Novel zona pellucida gene variants identified in patients with oocyte anomalies

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Objective: To detect *ZP* (zona pellucida) gene (*ZP1–ZP4*) mutations in patients with oocyte anomalies.
Design: Case-control genetic study.
Setting: University-based reproductive medicine center.
Patient(s): A total of 92 infertile patients with repeated cycles of oocyte maturation arrest (group I, n = 49) or oocyte morphologic defect (group II, n = 43) as well as 373 healthy controls.
Intervention(s): Genomic DNA extracted from peripheral blood and coding regions of *ZP* genes amplified by polymerase chain reaction and sequenced by a DNA analyzer.
Main Outcome Measure(s): Variant prediction of *ZP* genes with software.
Result(s): In group I with oocyte maturation arrest, no novel variants were found. In group II with oocyte morphologic defects, four novel variants, two in the *ZP1* gene [c.247T>C (p.W83R) and c.1413G>A (p.W471X)] and two in the *ZP2* gene [c.1599G>T (p.R533S) and c.1696T>C (p.C566R)] were detected in 4 of 43 patients (approximately 9%) but were absent from the controls. Protein alignments showed that the four variants were highly conserved among different species, and all four variants were predicted to be deleterious by gene software predictions.
Conclusion(s): *ZP* gene variants may account for patients with oocyte morphologic abnormalities but not for those with oocyte maturation arrest. (Fertil Steril® 2017;107:1364–9. ©2017 by American Society for Reproductive Medicine.)
Key Words: Genetic variants, infertility, oocyte maturation, zona pellucida

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It is generally known that a mature oocyte is a prerequisite for successful fertilization, the growth and development of embryos, and infertility treatment. Oocyte maturation, which refers to the completion of the first meiotic division and the accompanying processes essential for subsequent fertilization and embryo development, consists of two general components: nuclear maturation and cytoplasmic maturation (1–3). Morphologic evaluation of oocytes retrieved from ovarian mature follicles after ovulation induction for in vitro fertilization (IVF) treatment is a critical task. Mature oocytes exhibit a single first polar body, clear cytoplasm of homogeneous texture, a zona pellucida (ZP) with appropriate shape and thickness, and a proper perivitelline space (PVS) (1). In this context, the maturation of an oocyte reflects its intrinsic developmental competence and is a key factor in female fertility.

During the course of oocyte maturation, *ZP* genes, which code for the ZP glycoproteins, are expressed exclusively in growing and fully grown oocytes (4). The ZP, an extracellular, transparent, glycoproteinaceous matrix, is crucial for oogenesis, fertilization, and development of preimplantation

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embryos (5, 6). It is first observed from primary follicles and increases in thickness concomitantly with oocyte diameter increase. The width of the ZP is about 3 μm in secondary follicles, and it increases up to 7 μm in early antral follicles (7). During the growth of mouse oocytes, the absolute rate of protein synthesis increases about 40-fold, and the number of copies of *ZP* messenger RNAs (mRNAs) increases dramatically (6). The expression of *ZP* genes is postulated to be a potential marker of oocyte growth and maturation.

In mice, the ZP matrix is composed of three glycoproteins that are synthesized and secreted solely by growing oocytes, including ZP1, ZP2, and ZP3 (5, 6). Among them, ZP1 is regarded as an important component conductive to the integrity of the ZP structure (8); ZP3 serves as the primary sperm receptor in charge of intact sperm binding and induces an acrosome reaction (7, 9); and ZP2 acts as the secondary sperm receptor and contributes to the binding of an acrosome-reacted spermatozoon to egg ZP (10). The momentous functions of the ZP matrix during oocyte growth and fertilization have been further demonstrated by knockout mice. In *Zp1* knockout oocytes, the ZP is thinner than normal, with an obscure boundary and obvious PVS. The fecundity of *Zp1* null mice is lower than normal (11). Mice with a homozygous null mutant of *Zp2* or *Zp3* are infertile but could possess oocytes that lack a ZP (12, 13). Abnormal organization in the ZP may result in abnormal oocyte–cumulus cell interactions, thereby influencing oogenesis and oocyte maturation.

In humans, the ZP consists of four glycoproteins: ZP1–ZP4 (14, 15). The ZP plays an essential part in species recognition as well as induction of the sperm acrosome reaction during fertilization. It also prevents polyspermy after fertilization and protects early embryos until implantation (16). The ZP also could serve as an important morphologic predictor of oocyte maturation. Indeed, the thickness, shape, and birefringence of the ZP have been found to be connected with pregnancy outcomes (17–19).

A mode of autosomal recessive inheritance with a homozygous deletion in the *ZP1* gene was detected in a Chinese family with fertility problems; certain female members of the family possessed abnormal oocytes that lacked of a ZP (20). In a Finnish study, women with total fertilization failure were screened for *ZP* gene mutations and were compared with two control groups: women with at least one fertilized embryo via IVF and women with proven fertility (WPFs). Two variations in *ZP3*, c.1–87T>G and c.894G>A, were found at a higher frequency (33% and 50%, respectively) in the women with total fertilization failure as compared with the women with fertilized embryos (13% and 0, respectively) and the women with proven fertility (9% and 24%, respectively) (21). Another study also in Finland, suggested that variations in *ZP* genes, particularly in *ZP2* (c.116T>G, 39%) and *ZP3* (c.1–87T>G, 32%), may explain common ZP anomalies such as thin ZP (22).

To our knowledge, sequencing of the *ZP1–4* genes has rarely been reported in sporadic patients with repeated cycles of oocyte maturation arrest or morphologic defects, especially in Han Chinese women. Our assumption is that the ZP is connected to oocyte maturation, and we examined variations in the *ZP* genes that may be implicated in oocyte maturation arrest or oocyte morphologic defects.

MATERIALS AND METHODS

Patients

This study consisted of 92 Han Chinese women undergoing IVF and/or ICSI (intracytoplasmic sperm injection) treatment, with repeated cycles featuring oocyte maturation arrest (group I, n = 49) or oocyte morphologic defects (group II, n = 43). Human oocyte maturation arrest occurred in different phases of the cell-division cycle including germinal vesicle arrest, metaphase I arrest, or a combination of both. The oocyte morphologic parameters mainly comprised the polar body, cytoplasm, PVS, and ZP. A total of 373 Han Chinese women undergoing IVF/ICSI treatment to treat male factor infertility who had previously given birth to at least one healthy child were recruited as the controls. All patients were ≤40 years of age, and their blood samples were collected from the Center for Reproductive Medicine, Shandong University. The study was approved by the reproductive medicine institutional review board of Shandong University. All participants provided written, informed consent.

Variation Screening of *ZP* Genes

Genomic DNA was extracted from blood by the use of QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer's instructions. Afterward, the exons of the *ZP1–ZP4* genes (*ZP1*: NM_207341.3; *ZP2*: NM_003460.2; *ZP3*: NM_001110354.1; *ZP4*: NM_021186.3) were amplified by polymerase chain reaction. Similar to other studies (22–24), we analyzed the first four exons of *ZP3* to avoid false interpretation of the sequencing results by reason of a polymorphic locus, *POM-ZP3* (25). The polymerase chain reaction conditions were as follows: initial denaturation at 95°C for 5 minutes; then denaturation at 95°C for 30 seconds, annealing at 58°C or 60°C for 30 seconds, and extension at 72°C at 45 seconds for 35 cycles; finally, extension at 72°C for 7 minutes. The polymerase chain reaction products were sequenced by an automatic sequencer ABI 3730XL DNA analyzer (Applied Biosystems) after initial analysis by agarose gel electrophoresis and purification twice. The exons and exon-intron boundaries of the four *ZP* genes were analyzed with Sequencher 4.9 (Gene Codes Corporation). All novel variants were verified at least three times by forward and reverse sequencing. Clustal Omega (Science Foundation Irelan) was used to perform protein sequence alignments of ZP1 and ZP2 among different species. The possible functional effects of novel variants were assessed with the following: Polymorphism Phenotyping v2 (Polyphen-2: http://genetics.bwh.harvard.edu/pph2/), a tool to predict the possible impact of an amino acid substitution on the stability and function of human proteins; SNPs&GO (http://snps-and-go.biocomp.unibo.it/), a server for the prediction of human disease-related mutations; and Sorting Intolerant From Tolerant (SIFT: http://sift.jcvi.org/), software to predict whether an amino acid substitution is deleterious to protein function.