis kit (Stratagene/Agilent, Santa Clara, CA, USA). Mutations were confirmed by sequencing the complete open reading frame of each mutant clone. The primer sequences used for site-directed mutagenesis are as follows, with the mutated site in upper case:

T251I_F: 5′-ccctggaggtccctaTtggtgccagcag-3′
T251I_R: 5′-ctgctggcaccaAtagggacctccaggg-3′
P587L_F: 5′ tgcatggacccTgggccccagcc 3′
P587L_R: 5′-ggctggggcccAgggtccatgca-3′
D1135A_F: 5′-gcatcagcatccatgCGgaggttcgctacctgg-3′
D1135A_R: 5′-ccaggtagcgaacctcCGcatggatgctgatgc-3′
E1143G_F: 5′-cctggtgcgggGggaggaccgct-3′
E1143G_R: 5′-agcggtcctccCcccgcaccagg-3′.

POLG gene mutational analyses

DNA was isolated from tumors and cell lines using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). All 23 exons and flanking intron/splice junctions of POLG were amplified by PCR with AmpliTaq Gold-polymerase (Applied Biosystems, Foster City, CA, USA). Primers and PCR conditions are given in Supplementary Table 1. The PCR products were checked by agarose gel electrophoresis, purified by a QIAEX II Gel Extraction Kit (Qiagen) and sequenced using a BigDye terminator Ready Reaction Kit v.3 on a 3100 Genetic Analyzer Automatic Sequencer (Applied Biosystems).

Mitochondrial whole-genome sequencing

Complete mtDNA of four representative samples was amplified using 24 sets of overlapping primers. Direct sequencing of PCR products was carried out using 100 ng of the PCR product. Mitochondrial DNA mutations were identified by comparing the sequences with revised Cambridge Reference Sequence.

Analysis of mtDNA content

Mitochondrial DNA content was measured in breast tumor samples and cell lines by the SYBR green method (SA biosciences, Frederick, MD, USA) in a 7900HT Fast Real-time PCR system (Applied Biosystems). Standard curves were obtained using the MCF12A cell line DNA, and reactions were performed in triplicates. Two sets of primers, one amplifying the mtDNA tRNA (Leu) gene and the other amplifying the nDNA (β2-microglobulin), were used. The ratio of mtDNA to nDNA was used as an index for measuring the mtDNA content.[42]

MCF7 Tet-On Advanced cells were transiently transfected with a pTRE-Tight-BI-AcGFP1 POLG D1135A plasmid according to the Fugene HD Transfection Reagent protocol (Roche, Basel, Switzerland). Media containing 1000 ng ml^{-1} doxycycline (Clontech) were added 4 h after transfection. Transiently transfected cells were harvested 2 days after doxycycline treatment and were sorted for green fluorescent protein (GFP)-positive cells on a BD FACAria cell sorter (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). GFP-positive cells were replated with 1000 ng ml^{-1} doxycycline. DNA was isolated with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. MtDNA content was analyzed as described above.

GFP induction

The mean fluorescent intensity of GFP was determined reading the fluorescence of pTRE-Tight-BI-AcGFP1-transfected cells on the FL1 channel of a FACSCalibur (Becton Dickinson Biosciences). Values are represented as mean fluorescence intensity.

Mitochondrial functional analyses

MCF7 Tet-On Advanced cells were transiently transfected with the pTRE-Tight-BI-AcGFP1 POLG D1135A plasmid according to the Fugene HD Transfection Reagent protocol. Media containing 1000 ng ml^{-1} doxycycline were added 4 h after transfection. Expression of POLG D1135A was induced by 1000 ng ml^{-1} doxycycline for up to 5 days. Cells were analyzed for production of reactive oxygen species (ROS) by labeling with 10 μM dihydroethidium (DHE) for 40 min. Mitochondrial membrane potential was assessed by labeling the cells with 100 nM tetramethylrhodamine, ethyl ester, perchlorate (TMRE) for 35 min. Fluorescence of both dyes was analyzed on a FACSCalibur and gated for GFP-positive cells.

Mitochondrial respiratory activity was measured by the rate of resazurin reduction as previously described.[43,44] MCF7 Tet-On Advanced cells were transiently transfected with the pTRE-Tight-BI-AcGFP1 POLG D1135A vector, treated with 1000 ng ml^{-1} doxycycline and sorted for GFP-positive cells as described above. Cells assayed for mitochondrial respiratory activity as measured by the change in resazurin reduction.

Matrigel invasion assay

MCF7 Tet-on cells were transfected with mutant or wild type POLG plasmid, were treated with 1000 ng ml^{-1} doxycycline and were sorted for GFP as described above. Five days after doxycycline treatment, the cells were analyzed for *in vitro* Matrigel invasion. The cells were plated in serum-free media in an upper Boyden chamber with a Matrigel membrane. Complete media containing 10% FBS were added to the bottom well as a chemoattractant. The cells in

the chamber were incubated for 24 h and the membrane was fixed and stained with the Diff-Quick Stain Set (Dade Behring, Newark, DE, USA).

RESULTS

Mutation in POLG polymerase increases tumorigencity of breast cancer cells

To determine the functional as well as the tumorigenic role of POLG, a mutant defective in the polymerase domain (D1135A) was cloned under a tetracycline-inducible promoter and expressed in the MCF7 breast cancer cell line. A bicistronic promoter provided the expression of both GFP and POLG simultaneously. GFP expression was used as a guide to identify cells expressing the mutant POLG gene (Figure 1a). MtDNA content was drastically reduced when expression of the D1135A POLG mutant was turned on with the addition of doxycycline (Figure 1b).

These studies demonstrate that mutation(s) in the POLG polymerase domain lead to a reduced mtDNA content. We therefore characterized the effect of POLG mutations on mitochondrial function. As

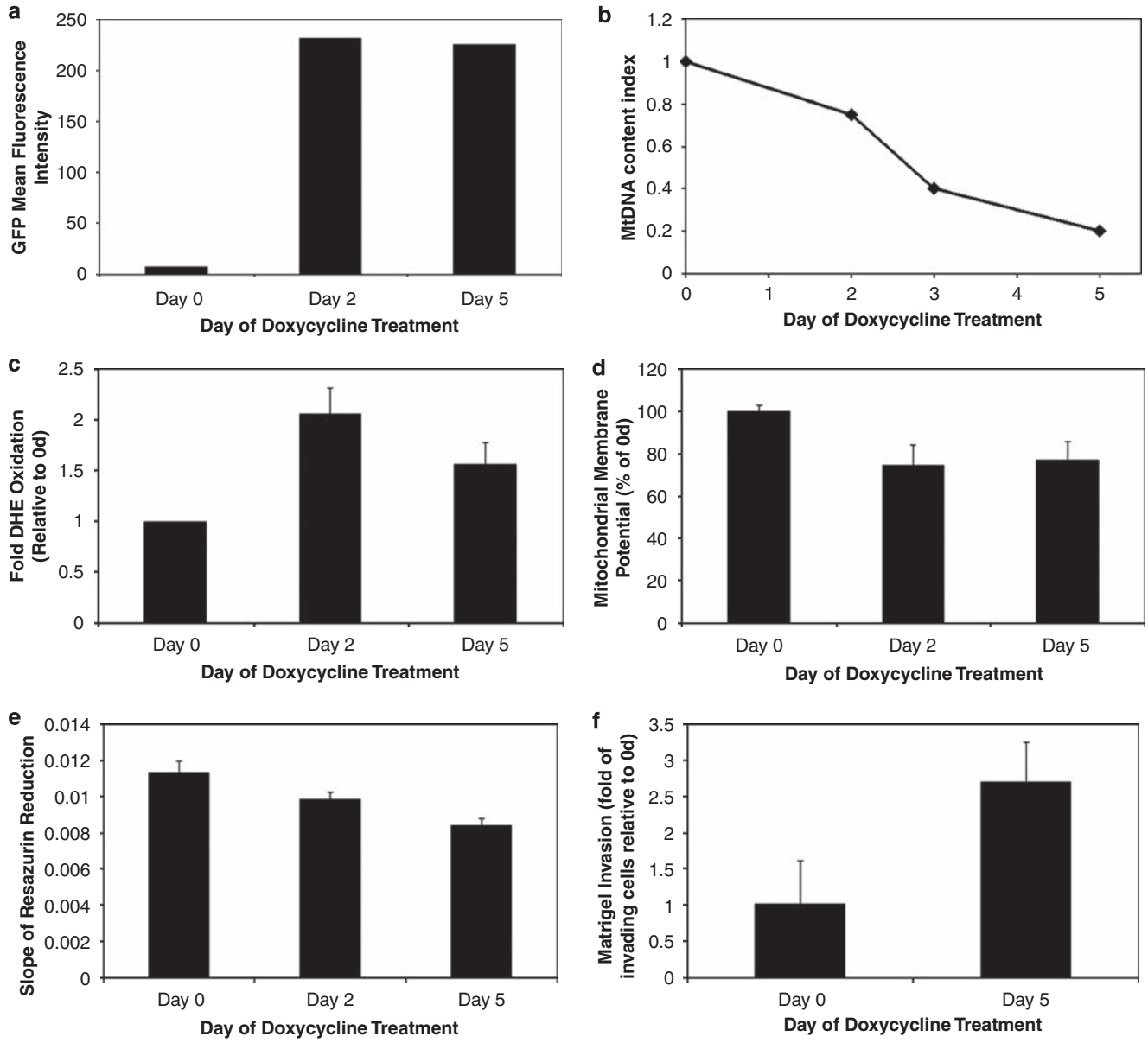

Figure 1 POLG D1135A mutant depletes mtDNA and promotes tumorigenicity in breast cancer cells. POLG D1135A cDNA was cloned in the tetracycline-inducible plasmid, pTRE-Tight-BI-AcGFP1. A bicistronic promoter provided the expression of both GFP and POLG simultaneously. Transfected MCF7 Tet-on Advanced cells were treated with 1000 ng ml^{-1} doxycycline for up to 5 days and were sorted by fluorescence-activated cell sorting. (**a**) GFP fluorescence was used as a guide to sort cells expressing the mutant POLG gene. Mean fluorescent intensity was determined on the FL1 channel of a FACSCalibur flow cytometer. Data represent geometric mean fluorescence intensity. (**b**) The MtDNA index in MCF7 Tet-on Advanced cells expressing POLG D1135A. The ratio of mtDNA to nuclear DNA was used as an index for measuring mtDNA content. (**c**) Dihydroethidium (DHE) oxidation of MCF7 Tet-on Advanced cells containing POLG D1135A was measured. The mean fluorescence intensity of each treatment group was normalized to day 0 and expressed as fold DHE oxidation +1 s.d. (**d**) Mitochondrial membrane potential was measured by tetramethylrhodamine, ethyl ester, perchlorate fluorescence. Data represent mitochondrial membrane potential as a percentage of control (day 0) +1 s.d. (**e**) Mitochondrial respiratory activity was measured by the rate of resazurin reduction. (**f**) Tumorigenicity was measured by Matrigel invasion assay.

Figure 1c shows, there is a twofold increase in the level of ROS as measured by DHE oxidation 2 days after POLG D1135A expression. This change in DHE oxidation decreases by day 5, potentially indicating a shift away from OXPHOS as a metabolic source. The majority of ROS production in the cell is derived from complex I and complex III of OXPHOS. Figure 1d shows that there is a 25% decrease in mitochondrial membrane potential in response to POLG D1135A expression. Mitochondrial respiratory activity was measured by the rate of resazurin reduction as previously described.[43,44] Resazurin is a redox-active dye that has an effect as an electron acceptor at complex IV of the ETC and fluoresces upon reduction.[44] Expression of POLG D1135A causes a decrease in OXPHOS when mtDNA is depleted from the POLG D1135A mutation (Figure 1e). As mtDNA encodes for 13 subunits of OXPHOS, loss of mtDNA would be expected to decrease oxidative metabolism. We then measured the *in vitro* tumorigeneic phenotype of cells expressing mutant POLG by Matrigel invasion assay. Figure 1f shows that cells expressing D1135A mutant POLG were more invasive than was the vector alone control. We conclude that mutations in the polymerase domain of the POLG gene cause a depletion of mtDNA, decrease mitochondrial membrane potential, decrease mitochondrial activity and increase oxidative stress, which together promote tumorigenesis.

POLG mutations identified in primary breast tumors

We screened all the coding exons and intron/splice junctions of POLG in 19 breast tumor samples and in three cancer cell lines (Supplementary Table 1). The sequence variants found are summarized in Table 1 and are depicted in Figures 2a and b. We identified novel as well as previously described pathogenic mutations in POLG.[33,45] The electropherograms of key mutations are given in Supplementary Figure 1. In exon 2 of POLG, CAG repeats were found to be extended in four breast tumor samples. We detected c.752C>T in exon 3 that affects the exonuclease domain of the protein (T251I), which was reported in PEO and infantile hepatocerebral syndrome.[33] Four mutations were detected in exons 9 and 10, which encode the linker region, including two novel and two previously reported mutations. The novel variants include c.1883G>A, a missense mutation causing a change in the conserved amino acid Arginine to Glutamine at the 628 residue of the POLG protein (Figure 2c), and another that is a silent mutation. Mutations were found in exon 16 (c.2492A>G) and exon 21 (c.3428A>G), which encode the POLG polymerase domain. In addition, we identified three novel variants in the intron/splice junctions of POLG. These results suggest that the POLG gene is a frequent target of mutation in breast tumors.

MtDNA mutations in primary breast tumors

Mutations in the POLG gene are known to result in the accumulation of mutations in mtDNA;[46] therefore, we sequenced the entire mitochondrial genome of four representative tumors samples. Interestingly, in all four samples analyzed, mutations were concentrated in the control D-loop region (Table 2). These mutations have previously been shown to occur in a variety of tumors.[47–51] These results suggest that the identified POLG mutation in breast tumors frequently targets the D-loop region.

Reduced mtDNA content in primary breast tumors and cell lines

In addition to mutations in mtDNA, a common consequence of the POLG mutation in mitochondrial diseases is mtDNA depletion.[33] MtDNA depletion is also found in breast tumors and is associated with the prognosis of breast cancer.[21] To identify the effect of the POLG mutations described above in breast tumors, we measured mtDNA content by real-time PCR. Single-copy nuclear gene β2-microglobulin was used to normalize the mtDNA content. Rho0 cells devoid of mtDNA served as a negative control. Figure 3a shows the mtDNA content index in primary breast tumors. MtDNA content was reduced in samples containing the POLG mutation. Interestingly, a similar observation was made in breast cancer cell lines (Figure 3b). We conclude that POLG mutation leads to a decrease in mtDNA content.

Breast tumor POLG mutations promote tumorigenesis

The above study demonstrates that the POLG gene is frequently mutated in primary breast tumors (Figure 1). Therefore, using site-directed mutagenesis, we mutagenized the cDNA-encoding representative mutations identified in breast tumors in all three functional domains of POLG (the E1143G—polymerase domain, the P587L—linker domain and the T251I—exonuclease domain), as well as the double mutations P587L and T251I that are often found in *cis*. Each mutant was tested for *in vitro* invasion 5 days after doxycycline treatment. Using the Matrigel invasion tumorigenicity assay, we demonstrate that expression of mutant POLG leads to increased invasiveness *in vitro* (Figure 4). These results suggest that POLG mutations identified in breast tumor indeed promote tumorigenesis by increasing the invasive potential of breast cancer cells.

DISCUSSION

Although mutation(s) in the POLG gene are shown to result in decreased OXPHOS, in decreased mtDNA content and in the pathogenesis of human mitochondrial diseases, its role in the pathogenesis of cancer is unclear. Therefore, we screened all coding exons and intron/splice junctions of POLG for mutations in breast tumors. Our analysis identified novel mutations in POLG. We also identified previously described mutations that are known to be involved in the pathogenesis of many mitochondrial diseases. Mutations were found in all three domains of the POLG protein. We identified a mutation in the exonuclease domain (C752T) of breast tumor that is associated with PEO and infantile hepatocerebral syndrome.[52–54]

Several mutations in the POLG linker region that lead to neuromuscular diseases, including Alpers' disease and Parkinson's disease, have been described.[35,55] However, we identified two novel linker region mutations in breast tumors. These include (a) a missense mutation in the evolutionarily conserved (R628Q) linker region and (b) a silent linker region mutation (A703A). An earlier functional analysis of the linker region mutants shows decreased enzyme activity, DNA binding and processivity of polymerase.[56] Mutants in the linker region of the fruit fly enzyme also affect its enzyme activity, processivity and DNA-binding affinity.[57] Codon usage analysis for human POLG suggests that 56/103 Alanines use the GCC codon, but only 13/103 alanines use the GCA codon. This is important in the context of the identified c.2109C>A (A703A) substitution in the linker region. It is conceivable that base substitution causes ribosome stalling because Alanyl-tRNAs do not recognize the GCA codon as well, which may slow down the synthesis of protein. In some proteins, this type of substitution results in an improper folding of protein, leading to reductions in activity.

Breast tumors also harbored mutations in the polymerase domain (Y831C and E1143G) of POLG. Previous studies suggest that these mutations inhibit mtDNA polymerase activity and, hence, may lead to mtDNA depletion.[58] Targeting POLG polymerase mutations in mice hearts also provides *in vivo* evidence for the depletion of mtDNA.[59]

One of the common features associated with mitochondrial diseases is the co-occurrence of mutations in POLG. The mutation T251I is

Table 1 POLG mutations in breast tumors

Number of tumors	*Mutant percentage*	*POLG1 mutations*															
Breast tumor samples																	
2	10.5		752 C>T*		1760C>T*												
2	10.5	Ins CAG 127-156*								3708G>T*	659+91 G>T*		2734+39 insGTAG**	3104-36 A>G*	3104-11 T>C*	3483-19 T>G*	3720+49 insG*
2	10.5										659+91 G>T*		2734+39 insGTAG*	3104-36 A>G*	3104-11 T>C*	3483-19 T>G*	3720+49 insG*
1	5.3									3708G>T*	659+91 G>T*		2734+39 insGTAG*	3104-36 A>G*	3104-11 T>C*	3483-19 T>G*	3720+49 insG*
1	5.3											1251-23delA*					
1	5.3	Ins CAG 127-156*											2734+39 insGTAG*				
1	5.3			1636 C>T*		1883 G>A*	2109 C>A*				659+91 G>T*		2734+39 insGTAG**			3483-19 T>G*	
1	5.3									3708G>T*	659+91 G>T**		2734+39 insGTAG**	3104-36 A>G**	3104-11 T>C**		3720+49 insG**
1	5.3	Ins CAG 127-156*							3428A>G*		659+91 G>T*		2734+39 insGTAG**	3104-36 A>G*	3104-11 T>C*	3483-19 T>G*	3720+49 insG*
Breast cancer cell lines																	
1	50										659+91 G>T*		2734+39 insGTAG*	3104-36 A>G*	3104-11 T>C*	3483-19 T>G*	3720+49 insG*
1	50							2492 A>G**									
Genomic region		Exon 2	Exon 3	Exon 9	Exon 10	Exon 10	Exon 12	Exon 16	Exon 21	Exon 23	Intron 2	Intron 6	Intron 17	Intron 19	Intron 19	Intron 21	3′UTR
Protein domain			Exonuclease	Linker	Linker	Linker	Linker	Polymerase	Polymerase								
Amino-acid change		Ins Gln 43-55	Thr251Ile	Arg546Cys	Pro587Leu	Arg628Gln	Ala703Ala	Tyr831Cys	Glu1143Gly	Gln1236His							
Disease association/ significance		Testicular cancer, Parkinson's disease	PEO, infantile hepatocerebral syndrome	Polymorphism	PEO, infantile hepatocerebral syndrome	Novel, missense	Novel, silent	PEO and Parkinsonism	Ataxia, Neuropathy	Polymorphism	Novel, Intronic	Novel, Intronic	Polymorphism	Polymorphism	Polymorphism	Polymorphism	Novel, Intronic

Abbreviation: PEO, progressive external ophthalmoplegia.
The table lists POLG mutations identified in primary breast tumor tissues (19) and breast cancer cell lines. The heterozygous mutations are marked by single asterisk and homozygous mutations are marked by double asterisks.

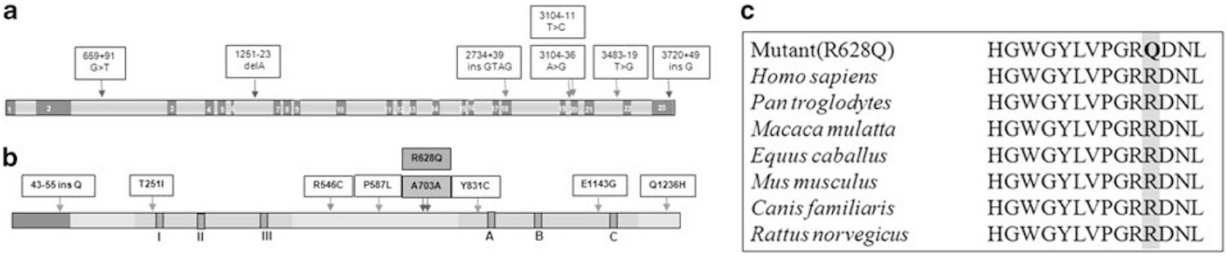

Figure 2 POLG mutations in breast tumors and breast cancer cell lines: (**a**) Intron/splice variants in the POLG genome; (**b**) Mutations in the POLG protein with amino-acid change. Green and red arrows indicate novel variants and disease-associated mutations/polymorphisms, respectively. Gray and orange boxes indicate novel silent and missense mutations, respectively; (**c**) Amino-acid conservation at the mutant residue of R628Q, a novel missense mutation observed in the linker region. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 2 Mitochondrial DNA mutations in breast tumors

Variant	*Mutant percentage (n=4)*	*Region*	*Amino-acid change*	*Association with cancer and other diseases*
A93G	50	D-loop	NA	Colorectal and gastric tumors
T152C	50	D-loop	NA	Ovarian/squamous-cell carcinoma
A263G	100	D-loop	NA	Oral cancer
C309CC	100	D-loop	NA	Multiple tumors
C315CC	100	D-loop	NA	Multiple tumors
C16169T	50	D-loop	NA	Polymorphism
T16172C	50	D-loop	NA	MNGIE/oral cancer
C16261T	50	D-loop	NA	Oral cancer
T16311C	50	D-loop	NA	Oral cancer
C16320T	50	D-loop	NA	Oral cancer
A750G	100	12S rRNA	NA	Polymorphism
A1438G	100	12S rRNA	NA	Polymorphism
A2706G	50	16S rRNA	NA	Oral cancer
A4769G	100	ND2	Syn	Polymorphism
G6755A	50	COI	Syn	Polymorphism
A8860G	100	ATPase 6	T-A	Polymorphism
A8869G	50	ATPase 6	M-V	Polymorphism
G13759A	50	ND5	A-T	Polymorphism
T15214C	50	CYT B	Syn	Polymorphism
A15326G	100	CYT B	T-A	Polymorphism

Abbreviation: NA, not applicable.
Complete mtDNA was sequenced in representative samples (n=4), containing POLG mutations T251I (exonuclease domain) and P587L (linker domain); ins Gln 43-55 and intronic variants.

found to occur in *cis* with P587L in many mitochondrial diseases.[34] Similarly, T251I was found in *cis* with P587L in two breast tumors. However, the E1143G mutation, frequently found in conjunction with W748S in ataxia,[60] was uniquely present in breast tumors. POLG contains trinucleotide repeats (CAG) in the coding region.[37] CAG trinucleotide repeat sequences are highly unstable, leading to an expansion or contraction of the repeat sequence, and are known to be involved in the pathogenesis of many human diseases.[61] Our study revealed an expansion of CAG repeats in more than 20% of the breast tumors analyzed.

We also identified novel intron/splice junction variants in conjunction with CAG repeats. Mutations in the intron/splice junctions of other genes are known to induce exon skipping, activation of cryptic splice sites or an alteration of the balance of alternative spliced isoforms.[62] Variants in splice junctions, particularly the GTAG insertion into intron 17, are predicted to alter splicing and POLG activity, as is also observed in PEO patients.[63,64] The CAG in 43–55Q was found to co-occur with seven variants in the intron/splice junction in two breast cancer cases. Interestingly, all breast tumors with CAG repeat expansion contained at least one splice site variant, c.2734+39 insGTAG. POLG repeat expansion is reported to be associated with testicular cancer.[65] POLG CAG repeat variation is also a predisposing genetic factor in idiopathic sporadic Parkinson's disease.[55] The expansion of CAG located in a number of genes has been shown to cause many dominantly inherited neurodegenerative diseases, described as polyglutamine diseases.[66] CAG repeats variation in other genes, such as androgen and estrogen receptors, has an important role in breast and other cancers.[67–69] The contraction of CAG repeats in POLG affects its expression.[58] However, it is unknown at this time whether the expansion of CAG repeats in the POLG gene described in this paper affects its expression. An expanded CAG tract seems to affect the function of the host protein through protein–protein interaction.[66] It is conceivable that CAG expansion in POLG affects its function and may contribute to tumorigenesis. However, further studies are

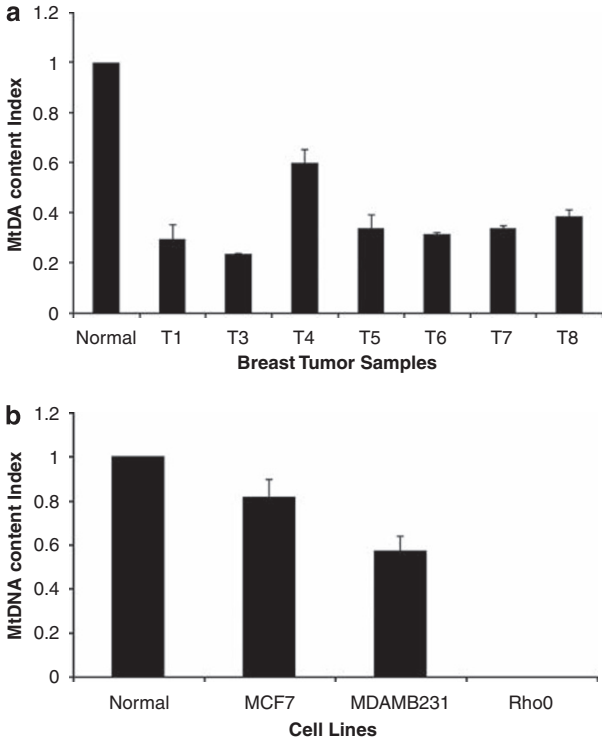

Figure 3 Decreased mtDNA content (**a**) in breast tumor samples and (**b**) in breast cancer cell lines. The ratio of mtDNA to nuclear DNA was used as an index for measuring mtDNA content (described in Materials and methods section).

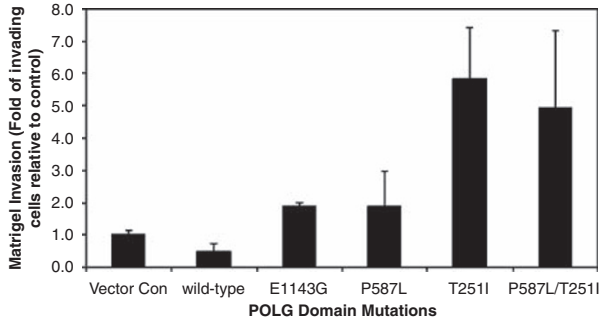

Figure 4 Breast tumor POLG mutations lead to increased tumorigenicity. Matrigel invasion of MCF7 Tet-on Advanced cells expressing representative mutations in POLG identified in primary breast tumors. Cell were treated with 1000 ng ml^{-1} doxycycline and sorted for GFP fluorescence. Cells were grown in the presence of doxycycline for 5 days and Matrigel invasion was carried out. Data represent the mean percentage of invading cells normalized to negative vector control ± 1 s.e.m.

required to identify the exact function of POLG CAG expansion in cancer.

Mutations in POLG are known to deplete mtDNA in multiple tissues of mitochondrial disease patients.[70] Interestingly, our analysis also revealed (1) a decreased mtDNA content in primary breast tumors and (2) when mutant POLG was expressed in breast cancer cells, it led to a depletion of mtDNA. Furthermore, we identified mutations that were predominantly present in the D-loop control region of mtDNA. An increased incidence of novel mtDNA point mutations has been demonstrated in patients with POLG mutations.[71,72] The highest incidence of the mtDNA D-loop mutations could be due to mutations affecting exonuclease and the polymerase domains of POLG. These findings suggest that reduced mtDNA content in breast tumors may arise because of (1) inefficient enzyme activity associated with POLG mutations and/or (2) mutations in the D-loop region affecting the binding of nuclear factors involved in mtDNA replication. Irrespective of POLG-induced depletion, our studies[11,73] and those of others[74,75] suggest that mtDNA depletion leads to tumorigenicity. Indeed, we recently demonstrated that depletion of mtDNA in breast epithelial cells leads to neoplastic transformation, and that this process is mediated by p53.[9] These studies led us to ask whether POLG mutations, particularly the one in the polymerase domain that causes mtDNA depletion, have a role in tumorigenesis. Studies presented in this paper demonstrate that the D1135A polymerase domain mutant, when expressed in MCF7 cells, functions as dominant negative and promotes tumorigenesis *in vitro*. We also show that expression of mutant protein results in a decreased mtDNA content, in decreased OXPHOS, decreased mitochondrial membrane potential and in increased oxidative stress, which together contribute to an increased tumorigenic phenotype. We also asked whether other POLG mutations have a role in tumorigenesis. The data presented in this paper show that, with the exception of the linker domain mutation (P587L), all other mutants (polymerase domain E1143G and exonuclease domain T251I) show increased tumorigenicity in breast cancer cells. As mutations P587L and T251I are often found in *cis* in many mitochondrial diseases, we also determined the effect of a double mutant on Matrigel invasion. Our results show lack of synergistic effects on tumorigenicity in double mutants. The single T251I mutant was as invasive as the double P587L/T251I mutant. These studies suggest that P587L does not have a significant effect in the increased invasive property of MCF7 cells.

Apart from depletion, breast tumors contained mutations in mtDNA. Mutations in POLG are known to cause mutations in mtDNA. mtDNA mutator mice that harbor a mutation in the exonuclease domain (which abolishes the POLG proofreading activity) show a marked reduction in their lifespan because of an increased rate of mtDNA mutation.[46,76] To date, there is no published report that describes the incidence of tumor development in these mice. It is possible that mtDNA mutations observed in these mice do not initiate tumorigenesis, that is, transform normal cells, but rather are involved in promoting tumoringenesis (as described in this paper) once cells are transformed. This argument is substantiated by our report, which demonstrates that mtDNA mutations in normal cells do not confer tumorigenicity. In contrast, mutant mtDNA from breast tumors, when transferred to transformed cells, shows metastasis.[77] In summary, our studies described in this paper provide the first comprehensive analyses of POLG gene mutations in human cancer that suggest a role for POLG in human tumorigenesis.

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