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Mutation Analysis of NANOS3 in 80 Chinese and 88 Caucasian Women with Premature Ovarian Failure

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Abstract

NANOS3 encodes an RNA binding protein and has a conserved function in germ cell development. Our objective was to investigate whether mutations in *NANOS3* were present in Chinese and Caucasian women with premature ovarian failure (POF). A known synonymous single nucleotide polymorphism (SNP) (rs 2016163) in exon 1 was identified through sequencing 80 Chinese and 88 Caucasian women with POF. No additional SNPs or mutations were found in exons encoding for *NANOS3*. Our findings suggest that mutations in *NANOS3* exons are rare in both Chinese and Caucasian women with POF.

Keywords

Premature Ovarian Failure; Mutation; NANOS3; DHPLC

Premature ovarian failure (POF) is characterized by secondary amenorrhea, infertility, hypoestrogenism and elevated gonadotropins (FSH>40 U/L). Affecting 1–2% of women under 40 years of age, POF is heterogeneous in etiology (1). Mechanisms involved in its pathogenesis include chromosomal abnormalities of the X or autosomes, autoimmune disorders, environmental toxins and iatrogenic causes (2). POF may be heritable in up to 30% of women with POF (3), and is predicted to be a complex genetic disorder. No single mutation appears responsible for more that 10% of cases (4). Premutation of the FMR1 gene is associated with some cases (5). Other cases are accounted for by mutations in genes preferentially expressed in the ovaries, such as FSHR (6), POF1B (7), FOXL2 (8) and BMP15 (9), and functional data verify that these genes are causative. Heterozygous missense mutations and polymorphisms

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Capsule A single nucleotide polymorphism of NANOS3 was found among women with premature ovarian failure. Our results indicate mutations in NANOS3 are rare in both Chinese and Caucasian women with POF.

have also been described for GDF9 (10), FOXO3A (11), FOXO1A (11), and INHA (12), however, functional data corroborating adverse role for these variations is lacking. Other genes preferentially expressed in the ovaries are thus likely to be involved in the development of POF.

NANOS3 encodes an RNA binding protein and has been recently proposed as a candidate gene for POF (13). *NANOS3* was first identified as a maternal effect gene in Drosophila and has a conserved function in germ cell development. Male and female mice deficient in *Nanos3* are infertile. *NANOS3* knockout female mice have no other known phenotype and represent a model for non- syndromic ovarian failure. Human *NANOS3*, encoded by two exons and preferentially expressed in germ cells, has a high degree of homology to the mouse.

Several molecular surveys of POF studies have been reported in different populations, but very few have been conducted in Chinese women. In fact no candidate gene has been positively correlated with POF in Chinese patients (14,15). The objective of the present study was to determine the involvement of *NANOS3* variations in both Chinese and Caucasian POF populations.

Our study subjects comprised a cohort of 80 POF patients from Jinan, Shandong Province, China and 88 from American Caucasians. Ages of the women at presentation ranged from 14 to 39 years old. Recruitment criteria were defined as menopause occurring before 40 years with at least two serum follicle stimulating hormone (FSH) concentrations that exceeded 40 IU/L. Women with chromosomal abnormalities were excluded. Sixty-three normal control samples were collected from each of the general populations of Chinese and Caucasian. Informed consent for molecular studies was obtained from all subjects. The study was approved by the Ethics Committees of Baylor College of Medicine and Shandong University.

Peripheral blood was obtained for genomic DNA. The two exons and exon- intron boundaries of *NANOS3* gene were amplified using an optimized polymerase chain reaction (PCR) protocol. Detailed information of the primer sequences and PCR conditions are available upon request. PCR products were denatured and reannealed to form potential heteroduplexes (wild-type strand paired with mutant strand). PCR products were mixed pair wise before annealing to enhance heteroduplex formation. Heteroduplexes were detected using denaturing high-performance liquid chromatography (DHPLC) on the WAVE System 3500 (Transgenomic Ltd, Omaha, NE) and interpreted as indicative of a mismatch in the analyzed PCR fragment. Samples with heteroduplex formation on DHPLC were then sequenced directly after PCR amplification on an automated sequencer, ABI PRISM 310 (Applied Biosystems, Foster City, CA).

We analyzed the two exons that comprise *NANOS3* gene for mutations and variations. Of 168 POF samples, 66 subjects showed abnormal DHPLC formations. DNA sequencing revealed a nucleotide substitution of A to G at position 353 (c.353 A>G) that accounted for all the abnormal DHPLC. This substitution did not alter the amino acid sequence and has been reported in the NCBI single nucleotide polymorphism (SNP) database as a synonymous SNP (rs2016163). The other 102 samples showed normal homoduplexes on DHPLC.

Samples with abnormal DHPLC patterns were interrogated further utilizing an optimized PCR-restriction fragment length polymorphism (RFLP) protocol based on c.353 A>G creating a restriction enzyme site recognized by MluI. Of the 66 POF cases showing abnormal DHPLC, we confirmed that 47 cases were heterozygous and 19 were homozygous at position 353 (Table 1). No additional SNPs or mutations were identified in the coding exons of *NANOS3*. Search of the NCBI SNP Database revealed known population diversity for this SNP (rs2016163) (c. 353 A>G) (Table 1). Comparison of genotype and allelic frequencies between the general

population and POF cases showed significant difference in the frequency of rs2016163in Caucasians (Chi square, $p<0.05$), however, the clinical meaning of this difference is not clear.

The current study is the first to investigate the role of human *NANOS3* gene in 46, XX women with premature ovarian failure. Although *NANOS3* is an excellent candidate gene for non-syndromic POF, our results indicate that mutations in the coding exons of *NANOS3* gene are not common in either Chinese or Caucasian women with POF. The clinical significance of being heterozygous for the rs2016163 SNP in the Caucasian population is unclear, and future functional studies are necessary to determine its effect, if any, on ovarian function.

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Table 1

Genotype Frequencies of SNP rs2016163 in *NANOS3*

	Wild-type AA	Homozygote GG	Heterozygote A/G
General Population			
European Caucasian[a]	0.533	0.033	0.434
Japanese[a]	0.545	0.159	0.396
Chinese (Han Chinese)[a]	0.533	0.089	0.379
American Caucasian[b*]	0.54(34/63)	0.0317(2/63)	0.429(27/63)
Premature Ovarian Failure			
Chinese (Han Chinese)[b]	0.438(35/80)	0.137 (11/80)	0.425(34/80)
American Caucasian[b*]	0.761(67/88)	0.091(8/88)	0.148(13/88)

Note:

[a] International HapMap Project Database (CSHL- HAPMAP)

[b] Present study

[*] Caucasian alleles between POF and normals show significant difference with p less than 0.05