Published in final edited form as:

Fertil Steril. 2010 December ; 94(7): 2932–2934. doi:10.1016/j.fertnstert.2010.06.049.

Five mutations of mitochondrial DNA polymerase-gamma (*POLG*) are not a prevalent etiology for spontaneous 46,XX primary ovarian insufficiency (sPOI)

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Abstract

The etiology of most cases of spontaneous 46,XX primary ovarian insufficiency (sPOI) is unknown, however, associations have been made between mitochondrial diseases due to mutated mitochondrial DNA polymerase gamma (*POLG*) and sPOI. Here, we show that in 201 women with 46,XX sPOI, RFLP analysis of genomic DNA shows only one case (0.5%, 95% CI <3%) of heterozygosity for a mtDNA *POLG* mutation, suggesting that this is not a common genetic etiology for this form of infertility.

Spontaneous 46,XX primary ovarian insufficiency (sPOI), also known as premature ovarian failure (POF), affects ~1% of women before age 40. sPOI is characterized by abnormal menses, menopausal FSH, and estrogen deficiency due to primary ovarian dysfunction. In ~90% of cases, the etiology is unknown. Recently, several genetic abnormalities have been highly associated with sPOI, including mutations in mitochondrial DNA polymerase- γ (*POLG*) (1-4).

POLG is the only DNA polymerase responsible for mitochondrial DNA (mtDNA) replication and repair, thus it is essential for mitochondrial function. Several *POLG* mutations have been described, resulting in deletions or point mutations in mtDNA and decreased polymerase activity. Although there is a spectrum in the degree of insufficiency in polymerase activity, the most severe mutations lead to loss of >99% of polymerase function and cell death due to insufficient cellular energy production (5). Autosomal dominant progressive external ophthalmoplegia (PEO) was the first disease associated with *POLG* mutations (3,6). Additional *POLG*-driven mitochondrial diseases include Parkinson's disease (PD), epilepsy, ataxia, psychiatric illnesses (1,6-9), and primary gonadal insufficiency in both men and women (1-4). In women, sPOI co-segregates with PEO and Parkinson's in heterozygous *POLG* mutation carriers, suggesting mtDNA polymerase activity is required for both normal neurologic and ovarian function (1). Several described *POLG* mutations are associated with PEO: Y955C [indicating change from tyrosine (Y) to

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cystine (C) at the 955th peptide residue], R943H, G923D, A957S, and R953C (5,10). Of these, R943H has also been associated with sPOI (3).

To determine if *POLG* mutations known to be associated with PEO are also associated with sPOI, we screened the genomic DNA of women with 46,XX sPOI for the 5 *POLG* mutations described above (G923D, R943H, R953C, Y955C, and A957S). Women aged 18-42 with 46,XX sPOI (n=201) were recruited between August 2004 and March 2006 as part of an ongoing cross-sectional study evaluating the health of women with sPOI (11). Women with known iatrogenic cause of POI were excluded. 46,XX sPOI was defined as abnormal menses for at least four months, menopausal range FSH levels confirmed on two occasions at least one month apart, and age less than 40 at the time of diagnosis. This study was approved by the Institutional Review Board of the National Institute of Child Health and Human Development.

Demographics of this group was similar to that seen in other published groups of women with 46,XX sPOI (12): 82% Caucasian (164/201), 9% black (19/201), 5% Hispanic (11/201), and 2% Asian (5/201). FSH was elevated into the menopausal range in all women (mean FSH 77.4 $\pm$ 37.8 U/L), with corresponding low serum estradiol levels (mean E2 44.5 $\pm$ 42.7 pg/mL). 112 (56%) women were tested for a premutation in the Fragile X (*FMRI*) gene. 3 of these women (3%) carry the *FMRI* premutation (defined as 55-199 CGG repeats on the *FMRI* gene), which itself infers approximately a 20% risk of developing sPOI (13). 1 woman (0.5%) had steroidogenic cell autoantibodies, indicating autoimmune oophoritis as the primary cause of her sPOI. 45 women (22%) reported a family history of sPOI (or "POF" or "premature menopause"). Several patients reported a family history of neurologic disorders: Parkinson's disease (4.5%), tremor (10%), ataxia (4%), early onset hearing loss (3.5%), learning disability (14.4%), and mental retardation (1%).

Genomic DNA from each patient was isolated using a rapid non-enzymatic method and amplified using PCR as previously described (14,15). We performed DNA mutagenesis on a plasmid with the PCR products (460 bp) containing normal sequences of exon 18 of the *POLG* using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) and confirmed the expected mutant plasmids with the *POLG* mutations c2767G>A, c2828G>A, c2857C>T, c2864A>G and c2869G>T, individually. These mutant plasmids were used as templates for PCR reactions for positive controls of such DNA mutations. In RFLP analysis as shown in Table 1, the restriction enzymes *Mwo*I, *Ms*II, *Eag*I, *Alw*NI and *Bse*YI were used and the resultant DNA fragments were separated by gel electrophoresis to screen for the *POLG* mutations c2767G>A, c2828G>A, c2857C>T, c2864A>G and c2869G>T, respectively. These nucleotide changes lead to the *POLG* mutations of the peptide residues with G923D, R943H, R953C, Y955C and A957S, respectively.

Mitochondrial DNA *POLG* mutations are associated with sPOI in combination with other mitochondrial diseases, particularly PEO. We screened 201 women with 46,XX sPOI for five *POLG* mutations with known association with PEO, including Y955C and R943H, which have also been associated with sPOI (1,3,4). Only 1 of the 201 women we screened (0.5%) demonstrated heterozygosity for a single *POLG* mutation--c2857C>T (R953C) (Table 1). Based on this observation, 95% confidence level indicates the frequency of these *POLG* mutations accounts for <3% of cases of 46,XX sPOI.

The woman found to have a heterozygous *POLG* mutation was an otherwise healthy, 28-year old Caucasian. She had menarche at age 11 and normal monthly menses until age 28, at which time she developed oligomenorrhea (menses every 3 to 4 months). She was diagnosed with sPOI after fertility workup showed menopausal range FSH. She had been attempting pregnancy for approximately 5 years without success. In addition to sPOI, she had one co-

morbid medical condition, Meniere's syndrome, characterized by episodic vertigo, tinnitus, and hearing loss due to damage to the inner ear. She had no current or past history of anxiety or depression, and no autoimmune disorders. Family history was negative for infertility, sPOI or other menstrual irregularity; neurologic disorders, including PEO or PD; and, except for alopecia in a maternal grandmother, negative for autoimmune disease. At the NIH, this patient's mean serum FSH was 72.7 U/L, LH 53.7 U/L, estradiol 58.2 pg/mL, and progesterone 0.5 ng/mL. Anti-adrenal and 21-hydroxylase antibodies were negative. *FMRI* premutation testing was normal, ruling out Fragile X-associated POI. Transvaginal ultrasound showed an 8 mm endometrial stripe, a 2.4×2.5×0.9 cm left ovary without evident follicles and a 2.7×1.0×2.3 cm right ovary with one 9 mm follicle.

The *POLG* R953C mutation has not been definitively associated with sPOI, although such a relationship has been suggested by its association with a Parkinson's-like syndrome that co-segregates with POI within families (1). Thus, the *POLG* R953C mutation may be a rare cause of sPOI due to mitochondrial dysfunction. A direct relationship between the R953C mutation and sPOI cannot be determined from this study. However, previous work has shown that neither the R953C nor any other *POLG* mutations were present among 1640 healthy controls (1), suggesting the possibility of a causal association with this fertility disorder. However, whether R953C is a rare DNA sequence variant or a true functional mutation cannot be determined from this study or previous studies, given that impaired function due to the mutation has not been demonstrated *in vitro*. Further investigation is needed to clarify this.

Together, these data demonstrate that these five *POLG* mutations are not a prevalent etiology of sPOI. This is in agreement with similar studies in infertile males, in which patients were screened for *POLG* CAG repeat polymorphisms that had previously been associated with primary gonadal insufficiency (14,15). Only 3% of infertile males screened were found to be homozygous carriers of the mutations, and half of these reported successful pregnancies despite presence of the mutation. In addition, 1% of fertile males were found to be homozygous carriers, providing further evidence that *POLG* dysfunction may not be a factor in primary gonadal insufficiency in men (16).

The *POLG* mutations most commonly associated with sPOI to date, Y955C and R943H (1,3,4), were not seen in any of the women we screened. The prevalence of *POLG* mutations is estimated to be as high as 1% in certain populations, including the U.S. (17). We cannot exclude the possibility that screening a larger number of patients would have shown more cases of *POLG* mutations associated with sPOI. Further, we only tested for 5 of the >20 described *POLG* mutations, thus we are unable to exclude the possibility that mutations other than those we tested associate with sPOI.

Whether ovarian dysfunction due to *POLG* mutations is due to follicle depletion or follicle dysfunction is not known. Oocytes have the highest mtDNA copy number of all cells (18), which increases their susceptibility to mtDNA depletion, and suggests a role for follicular depletion. On the other hand, oxidative phosphorylation, and thus mtDNA, is required for follicular development (19), suggesting follicle dysfunction may be responsible. Indeed, infertility in men believed to be due to *POLG* mutations is never due to azoospermia, but seems to be associated with defective spermatogenesis and/or steroidogenesis (14).

We conclude that the 5 *POLG* mutations tested here are not a prevalent genetic etiology for sPOI. Thus, testing all patients diagnosed with 46,XX sPOI for *POLG* mutations, especially in the absence of clinical suspicion of other mtDNA disorders, is unlikely to be of clinical usefulness or cost-effective. Further analysis of other *POLG* mutations for an association with sPOI is warranted.

Capsule

Spontaneous 46,XX primary ovarian insufficiency has been associated with mutations in mitochondrial DNA polymerase- γ (*POLG*). Of 201 women with 46,XX sPOI screened, we found only one with a *POLG* mutation.

Acknowledgments

Supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health. Vien H. Vanderhoof and Lawrence M. Nelson are Commissioned Officers in the United States Public Health Service.

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Table 1

RFLP Analysis of *POLG* Mutations

Mutation	Enzyme	PCR Products w/o Mutation		PCR Products w/ Mutation		DNA of Patients w/ POI (n=201)	
		No. of cuts	Fragments (bp)	No. of cuts	Fragments (bp)	No. of cases w/ Mutation	
c2767G>A	<i>Mwo</i> I	3	19, 62, 148 & 201	2	19, 201 & 210	0	
c2828G>A	<i>Msl</i> I	2	32, 100 & 298	3	19, 32, 100 & 279	0	
c2857C>T	<i>Eag</i> I	1	141 & 289	0	430	1 (heterozygous)	
c2864A>G	<i>Alw</i> NI	0	300	1	19 & 281	0	
c2869G>T	<i>Bse</i> YI	2	76, 157 & 197	1	197 & 233	0	