No Mutations in the *PSMC3IP* Gene Identified in a Swedish Cohort of Women with Primary Ovarian Insufficiency

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Key Words

Disorders of sex development · Gonadal dysgenesis · Premature ovarian failure · Primary ovarian insufficiency · *PSMC3IP*

Abstract

Ovarian dysfunction before the age of 40 years, characterized by hypergonadotropic hypogonadism and presenting with either primary or secondary amenorrhea, is called primary ovarian insufficiency (POI). POI has a significant genetic component, but the specific genetic cause is often unknown. A novel candidate gene for POI, *PSMC3IP*, has recently been identified. The aim of this study was to investigate a group of patients with POI for possible *PSMC3IP* mutations. Therefore, DNA samples from 50 patients with POI of primarily Swedish origin were used in the study, 27 with secondary amenorrhea (median age of diagnosis 23 years) and 23 with primary amenorrhea. Control material consisting of DNA samples from 95 women without POI was used for investigation of novel sequence variants. All exons and intron/exon boundaries of the *PSMC3IP* gene were analyzed by PCR and sequencing. As a result, no pathogenic mutation in the *PSMC3IP* gene was detected in the cohort. A previously un-

reported variant, NM_016556.3:c.337+33A>G, was detected in heterozygous form in 1 patient with secondary amenorrhea, likely constituting a normal variant. Two reported single nucleotide polymorphisms were detected in the cohort at the expected frequency. In conclusion, *PSMC3IP* gene mutations are not common causes of POI in this Swedish cohort.

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Primary ovarian insufficiency (POI) is the suggested term to describe ovarian dysfunction before the age of 40 years [Nelson, 2009]. This condition can present with either primary, or more commonly, secondary amenorrhea and is characterized by hypergonadotropic hypogonadism with serum FSH levels in the menopausal range [Simpson and Rajkovic, 1999; Nelson, 2009]. The suggested causative mechanisms for POI are follicle depletion or follicle dysfunction. A small number of initial germ cells, increased apoptosis, or destruction of primordial follicles cause follicle depletion, and a failure of follicles to respond to hormonal stimuli constitutes follicle dysfunction [Nelson, 2009; De Vos et al., 2010]. POI is heterogeneous, and follicle depletion can also be caused

Table 1. Patient overview

Clinical data	Primary amenorrhea (n = 23)	Secondary amenorrhea (n = 27)
Median age at diagnosis, years	16 (13–18)	23 (13–37)
Median age at menarche, years	–	13 (10–15)
Median FSH level at diagnosis, IU/l (reference value: <30 IU/l)	89 (39–150)	84 (34–155)
Swedish, Caucasian, n (%)	17 (74)	23 (85)
Known heredity for POI, n (%)	1 (4.3)	5 (18.5)
Spontaneous conception, n (%)	0	10 (37)

Figures in parentheses represent total range unless indicated otherwise.

by autoimmune or toxic follicle destruction, pelvic surgery, radiation therapy, and other external factors [MacLaran and Panay, 2011]. However, POI has a significant genetic component, although the specific genetic cause is often not identified [Simpson and Rajkovic, 1999; Beck-Peccoz and Persani, 2006; Woad et al., 2006; Nelson, 2009]. In addition, there are syndromic forms of POI, the most common of which is Turner syndrome due to monosomy X in complete or mosaic forms [Simpson and Rajkovic, 1999].

Chromosomal aberrations such as X chromosome deletions and translocations account for 5–10% of all cases with POI [Baronchelli et al., 2012]. Premutations of the *FMR1* gene on the X chromosome are overrepresented in patients with POI, with a POI incidence of 15–20% in premutation carriers [Saul and Tarleton, 1993; Wittenberger et al., 2007]. In comparison, the general population incidence of POI is 1% [Coulam et al., 1986]. Approximately 2–5% of sporadic cases with POI can be explained by *FMR1* premutations but so far are only described in patients with secondary amenorrhea [Wittenberger et al., 2007; De Vos et al., 2010]. Mutations in the genes encoding the transcription factors *NOBOX* and *FIGLA* [Qin et al., 2007; Zhao et al., 2008], the oocyte secreted factors *GDF9* and *BMP15* [Dixit et al., 2006; Kovanci et al., 2007], and the hormone receptors *FSHR* and *NR5A1* [Aittomaki et al., 1995; Janse et al., 2012] have also been found in patients with POI.

Recently, a novel candidate gene for POI has been identified by Zangen et al. [2011]. The authors describe a consanguineous family with several members affected by 46,XX gonadal dysgenesis, a form of POI with primary amenorrhea and streak gonads. All affected patients shared a homozygous 3-bp deletion (c.600_602del,

p.Glu201del) in the *PSMC3IP* (*PSMC3 interacting protein*) gene detected by whole-exome sequencing. In cell line experiments, the mutant *PSMC3IP* protein exhibits a significantly decreased function as an estrogen co-activator. Estrogen is described to be important for primate prenatal development of the follicle pool as well as for the second stage of follicular development at puberty. Decreased estrogen-dependent transcription could possibly affect these developmental steps [Albrecht and Pepe, 2010; Zangen et al., 2011]. A defective function of *PSMC3IP* might therefore cause POI with either primary or secondary amenorrhea. Therefore, we have investigated our cohort of patients with POI for possible *PSMC3IP* mutations. To date, this is the first study to investigate the *PSMC3IP* gene in a group of unrelated patients with POI.

Material and Methods

Patients

Fifty cases selected among the patients referred to the Clinical Genetic Laboratory of Karolinska University Hospital Stockholm, Sweden were included in the study. The cohort consists of 23 patients with primary amenorrhea and 27 patients with secondary amenorrhea. Written informed consent was collected from all participants of age and from parents on behalf of minors included in the study. The regional Ethics Committee at Karolinska Institutet, Sweden approved the study and consent procedure.

The diagnosis of POI was established by the presentation of primary or secondary amenorrhea in individuals with female external genitalia as well as internal Müllerian structures (uterus) and hypergonadotropic hypogonadism (FSH >30 IU/l) in at least 2 independent measurements. See table 1 for an overview of patients and clinical data.

Family history was negative for POI for all except 3 patients with secondary amenorrhea whose mothers also had menopause before 40 years of age. One patient with primary amenorrhea also has an affected sister with primary amenorrhea. Two patients within the cohort are related, paternal aunt and niece, with development of secondary amenorrhea at 34 and 22 years of age, respectively.

For all patients, previous surgery, chemotherapy, and radiotherapy were excluded. Five patients, 4 with secondary and 1 with primary amenorrhea, have hypothyroidism with anti-thyroid autoantibodies. None have antibodies against adrenal cortex or 21-hydroxylase proteins. The 4 patients with secondary amenorrhea had debut of hypothyroidism after POI diagnosis. The patient with primary amenorrhea was diagnosed with anti-thyroid autoantibodies at 11 years of age.

Four patients with primary amenorrhea have been investigated with ovarian biopsies. These confirm streak gonads with no visible follicles. Five patients with secondary amenorrhea have also been investigated with ovarian biopsies, in 3 cases exhibiting streak gonads with no visible follicles. The 4th patient's biopsy showed multiple primordial follicles but no mature follicles. This patient pre-

Table 2. Sequencing results

SNP ID	Minor allele frequency	Location	Nucleotide change	Amino acid change	Primary amenorrhea (23)	Secondary amenorrhea (27)	Total (50)
Novel change	–	intron 4	NM_016556.3:c.337+33A>G	–	A/A: 23 A/G: 0 G/G: 0	A/A: 26 A/G: 1 G/G: 0	A/A: 49 A/G: 1 G/G: 0
rs75716493	C = 0.018/40	intron 4	NM_016556.3:c.338–101T>C	–	T/T: 23 T/C: 0 C/C: 0	T/T: 26 T/C: 1 C/C: 0	T/T: 49 T/C: 1 C/C: 0
rs2292752	G = 0.465/1015	intron 4	NM_016556.3:c.338–15C>G	–	C/C: 5 C/G: 10 G/G: 8	C/C: 3 C/G: 13 G/G: 11	C/C: 8 C/G: 23 G/G: 19

sented with secondary amenorrhea at 23 years of age. The 5th patient had a few visible primordial and primary follicles and presented with secondary amenorrhea at 15 years of age.

Genetic Investigation

DNA was extracted from peripheral blood leukocytes or from EBV-transformed lymphocytes. A 46,XX karyotype in peripheral blood was confirmed in all but in patient 24 in whom a balanced Robertsonian translocation was identified (45,XX,der(13;14)), not considered causative. Sex chromosome mosaicism was excluded using fluorescence in situ hybridization (FISH) and DNA probes from chromosome X and Y on peripheral blood smears and, when available, on touch preparations from gonadal tissue. Genetic investigation included sequencing of *NR5A1*, *BMP15*, *FSHR*, *NOBOX*, and *GDF9*. *FMR1* mutations were also excluded in patients with secondary amenorrhea.

Control Material

As control material DNA samples were prepared from peripheral blood lymphocytes of 95 healthy women. All were above the age of 40 years at sample collection and had given birth to at least 1 child. Exclusion criteria for participation were previous egg donation, in vitro fertilization, fertility treatment, or menopause before the age of 40 years.

PCR and Sequencing

The 8 exons and all exon-intron boundaries of the *PSMC3IP* gene were amplified by PCR and sequenced. Primers were designed using Primer 3 software (v3.0.0, <http://primer3.wi.mit.edu/>) [Koressaar and Remm, 2007; Untergasser et al., 2012]. See online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000357605) for primer sequences. PCR conditions are available on request.

PCR products were cleaned with Exonuclease 1 and Shrimp Alkaline Phosphatase (Thermo Scientific, Fermentas, St. Leon-Rot, Germany) and sequenced using the ABI BigDye Terminator v3.1 kit (Applied Biosystems, Life Technologies Europe BV, Stockholm, Sweden) according to the manufacturers' instructions. Fragments were separated on a 3730 DNA Analyzer (Applied Biosystems). Electropherograms were analyzed against the reference sequence NG_031960.1 using the SeqScape v2.5 program (Applied Biosystems).

Statistical Analysis

Binomial parameters were calculated using StatXact 4, CYTEL Software corporation (Cambridge, USA). A 95% confidence interval was calculated according to Clopper-Pearson.

In silico Analysis

The analysis of splicing efficiency for the novel variant as well as the normal sequence was carried out using 3 different splice site prediction tools: The Splice Site Prediction by Neural Network tool from the Berkeley Drosophila Genome Project (http://www.fruit-fly.org/seq_tools/splice.html); NetGene2 v. 2.4 (<http://www.cbs.dtu.dk/services/NetGene2/>); FSPLICE 1.0 (<http://linux1.softberry.com/berry.phtml?topic=fsplce&group=programs&subgroup=gfind>).

Results

Samples from all 50 patients were successfully sequenced. No mutations or sequence variations in the coding region of the *PSMC3IP* gene were detected. In intron 4, a novel heterozygous change was identified in 1 patient with secondary amenorrhea at 37 years of age which was not detected in any of the 190 control alleles investigated. The transition NM_016556.3:c.337+33A>G has not been previously reported. In addition, 2 single nucleotide polymorphisms were found, rs75716493 and rs2292752, presenting at a frequency concordant with the reported minor allele frequency. See table 2 for sequencing results.

Discussion

POI, characterized by hypergonadotropic hypogonadism, can present with primary amenorrhea, or more commonly with secondary amenorrhea before 40 years of age. POI is heterogeneous and has several described causative

genetic mechanisms, both at the chromosomal and single gene level. Still, the majority (90%) of patients with POI does not receive a molecular diagnosis [Nelson, 2009], and many involved genes are still unknown.

The most common known single gene cause of POI is an *FMR1* premutation, with a detection rate between 2 – 5% in sporadic cases and up to 13% in familial cases [Wittenberger et al., 2007; De Vos et al., 2010]. A French study reported a high mutation frequency (6.2%) in the *NOBOX* gene in a cohort of patients with POI [Bouilly et al., 2011]. Mutations in other genes, such as *FSHR*, *BMP15*, and *GDF9*, have so far only been described in a limited number of cases with POI [Aittomaki et al., 1995; Di Pasquale et al., 2004; Zhao et al., 2007; Lakhal et al., 2010].

The *PSMC3IP* protein binds to the alpha- and beta-estrogen receptors as well as the glucocorticoid, thyroid, androgen, and progesterone receptors and acts as a co-activator of hormonally dependent transcriptional activation [Ko et al., 2002]. It is therefore an interesting candidate gene for ovarian function and development and has been proposed as a novel candidate gene for POI by Zangen et al. [2011]. In this study, a decreased transcriptional activity was reported for the mutant *PSMC3IP* protein in cell lines, supporting its possible causative role. The described homozygous c.600–602del mutation could, however, not be detected in any of the samples in our study, nor was any other mutation in the coding region of the *PSMC3IP* gene discovered. In intron 4, a previously unreported variant, NM_016556.3:c.337+33A>G, was detected in heterozygous form in a single patient with secondary amenorrhea. In silico analysis of splicing efficiency using 3 different prediction tools did not support an effect of the variant on splicing. Although we could not detect this change in any of the 190 control alleles investigated, it is most likely to constitute a rare normal variant.

POI is a severe condition affecting approximately 1% of women, and for each new candidate gene it is important to investigate the possible frequency of pathogenic mutations in patients with POI. This should be done both for diagnostic purposes and for strengthening the understanding of gene function in ovarian development and maintenance. The *PSMC3IP* gene has so far only been investigated in a single consanguineous family with hereditary POI. The present study is based on a group of 50 patients with POI and primarily Swedish Caucasian ethnic background, where no mutation of the *PSMC3IP* gene was detected. Using binomial probability calculation, we can note that the probability of detecting mutations in 0

out of 50 patients with POI with an assumed POI population prevalence of possible causative *PSMC3IP* mutations of 5.8% is only 5% (exact confidence interval 0–7.11%). This is less than reported for mutations in the autosomal gene *NOBOX* in Caucasian patients with POI and some reported studies of *FMR1* premutations. Therefore, we can conclude that *PSMC3IP* gene mutations are not a common cause of POI in this Swedish cohort. Conversely, to detect a *PSMC3IP* mutation prevalence of 1.5%, i.e. below the prevalence of *FMR1* premutations, with the same 5% probability, a sample size of 200 patients would be necessary, larger than any reported single gene study for POI.

Our patients are mostly Swedish, whereas the family investigated by Zangen et al. [2011] was of Palestinian descent. It remains possible that the discovered mutation can be found in a higher prevalence in patients of this ethnic background. For comparison, *FSHR* mutations are primarily found in the Finnish population [Aittomaki et al., 1995; Conway et al., 1999], and the aforementioned *NOBOX* mutations have been detected in Caucasians but could not be found in a large group of Chinese women [Qin et al., 2007, 2009; Bouilly et al., 2011]. In addition to the population differences, causative mutations have so far mostly been described only in a small number of cases each, indicating that there is a large degree of heterogeneity in POI. Thus, several unknown genes most likely remain to be identified. It is also possible that there could be synergistic effects with combinations of gene variants causing the phenotype.

For clinical genetic investigation of POI, a single gene approach with consecutive screening by conventional sequencing of candidate genes has so far been quite unsuccessful and expensive, with a low detection rate per gene investigated. With the development of next-generation sequencing techniques, a multiple gene approach where a panel of known candidate genes is analyzed simultaneously, screening could be more successful. Whole-exome sequencing can also be considered, with proper caution concerning interpretation of unknown variants. Also, as many developmentally important genes act in a dosage dependent manner, array-CGH could be another useful tool for the identification of both known causes and novel candidate genes for POI. In the future, whole-genome sequencing may allow the simultaneous detection of gene dosage aberrations and small DNA sequence aberrations, hopefully improving diagnostics and knowledge on pathogenetic mechanisms for this group of patients.

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References

- Aittomäki K, Lucena JL, Pakarinen P, Sistonen P, Tapanainen J, et al: Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell* 82:959–968 (1995).
- Albrecht ED, Pepe GJ: Estrogen regulation of placental angiogenesis and fetal ovarian development during primate pregnancy. *Int J Dev Biol* 54:397–408 (2010).
- Baronchelli S, Villa N, Redaelli S, Lissoni S, Saccheri F, et al: Investigating the role of X chromosome breakpoints in premature ovarian failure. *Mol Cytogenet* 5:32 (2012).
- Beck-Peccoz P, Persani L: Premature ovarian failure. *Orphanet J Rare Dis* 1:9 (2006).
- Bouilly J, Bachelot A, Broutin I, Touraine P, Binart N: Novel *NOBOX* loss-of-function mutations account for 6.2% of cases in a large primary ovarian insufficiency cohort. *Hum Mutat* 32:1108–1113 (2011).
- Conway GS, Conway E, Walker C, Hoppner W, Gromoll J, Simoni M: Mutation screening and isoform prevalence of the follicle stimulating hormone receptor gene in women with premature ovarian failure, resistant ovary syndrome and polycystic ovary syndrome. *Clin Endocrinol (Oxf)* 51:97–99 (1999).
- Coulam CB, Adamson SC, Annegers JF: Incidence of premature ovarian failure. *Obstet Gynecol* 67:604–606 (1986).
- De Vos M, Devroey P, Fauser BC: Primary ovarian insufficiency. *Lancet* 376:911–921 (2010).
- Di Pasquale E, Beck-Peccoz P, Persani L: Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (*BMP15*) gene. *Am J Hum Genet* 75:106–111 (2004).
- Dixit H, Rao LK, Padmalatha VV, Kanakavalli M, Deenadayal M, et al: Missense mutations in the *BMP15* gene are associated with ovarian failure. *Hum Genet* 119:408–415 (2006).
- Janse F, de With LM, Duran KJ, Kloosterman WP, Goverde AJ, et al: Limited contribution of *NR5A1* (*SF-1*) mutations in women with primary ovarian insufficiency (POI). *Fertil Steril* 97:141–146 e142 (2012).
- Ko L, Cardona GR, Henrion-Caude A, Chin WW: Identification and characterization of a tissue-specific coactivator, GT198, that interacts with the DNA-binding domains of nuclear receptors. *Mol Cell Biol* 22:357–369 (2002).
- Koressaar T, Remm M: Enhancements and modifications of primer design program primer3. *Bioinformatics* 23:1289–1291 (2007).
- Kovanci E, Rohozinski J, Simpson JL, Heard MJ, Bishop CE, Carson SA: Growth differentiating factor-9 mutations may be associated with premature ovarian failure. *Fertil Steril* 87:143–146 (2007).
- Lakhal B, Laissue P, Braham R, Elghezal H, Saad A, et al: *BMP15* and premature ovarian failure: causal mutations, variants, polymorphisms? *Clin Endocrinol (Oxf)* 72:425–426 (2010).
- Maclaran K, Panay N: Premature ovarian failure. *J Fam Plann Reprod Health Care* 37:35–42 (2011).
- Nelson LM: Clinical practice. Primary ovarian insufficiency. *N Engl J Med* 360:606–614 (2009).
- Qin Y, Choi Y, Zhao H, Simpson JL, Chen ZJ, Rajkovic A: *NOBOX* homeobox mutation causes premature ovarian failure. *Am J Hum Genet* 81:576–581 (2007).
- Qin Y, Shi Y, Zhao Y, Carson SA, Simpson JL, Chen ZJ: Mutation analysis of *NOBOX* homeodomain in Chinese women with premature ovarian failure. *Fertil Steril* 91:1507–1509 (2009).
- Saul RA, Tarleton JC: FMR1-related disorders, in Pagon RA, Bird TD, Dolan CR, Stephens K, Adam MP (eds): *GeneReviews™* [Internet]. (University of Washington, Seattle 1993). <http://www.ncbi.nlm.nih.gov/books/NBK1384/>
- Simpson JL, Rajkovic A: Ovarian differentiation and gonadal failure. *Am J Med Genet* 89:186–200 (1999).
- Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, et al: Primer3 – new capabilities and interfaces. *Nucleic Acids Res* 40:e115 (2012).
- Wittenberger MD, Hagerman RJ, Sherman SL, McConkie-Rosell A, Welt CK, et al: The *FMR1* premutation and reproduction. *Fertil Steril* 87:456–465 (2007).
- Woad KJ, Watkins WJ, Prendergast D, Shelling AN: The genetic basis of premature ovarian failure. *Aust N Z J Obstet Gynaecol* 46:242–244 (2006).
- Zangen D, Kaufman Y, Zeligson S, Perlberg S, Fridman H, et al: XX ovarian dysgenesis is caused by a *PSMC3IP/HOP2* mutation that abolishes coactivation of estrogen-driven transcription. *Am J Hum Genet* 89:572–579 (2011).
- Zhao H, Qin Y, Kovanci E, Simpson JL, Chen ZJ, Rajkovic A: Analyses of *GDF9* mutation in 100 Chinese women with premature ovarian failure. *Fertil Steril* 88:1474–1476 (2007).
- Zhao H, Chen ZJ, Qin Y, Shi Y, Wang S, et al: Transcription factor *FIGLA* is mutated in patients with premature ovarian failure. *Am J Hum Genet* 82:1342–1348 (2008).