Mutation analysis of NANOS3 in Brazilian women with primary ovarian failure

Braian Lucas A. Sousa, Mirian Yumie Nishi, Mariza Gerdulo Santos, Vinicius Nahime Brito, Sorahia Domenice, Berenice B. Mendonca*

Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Disciplina de Endocrinologia, Unidade de Endocrinologia do Desenvolvimento, Laboratório de Hormônios e Genética Molecular LIM/42, São Paulo/SP, Brazil.

OBJECTIVES: Primary ovarian failure is a rare disorder, and approximately 90% of cases are of unknown etiology. The aim of this study was to search for mutations in NANOS3, a gene that was recently related to the etiology of primary ovarian failure, in a group of Brazilian women.

METHODS: We screened for NANOS3 DNA variants in 30 consecutive women who were previously diagnosed with primary ovarian failure, of unknown etiology and compared the results with those from 185 women with normal fertility. The NANOS3 gene was amplified by polymerase chain reaction using pairs of specific primers and then sequenced. The resulting sequences were compared with control sequences available in the National Center for Biotechnology and Information database.

RESULTS: No mutations in NANOS3 were found in primary ovarian failure patients, but four previously described polymorphisms were identified at a similar frequency in the control and primary ovarian failure groups.

CONCLUSIONS: Mutations in NANOS3 were not associated with primary ovarian failure in the present cohort.

KEYWORDS: Primary Amenorrhea; NANOS3; Gonadal Dysgenesis; Primary Ovarian Failure; Sexual Differentiation.

INTRODUCTION

Primary ovarian failure (POF), also known as premature ovarian failure, premature ovarian insufficiency, premature menopause, or hypergonadotropic hypogonadism, is defined as the loss of function of the ovaries before age 40 (1). POF is phenotypically characterized by the triad of primary or secondary amenorrhea, hypergonadotropic hypogonadism and hypoestrogenism.

There are several causes of POF, and the genetic causes are associated with a defect in ovarian development.

Sex determination in mammals is a dynamic and complex process that requires the interaction of multiple factors, and both primordial germ cells (PGCs) and somatic cells acquire sex-specific characteristics.

PGCs are essential for the differentiation and maintenance of the fetal ovary: when few germ cells enter the genital ridge during embryogenesis, the supporting lineage cells do not differentiate, generating strike ovaries (2-4). Because the differentiation of ovarian somatic cells relies on PGCs, genes involved in the differentiation and subsequent maintenance of PGCs are potential candidates for the etiology of 46,XX gonadal development disorders (5).

Nanos homolog 3 was identified in PGCs of Drosophila, and the NANOS3 protein has anti-apoptotic activity during the migration of PGCs toward the gonad (6). Male and female mice that do not express Nanos3 experience a complete loss of PGCs during gonadal development (7). The human NANOS3 gene is located on the short arm of chromosome 19 (region 13.13) and has two exons.

A preliminary study in a Brazilian population identified a NANOS3 c.385G>A homozygous mutation in two sisters with POF. In vitro analysis showed that the NANOS3 protein in these two patients had an impaired ability to prevent the apoptosis of PGCs, suggesting a possible etiology for the disorder (8).

Our objective was to search for inactivating NANOS3 mutations in an additional group of 30 women with POF without apparent cause.

MATERIALS AND METHODS

This was a cohort study performed in accordance with the ethical standards of the Ethics Committee of Hospital das Clínicas, University of São Paulo School of Medicine, Brazil (Protocol number 1226/07).
POF was defined as the loss of function of the ovaries before age 40 (1). We invited 30 consecutive patients with the diagnosis of POF of unknown etiology who were followed in the Developmental Endocrinology Unit of Hospital das Clínicas, São Paulo, Brazil, to undergo screening for NANO3 mutations. Thyroid, adrenal and ovarian autoimmune disorders, as well as mutations in FSHR, NR5A1, BMP15, GDF9, GATA4, DMRT1, FOXL2, LHCG, and STRA8 and premutation of FMR1, had been ruled out in all these patients. All the patients had a normal 46,XX karyotype obtained by analysis of 50 metaphases.

Hormonal tests required for the POF diagnosis were performed at the Laboratory of Hormones and Molecular Genetics, LIM/42 of the HCFMUSP, using immunofluorometric assays (Delfia, Turku, Finland).

Basal gonadotropin levels were elevated in all patients, with predominantly high serum FSH levels ranging from 32 to 111 U/L and serum LH levels ranging from 13 to 107 U/L. Serum estradiol levels were low or within the follicular phase range in all patients.

The control group was composed of 185 women aged 18 to 65 years, all of whom had normal menstrual cycles and no history of menopause before 40 years of age or treatment for subfertility. The control group was screened for the NANO3 allelic variants found in the POF group.

Genomic DNA was extracted from peripheral blood leucocytes using the salting out procedure (9). The DNA concentration and purity were determined using the NanoPhotometer (Implen GmbH, Schatzbogen, Germany), and visualization with ultraviolet light. The samples were subjected to direct automated sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Life Technologies Corporation, CA, USA).

The samples were subjected to direct automated sequencing using the ABI Prism Genetic Analyzer 3130XL (Life Technologies Corporation, CA, USA). The sequences obtained were compared to those in the National Center for Biotechnology Information database (NCBI http://www.ncbi.nlm.nih.gov/).

We performed in silico analysis of the identified variants using Mutation Taster (available online at http://www.mutationtaster.org).

Comparisons between unrelated patients with POF and controls were performed using contingency tables and $\chi^2$ analysis for each polymorphism in SigmaStat for Windows v.3.5. $p$ values $<0.05$ were considered statistically significant.

### RESULTS

NANO3 mutation screening in 30 unrelated women with POF of unknown etiology identified the rs897790, rs2016163, rs369192674 and rs371590850 variants in 18 patients.

The rs897790 variant is located in the 5‘ untranslated region of exon 1, where it caused the replacement of a cytosine with a thymine at position -23 (c.-23C>T); this variant was found in 6 patients in the heterozygous state (Figure 1). In the control group, the rs897790 variant was identified in 59 women, of whom 54 were heterozygous and 5 were homozygous.

The rs2016163 polymorphism is located in exon 1 and causes the substitution of an adenine with a guanine at

| Table 1 | Sequences of the primers used to amplify NANO3 exons. |
|---------|-----------------------------------------------------|
| Exon    | Primer sequence                                    |
| 1       | Forward 5’ CTG CTC CTC CCT CTG CAC AC 3’                |
|         | Reverse 5’ GTC TTC CCC TAA CCC TGG GA 3’              |
| 2       | Forward 5’ GTC AC 5’ GGG TCG CTG TCT 3’              |
|         | Reverse 5’ AGT GGG GCC AGT CGT CAT AG 3’              |

| Table 2 | PCR amplification protocol for the NANO3 gene. |
|---------|---------------------------------------------|
| Exon    | H2O (µl)          | SX Buffer (µl) | dNTP (mM) | Forward Primer (pmol) | Reverse Primer (pmol) | Taq DNA Polymerase (U) |
|---------|-------------------|----------------|-----------|-----------------------|-----------------------|------------------------|
| 1       | 16.3              | 5              | 0.2       | 10                    | 10                    | 0.75                   |
| 2       | 16.3              | 5              | 0.2       | 10                    | 10                    | 0.75                   |

dNTP: deoxynucleoside triphosphate; µl: microliters; mM: millimolar; pmol: picomoles; U: units.
position 354 (c.354A>G). This substitution does not change the amino acid sequence. Ten of the 30 patients harbored this polymorphism, one in the homozygous state and 9 in the heterozygous state (Figure 2). This variant was present in 70 individuals in the control group, of whom 58 were heterozygous and 12 were homozygous.

Six patients presented with both variants. These previously identified variants are reported in control databases, such as the 1000 Genomes Project, at a frequency of greater than 1%, which classifies them as polymorphisms.

Two patients harbored a GAG duplication at position 512-514 (c.512_514dupGAG) in the transcribed region of exon 1 (rs369192674). This trinucleotide duplication led to the addition of a glycine between positions p.171 and p.172 (p.Gly171dup) (Figure 3). In silico analysis of this variant classified it as not deleterious. This variant is described in the NCBI database, although its population frequency is not known. This variant was found in three women in the control group in the heterozygous state.

Another patient harbored a heterozygous insertion of 18 nucleotides between positions c.134 and c.135 (c.134_135insGCCGGAGCCGGTGTCAGC) in the transcribed region of exon 1 (rs371590850). This insertion leads to the addition of six amino acids between positions p.45 and p.46 (p.Ala45_Leu46insProGluProValSerAla) (Figure 4). In silico analysis classified this variant as not harmful. This variant is described in the NCBI database, although its population frequency is not known. In the control group, this variant was identified as heterozygous in 6 women.

The screening results for the rs897790, rs2016163, rs369192674 and rs371590850 variants in the control and POF groups are summarized in Table 3.

The allele distributions of the rs897790, rs2016163, rs369192674 and rs371590850 variants were not significantly different between the POF patients and the control group (p=0.271, p=0.787, p=0.295 and p=0.597, respectively) (Table 3).

We did not identify any deleterious NANOSS3 variants in the studied population.

**DISCUSSION**

In the present study, we screened for NANOSS3 mutations in a cohort of women with POF and identified only four previously described variants, which were detected at a similar frequency in the control group.

Although most POF cases are sporadic, 10-15% are familial, corroborating the hypothesis that this disorder has a genetic component. However, despite recent advances in molecular biology, approximately 90% of POF cases are of unknown etiology (1,10). The role of NANOSS3 in inhibiting the apoptosis of PGCs and thus contributing to their maintenance makes this protein a natural candidate for the etiology of POF in humans (11). Loss of NANOSS3 function may lead to PGC death: Nanos3 knockout mice do not have PGCs (7). Based on this finding, three groups used Sanger sequencing to screen women with POF for NANOSS3 mutations (8,12-14). Qin et al. studied samples from 80 Chinese

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**Table 3 - Distribution of NANOSS3 variants in the control and patient groups.**

| Allelic Variant | POF Group | Control Group | p# |
|----------------|-----------|---------------|----|
| rs897790       | 6*/30**   | 59*/185**     | 0.271 |
| rs2016163      | 10*/30**  | 70*/185**     | 0.787 |
| rs369192674    | 2*/30**   | 3*/185**      | 0.295 |
| rs371590850    | 1*/30**   | 6*/185**      | 0.597 |

POF: Premature Ovarian Failure.  
*: number of individuals with the variant; **: total number of individuals;  
#: Chi-square test; p < 0.05 indicated significance.
and 88 Caucasian women by denaturing high-performance liquid chromatography (DHPLC), followed by Sanger sequencing, and did not identify any causative mutations in NANOS3 (12). Wu et al. evaluated 100 Chinese patients with POF and reported a heterozygous p.Arg153Trp mutation in NANOS3 in a 23-year-old patient born from consanguineous parents. Transfection assays in HEK293 cells showed that the half-life of the mutant p.Arg153Trp protein was significantly reduced. Using an animal model, these researchers also showed that the PGC population was directly related to NANOS3 protein levels. Together, these two findings implicated the c.457C > T mutation (p.Arg153Trp) in the etiology of POF in this patient (13).

Finally, a previous study in a Brazilian cohort evaluated 85 patients with primary or secondary ovarian failure and reported the novel homozygous c.358G > A mutation (p.Glu120Lys) in NANOS3 in two sisters with POF. In vitro analysis showed a significant loss of PGC viability, and in silico modeling of mutant NANOS3 suggested that this amino acid change might destabilize the protein-RNA interaction. This evidence supports the hypothesis that the c.358G > A mutation, which increases the apoptosis rate of PGCs during the embryonic period, is the etiological factor for POF in these two patients (8).

Considering these three studies involving the index case of 343 families, the NANOS3 mutation frequency in POF is less than 0.5% (8,12-14).

Our study included 30 additional Brazilian women with POF, but no mutations were found in the NANOS3 gene. The four variants that were identified in the present study have already been described, but only the rs897790 and rs2016163 variants were previously reported in POF women (8,12,13). The rs2016163 variant was also identified in normal women by Qin et al. (12). No significant difference was observed in the allele frequencies of the rs897790, rs2016163, rs369192674 and rs371590850 variants between POF patients and the control group. Our negative results should be interpreted cautiously due to the small number of patients; POF is a rare disorder. In addition, only the NANOS3 coding regions and exon boundaries, not the intronic and regulatory regions, were sequenced.

In conclusion, despite the prominent role of NANOS3 in ovarian development, our findings suggest that NANOS3 mutations were not associated with POF in the present cohort.

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■ AUTHOR CONTRIBUTIONS

Sousa BL, Santos MG and Nishi MY performed and analyzed the experimental assays. Brito VN performed the statistical analysis. Sousa BL was responsible for writing the manuscript. Domenice S followed and selected the patients. Mendonça BB designed the project, supervised the study execution and wrote the manuscript.

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