Growth differentiating factor-9 mutations may be associated with premature ovarian failure

Ertug Kovanci, M.D.,[a] *Jan Rohozinski, Ph.D.,*[a,b] *Joe Leigh Simpson, M.D.,*[a,b] *Michael J. Heard, M.D.,*[c] *Colin E. Bishop, Ph.D.,*[a,b] *and Sandra A. Carson, M.D.*[a]

[a] Department of Obstetrics and Gynecology and [b] Department of Molecular and Human Genetics, Baylor College of Medicine, and [c] The Women's Specialists of Houston, Houston, Texas

Objective: To determine whether perturbations of the growth differentiating factor-9 (*GDF9*) gene are associated with premature ovarian failure (POF).
Design: Mutational analysis of the *GDF9* gene in 61 women with POF.
Setting: Academic institution.
Patient(s): Sixty-one women with POF; 60 control women.
Intervention(s): Peripheral blood sampling, genomic DNA extraction, mutational screening, and DNA sequencing.
Main Outcome Measure(s): Genetic perturbations in *GDF9* that are associated with POF.
Result(s): A single missense mutation, substitution of a cytosine residue with thymidine in exon 1 of *GDF9*, was found in a white woman in whom POF developed at age 22. This mutation occurred in a highly conserved proprotein region and resulted in replacement of a nonpolar amino acid (proline) with a polar amino acid (serine) at position 103. Neither 60 control women nor 60 other women with POF demonstrated this genetic perturbation. Exon 2 showed only previously recognized single nucleotide polymorphisms.
Conclusion(s): *GDF9* mutations may be one explanation for POF, albeit uncommon. (Fertil Steril® 2007;87: 143–6. ©2007 by American Society for Reproductive Medicine.)

Key Words: Premature ovarian failure, growth differentiating factor-9, mutation, single nucleotide polymorphism

Premature ovarian failure (POF) affects approximately 1% of women (1). Multiple causes explain this relatively common condition. Problems may arise either during fetal development and maturation or with the normal menstrual cycle. Thus, many different genes are involved in normal follicular development and fertility. The role of chromosomal abnormalities that involve the X chromosome and autosomes has long been accepted (2). Very few women with monosomy X menstruate spontaneously, because of increased germ cell attrition. Deletions of both the short and long arm of the X chromosome may also cause POF. Phenotypes of women with X chromosome deletions range from primary amenorrhea with short stature and somatic anomalies to isolated secondary amenorrhea (2, 3). Autosomal chromosomal rearrangements and X/autosome translocations also cause POF.

The role of single X-linked and autosomal genes recently has become the focus of investigations. Perturbations of autosomal genes have been postulated to cause POF based on known involvement in human ovarian development and function and on the basis of null phenotypes in mice. Growth differentiating factor-9 (*GDF9*) is one of the genes inferred from mouse models (4).

GDF9 is a member of the transforming growth factor-β (TGF-β) family. Located on chromosome 5 (5q31.1), this gene consists of 2 exons encoding a 454 amino acid peptide. It is translated as a preproprotein composed of 3 subunits: the signal peptide, pro-domain, and mature region. This arrangement is similar to that of other members of the TGF-β family (5). Another prominent member of this family is bone morphogenetic protein-15 (*BMP15*), perturbations of which are known to be associated with POF in sheep and humans (6, 7). The *BMP15* and *GDF9* gene products can form heterodimers (8).

GDF9 is expressed specifically in oocytes and plays an essential role in both early and late folliculogenesis. The GDF9 protein in culture media promotes cumulus expansion in cumulus cell-oocyte complexes, whereas its suppression by RNA interference (double-stranded interfering RNA) prevents cumulus expansion (9, 10). Long-term immunization against GDF9 in sheep disrupts early folliculogenesis and leads to the absence of normal follicles beyond the primary stage of development (11). In addition, a naturally occurring mutation in this gene causes infertility in sheep (12).

In 2005, Dixit et al. (13) studied 127 Indian women with POF (onset before 35 years), of whom 4 women had the missense mutation A199C and 2 women had the G646A mutation. Another woman who had secondary amenorrhea,

Received February 24, 2006; revised and accepted May 18, 2006.
Supported in part by National Institutes of Health grants K12-HD01426 and K12-HD050128.
Presented in part at the 61st Annual Meeting of the American Society for Reproductive Medicine, Montreal, Canada, October 15–19, 2005.
The current address for Jan Rohozinski and Colin E. Bishop is The Wake Forest Institute for Regenerative Medicine, Winston Salem, NC 27157.
Reprint requests: Joe Leigh Simpson, M.D., Baylor College of Medicine, 1709 Dryden Road, Suite 1100, Houston, TX 77030 (FAX: 713-798-8410; E-mail: jsimpson@bcm.edu).

defined as cessation of menses after age 35 years but before age 40 years, also had the A199C mutation. All A199C and G646A mutations were heterozygous and occurred in the proprotein region. In aggregate, these findings make *GDF9* a strong candidate for POF.

The goal of this study was to determine whether perturbations of the *GDF9* gene were associated with POF in a different population.

MATERIAL AND METHODS

Study Population

Since 2001, DNA samples from 61 women with idiopathic POF have been collected at Baylor College of Medicine. These were ascertained from different geographic regions of the United States. *Idiopathic POF* was defined as cessation of menses before age 40 years, with measurements of serum follicle-stimulating hormone concentrations of >20 IU/mL on 2 occasions and absence of recognized chromosomal abnormalities.

Of these 61 women, 49 women were white (80%), and 12 of the women were of other ethnicity (20%). Our control group comprised 60 women with no evidence of POF. Of the control group, 34 women were white (57%); 16 women were black (27%), and 10 women were of other ethnicity (16%). The control group displayed normal menarche, normal menstrual cycles, and no clinical evidence of infertility.

DNA Analysis

Genomic DNA was extracted from peripheral blood samples collected in 5-mL ethylenediaminetetraacetic acid vacutainers with a DNA purification kit (Puregene; Gentra Systems, Minneapolis, MN). Both exons of the *GDF9* gene were amplified with the use of the polymerase chain reaction with exon specific primers (Table 1).

Denaturing high-performance liquid chromatography was used as an initial screen for genetic variations. Heteroduplexes that are formed by a wild-type strand annealing with a genetically different strand are detected by an ultraviolet detector on the WAVE System 3500 (Transgenomic Ltd., Omaha, NE). Sensitivity and specificity of this method approaches 100% for single or multiple base-pair substitutions, deletions, and insertions (14). Samples that demonstrate heteroduplex formation on denaturing high-performance liquid chromatography were then sequenced directly on the automated sequencer (ABI Prism 310; Applied Biosystems, Foster City, CA) with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems).

The study was approved by the Baylor College of Medicine Institutional Review Board.

RESULTS

Exon 1

A single white woman, in whom POF manifested at age 22 years, showed a novel heteroduplex formation for a polymerase chain reaction product transcribed from exon 1 and detected by denaturing high-performance liquid chromatography. No heteroduplexes of *GDF9* exon 1 were detected in the other 60 POF cases or in the 60 control women.

In the single subject who showed the perturbation, DNA sequencing revealed heterozygous substitution of the cytosine residue with a thymidine at position 307 (Fig. 1). This perturbation resulted in substitution of a nonpolar amino acid (proline) with a polar amino acid (serine) at position 103, in the proprotein region that is highly conserved across species (Fig. 2).

The 46,XX proband was 162-cm tall and had no autoimmune disorders. Somatic abnormalities were not evident, specifically neither skeletal anomalies nor hearing deficits. No other family members had POF. She had 2 male siblings. One sibling was healthy at age 31 years and had no offspring; the other sibling had died of a hematologic disorder at 1 month of age. Her mother died of lymphoma at the age of 53 years. Her father is alive and healthy at age 60 years.

Exon 2

Analysis of exon 2 demonstrated heteroduplex formation in 44 of the 61 women with POF (72.1%). Three known single nucleotide polymorphisms (SNPs) accounted for all cases. Two of these SNPs, C447T and G546A, do not result in an amino acid substitution and thus are silent (SNP database ID numbers rs254286 and rs10491279). The third (G to C) occurs 38 base-pairs upstream from exon 2 (ID number

TABLE 1

Primers used to amplify both exons of the *GDF9* gene.

Fragment	Size (base pair)	Forward primer	Reverse primer
Exon 1	461	TTCCTCACTAGTTCTCCCAAGC	CATCTTCCCTCCACCCAGT
Exon 2, first part	491	TTGACTTGACTGCCTGTTGTG	AGCCTGAGCACTTGTGTCATT
Exon 2, second part	686	ATGAAAGACCAGCTGGAGCA	TTTGCCAAATAGGCTCAAGG

Kovanci. GDF9 *missense mutation in POF. Fertil Steril 2007.*