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ORIGINAL ARTICLE

Male Infertility

Novel *PLCZ1* mutation caused polyspermy during *in vitro* fertilization

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Failure of oocyte activation, including polyspermy and defects in pronuclear (PN) formation, triggers early embryonic developmental arrest. Many studies have shown that phospholipase C zeta 1 (*PLCZ1*) mutations cause failure of PN formation following intracytoplasmic sperm injection (ICSI); however, whether *PLCZ1* mutation is associated with polyspermy during *in vitro* fertilization (IVF) remains unknown. Whole-exome sequencing (WES) was performed to identify candidate mutations in couples with primary infertility. Sanger sequencing was used to validate the mutations. Multiple *PLCZ1*-mutated sperm were injected into human and mouse oocytes to explore whether PN formation was induced. Assisted oocyte activation (AOA) after ICSI was performed to overcome the failure of oocyte activation. We identified three *PLCZ1* mutations in three patients who experienced polyspermy during IVF cycles, including a novel missense mutation c.1154C>T, p.R385Q. PN formation failure was observed during the ICSI cycle. However, injection of multiple *PLCZ1*-mutated sperm induced PN formation, suggesting that the Ca^{2+} oscillations induced by the sperm exceeded the necessary threshold for PN formation. AOA after ICSI enabled normal fertilization, and all patients achieved successful pregnancies. These findings expand the mutational spectrum of *PLCZ1* and suggest an important role for PLCZ1 in terms of blocking polyspermy. Furthermore, this study may benefit genetic diagnoses in cases of abnormal fertilization and provide potential appropriate therapeutic measures for these patients with sperm-derived polyspermy.

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INTRODUCTION

During fertilization, sperm entry induces oscillations in the levels of calcium ions (Ca^{2+}), and the oocyte Ca^{2+} concentration transiently increases. These oscillations trigger oocyte activation, including the blocking of polyspermy, followed by pronuclear (PN) formation.[1–4] Inadequate sperm-induced Ca^{2+} oscillations cannot block the entry of multiple sperm. Polyspermy during *in vitro* fertilization (IVF) usually triggers early embryonic developmental arrest.

Phospholipase C zeta 1 (PLCZ1) is a sperm-specific protein that enters oocytes during fertilization. In somatic cells, PLCZ1 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP3), in turn triggering Ca^{2+} release from the endoplasmic reticulum.[5–7] Microinjection of the recombinant protein revealed that PLCZ1 alone caused Ca^{2+} oscillations in oocytes. Therefore, a deficiency of sperm PLCZ1 would prevent oocyte activation because of inadequate sperm-induced Ca^{2+} oscillations. In 2012, Kashir *et al.*[8] identified compound heterozygous *PLCZ1* mutations in an infertile man diagnosed with total fertilization failure (TFF) after intracytoplasmic sperm injection (ICSI) and revealed that inadequate Ca^{2+} oscillations were the primary cause of the condition. Since then, many studies have confirmed that more than 20 pathogenic mutations in *PLCZ1* cause TFF.[9–15] Artificial oocyte activation (AOA) uses calcium ionophores to trigger Ca^{2+} increases and effectively rescues TFF.[16] In addition to TFF, recent studies on *Plcz1*-knockout mice reported a high incidence of polyspermy after IVF.[17,18] However, few clinical studies have yet demonstrated an association between *PLCZ1* mutation and polyspermy in humans.

Here, *PLCZ1* mutations in males who experienced polyspermy during IVF cycles were identified. Mutations were identified in three different individuals. Two individuals were from the same family, and had compound heterozygous mutations, the novel missense mutation c.1154C>T, and the previously reported frameshift mutation c.1234del;[10] the third individual had the homozygous mutation c.1733T>C. PN formation failed in all patients during the ICSI cycles. We also explored whether PN formation could be induced and performed ICSI-AOA treatment to overcome the failure of oocyte activation.

PARTICIPANTS AND METHODS

Ethical approval

This study was approved by the Institutional Review Board of the Chongqing Health Center for Women and Children (Chongqing, China; Approval No. 2020-RGI-04). We followed the guiding principles of the Ministry of Science and Technology (MOST) in regard to human genetic resources. All samples were collected after the participants gave

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written informed consent to the Center for Reproductive Medicine at the Chongqing Health Center for Women and Children.

Participants

Based on the clinical manifestations of polyspermy and abnormal pronuclear formation, a total of 38 couples with primary infertility were recruited by the Center for Reproductive Medicine at the Chongqing Health Center for Women and Children (Chongqing, China). Genomic DNA was extracted from these affected couples for whole-exome sequencing (WES). Of these, 9 couples experienced polyspermy at least once during IVF cycles. Semen analyses, reproductive hormone levels, and assisted reproductive technology (ART) outcomes were collected. Genetic counseling was given to all patients from whom informed consent was obtained. Genetic testing followed the dictates of the Helsinki Declaration.

WES and Sanger sequencing

Genomic DNA from blood was collected from affected couples with primary infertility. After fragmentation, connection, amplification, and purification, the DNA libraries were subjected to hybridization capture. The exonic and collateral intronic (20 bp) regions of 20 099 genes were screened via high-throughput sequencing and the sequences were aligned to the reference dataset of the human genome assembly GRCh37/hg19. All identified mutations were annotated using dbSNP, 1000 Genomes, and gnomAD data. Functional annotations were performed using the prediction tools of Mutation Taster, PolyPhen-2, SIFT, ROVEAN, GeneSplicer, and SpliceAI16. Meanwhile, the Integrative Genomics Viewer (IGV) software version 2.16.2 (https://igv.org/; last accessed on 8 Nov 2023) was used to observe the candidate variant sites manually. Candidate variants in probands and their family members were confirmed via Sanger sequencing.

Protein molecular modeling and structural analysis

Three-dimensional (3D) models of the PLCZ1 wild-type (WT) and mutant proteins were generated based on the reference template in the Protein Data Bank using the homology modeling software SWISS-MODEL.[19] Structural analysis and the effects of residue interactions on protein function were analyzed and visualized using PyMOL software version 2.3.4 (https://pymol.org/2/, last accessed on 13 May 2022).

Vector construction, cell culture, and transfection

The WT human *PLCZ1* and a novel mutant *PLCZ1* (p.R385Q) were synthesized and cloned into the pcDNA3.1 vector with an N-terminal FLAG-tag using services provided by GenScript Corp. (Nanjing, China). HEK-293T cells obtained from the American Tissue Culture and Collection (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin solution at 37°C in 5% CO_2. *PLCZ1* WT and mutant plasmids were transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA).

Western blotting analysis

WT sperm from a man with normal fertility and *PLCZ1*-mutated sperm from patient II-2 from family 1 were lysed by RIPA cell lysis buffer (Beyotime, Shanghai, China), and lysates were used for protein quantification with a BCA Protein Assay (Thermo Fisher Scientific). Forty micrograms of total proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 for 1 h at room temperature. Then, they were incubated overnight at 4°C with the following primary antibodies: anti-β-actin (GB11001; Servicebio, Wuhan, China), anti-FLAG-PLCZ1 (AF519; Beyotime), and anti-PLCZ1 (A65778; Epigentek, New York, NY, USA). After incubation with the appropriate secondary antibodies for 1 h at room temperature, the immune complexes were detected by enhanced chemiluminescence (PE0010; Solarbio, Nanjing, China).

Immunofluorescence assay

Spermatozoa from patient II-2 of family 1 were washed three times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min at room temperature. PLCZ1 localization was detected using an anti-PLCZ1 (A65778; Epigentek) antibody. After labeling with Hoechst 33342 (C1011; Beyotime) to visualize nuclei, immunofluorescence images were captured by a Leica SP8 Laser Scanning Confocal Microscope (Leica, Heidelberg, Germany).

In vitro maturation and ICSI of human oocytes

To perform *in vitro* maturation (IVM), germinal vesicle (GV) oocytes were voluntarily donated by the patients and cultured in G-1-plus medium (Vitrolife, Gothenburg, Sweden) at 37°C under 6% CO_2 (*v*/*v*) and 5% O_2 (*v*/*v*) for 24 h. *PLCZ1*-mutated sperm with normal morphology were collected from patient II-1 of family 2, who carried a homozygous p.M578T mutation. WT sperm from a fertile male patient was recruited as a control. ICSI was performed using a micromanipulation system (CellTram 4r; Eppendorf, Hamburg, Germany) under an inverted microscope (Olympus IX70; Olympus Optical Co. Ltd., Tokyo, Japan). During ICSI, oocytes were placed in pre-equilibrated culture droplets and covered with 6 ml mineral oil (Ovoil; Vitrolife). Each oocyte was positioned using a holding pipette. When the first polar body attained the 6- or 12-o'clock position, single and 6–9 *PLCZ1*-mutated sperm were injected via a micropipette into the cytoplasm, respectively.

AOA

After ICSI manipulation for 1 h, metaphase II (MII) oocytes were artificially activated by exposure to 10 μmol l^{-1} calcium ionophore solution (A23187; Sigma, St. Louis, MO, USA) for 10 min at 37°C under 6% CO_2 (*v*/*v*) and 5% O_2 (*v*/*v*), thoroughly washed in fresh culture medium, and cultured in G-1-plus medium. The qualities of zygotes and embryos were evaluated using the European Society of Human Reproduction and Embryology (ESHRE) consensus guidelines.[20]

Animals

All procedures strictly followed the 1988 guidelines of the State Scientific and Technological Commission of China for the use of laboratory animals. All protocols were approved by the Ethics Committee of the Chongqing Health Center for Women and Children (Approval No. 2022034). The Institute of Cancer Research (ICR) female mice (8 weeks old) were purchased from Charles River (Beijing, China) and kept under controlled temperature (20°C–23°C) and illumination (12 h light/dark cycle) conditions with *ad libitum* access to water and food. Mice were sacrificed via cervical dislocation and treated humanely. GV oocytes from superovulated mice were collected by cutting the ovaries with a clean surgical blade. After *in vitro* maturation, WT sperm from a man with normal fertility and mutated sperm from patient II-1 of family 2 were injected into the cytoplasm.

RESULTS

Clinical characteristics of patients

Candidate *PLCZ1* variants were identified in three different couples from two families who had experienced at least one episode of polyspermy after IVF. All patients had normal sperm counts, morphologies, and motilities (**Supplementary Table 1**). Their female partners exhibited normal ovarian reserve functions. During IVF cycles, the percentages of polyspermy ranged

from 50% to 100% (**Table 1**). Patient II-1 from family 1 yielded four MII oocytes; two failed to form PN, and the others formed one 3PN and one 5PN zygote after IVF. Patient II-2 from family 1 experienced one failed IVF cycle and one failed ICSI cycle. In all, thirteen MII oocytes were retrieved that formed four 5PN zygotes, and eight were retrieved that formed >6PN zygotes during the first IVF cycle. In the second ICSI cycle, nine MII oocytes were retrieved, but all failed to form PN (**Supplementary Movie 1**). Patient II-1 from family 2 yielded nineteen retrieved MII oocytes during the IVF cycle, but all were polyspermic, thus giving rise to fourteen 4PN zygotes, four 7PN zygotes, and one 8PN zygote (**Table 1**).

Identification of candidate variants in PLCZ1

To explore the cause of polyspermy, we performed WES and analysis. After stringent filtering according to the filter criteria of WES variants, as shown in **Supplementary Figure 1**, *PLCZ1* (highlighted) was the only gene reported to be associated with fertilization failure and expressed in the testis. Pathogenic variants in transducin-like enhancer of split 6 (*TLE6*) and wee1-like protein kinase 2 (*WEE2*) were not detected in the women (**Supplementary Table 2**). Patients II-1 and II-2 of family 1 were brothers and exhibited compound heterozygous mutations of a frameshift mutation (c.1234del, p.R412Efs*15) and a missense mutation (c.1154C>T, p.R385Q; **Figure 1a**). The novel c.1154C>T, p.R385Q mutation was inherited from their mother (**Figure 1b**). Patient II-1 from family 2 had a homozygous missense mutation (c.1733T>C, p.M578T), which caused TFF after ICSI.[12] IGV screenshots depicting analysis for these loci are shown in **Supplementary Figure 2**. All mutations were verified by Sanger sequencing. The allele frequencies of p.R385Q, p.R412Efs*15, and p.M578T in the gnomAD database were 0.00007 (19/282 170), 0.00001 (3/274 280), and 0.00002 (5/281 390), respectively (**Table 2**). PolyPhen-2 and Mutation Taster predicted that the two missense mutations, p.R385Q and p.M578T, were potentially deleterious. The distributions of the mutations in *PLCZ1* exons and the PLCZ1 protein are also shown in **Figure 1c**. The novel p.R385Q mutation was conserved among different species, with the exception of *Gallus gallus* (**Figure 1d**).

Prediction of the effects of PLCZ1 mutations on protein conformation

To explore the structural basis of human *PLCZ1* mutations associated with polyspermy, we constructed a 3D model of human PLCZ1 based on the homologous structure of rat PLCZ1 (**Figure 1e**). The mutation of arginine to glutamine at position 385 (R385Q) may alter the spatial relationship between Arg385 and the hydrogen bonds of Glu422 and Glu831, which potentially destabilizes the XY-link domain. The change in the reading frame after amino acid 412 (R412Efs*15) creates a stop codon at nucleotide position 427, disrupting the core region of the XY-link domain. A mutation of Met578 (p.M578T) to threonine might completely remove the hydrogen bonds to Lys580. The mutation also disrupts the interactions of nearby hydrophobic residues. Thus, the C2 domain and the C2-catalytic domain interaction would be affected.

Expression and localization of PLCZ1

To investigate the expression and localization of PLCZ1 in the sperm from patient II-2 of family 1, immunofluorescence assay and Western blotting were performed. Immunofluorescence and the Western blotting analysis showed the abnormal location and decreased expression level of PLCZ1 in the sperm of patient II-2 of family 1 (**Figure 2a** and **2b**), who carried compound heterozygous p.R385Q and p.R412Efs*15 mutations. The pathogenicity of the *PLCZ1*-p.M578T mutation has been previously confirmed in a study that demonstrated a decrease in catalytic activity by *in vitro* functional analysis.[12] To further investigate the effect of the *PLCZ1*-p.R385Q mutation *in vitro*, we examined its expression level in HEK-293T cells after transfection with WT or p.R385Q mutant constructs, revealing that the p.R385Q mutation resulted in a significantly reduced expression level (**Figure 2c**).

Multiple PLCZ1-mutated sperm can induce PN formation in humans but not mice

Using human IVM-MII oocytes, fertilization status was assessed after injection of WT or *PLCZ1*-mutated sperm. PN formation failed after injection of a single *PLCZ1*-mutated sperm, but multiple PN formation was observed when six *PLCZ1*-mutated sperm were injected (**Figure 3**), suggesting that Ca^{2+} oscillations triggered by multiple *PLCZ1*-mutated sperm might attain the threshold for PN formation. Numerous previous studies have shown that the activation capacity of human sperm can be evaluated by microinjection of human sperm into mouse oocytes.[21–24] We similarly performed ICSI using mouse oocytes and found that most oocytes injected with single *PLCZ1*-mutated sperm exhibited failed PN formation. Intriguingly, zygotes showed multiple PN formation after injection of multiple WT sperm, but PN formation was lacking after injection of multiple *PLCZ1*-mutated sperm (**Supplementary Figure 3**).

Treatment outcomes of ICSI-AOA

After identification of the *PLCZ1* mutations, AOA was combined with ICSI in the next cycles. As shown in **Table 3**, all patients yielded normal

Table 1: IVF/ICSI outcomes of the patients with phospholipase C zeta 1 mutations before artificial oocyte activation treatment

Case	*Treatment cycles*	*Total oocytes (n)*	*MII oocytes (n)*	*2PN (n)*	*≥3PN, n/total (%)*	*Available embryos (n)*
Family 1 II-1	IVF	5	4	0	2/4 (50.0)	0
Family 1 II-2	IVF	13	13	0	12/13 (92.3)	0
	ICSI	10	9	0	0/9 (0)	0
Family 2 II-1	IVF	20	19	0	19/19 (100.0)	0

IVF: *in vitro* fertilization; ICSI: intracytoplasmic sperm injection; MII: metaphase II; 2PN: two pronuclei; 3PN: three pronuclei

Table 2: Overview of the phospholipase C zeta 1 mutations identified in the two families

Case	*Genomic position on Chr12 (bp)*	*cDNA change*	*Protein change*	*Mutation type*	*GnomAD (EAS[a])*	*GnomAD (total[a])*	*Mutation Taster[b]*	*PolyPhen-2[b]*
Family 1	18852748	c.1154C>T	p.R385Q	Missense	0.00005 (1/19 934)	0.00007 (19/282 170)	Damaging	Possibly D
	18849141	c.1234del	p.R412Efs*15	Frameshift deletion	0.00015 (3/19 502)	0.00001 (3/274 280)	NA	NA
Family 2	18837072	c.1733T>C	p.M578T	Missense	0.00025 (5/19 858)	0.00002 (5/281 390)	Damaging	Probably D

[a]Frequency of corresponding mutations in the EAS and total population of GnomAD. [b]Mutation assessment by Mutation Taste and PolyPhen-2. NA: not available; bp: base pair; Chr: chromosome; EAS: East Asian; cDNA: complementary DNA

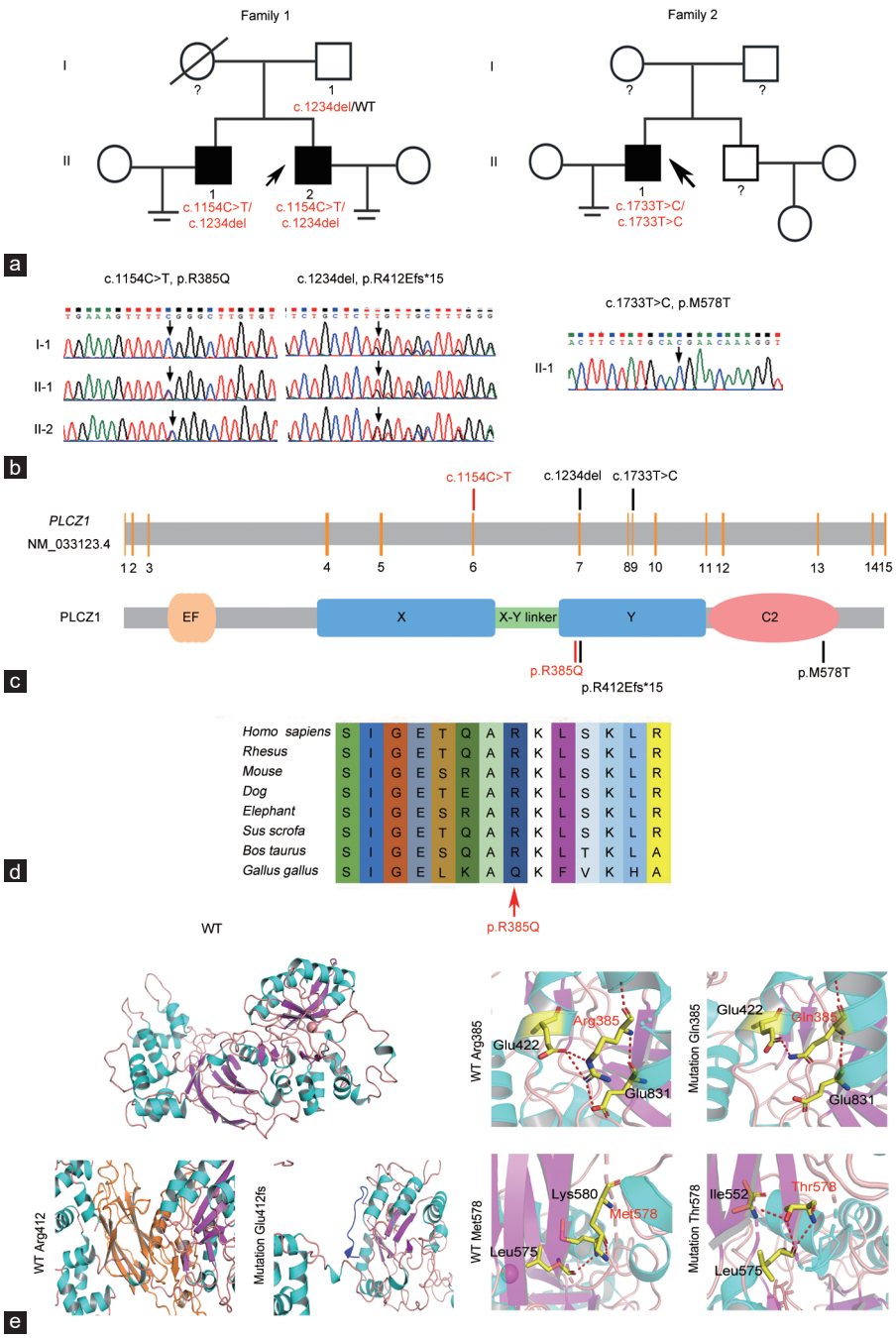

Figure 1: *PLCZ1* mutations in three patients with primary infertility. (**a**) The pedigrees of the three patients. Arrows indicate the probands and black solid squares are the affected individuals. (**b**) Sanger sequencing chromatograms of the two families. The black arrows indicate the positions of the mutations. (**c**) The locations of the three mutations in the genomic and protein structures of *PLCZ1*. The novel mutation is highlighted in red, and two known mutations are highlighted in black. (**d**) The R385 residue (red arrow) is almost conserved among species except birds. (**e**) Prediction of the conformations of mutant PLCZ1 proteins. The panel in the upper-right corner is an overall 3D structure of WT PLCZ1. Enlargements of the PLCZ1 structure are shown on the upper-left and lower panels, respectively. The WT, and mutated R385 and M578 residues are shown in red. WT: wild-type; *PLCZ1*: phospholipase C zeta 1; 3D: three-dimensional.

2PN zygotes. Patient II-1 of family 1 yielded twelve MII oocytes, eleven of which were normally fertilized and developed into embryos; two embryos were transferred, and two healthy babies were born. Patient II-1 of family 1 yielded nine MII oocytes. Seven embryos were obtained (**Supplementary Movie 2**); two embryos were transferred and yielded two full-term healthy babies. For patient II-1 of family 2, four of six oocytes were fertilized, but only two were available (**Supplementary Movie 3**). Both embryos were transferred, and the patient achieved pregnancy.

DISCUSSION

Previous studies have shown that *PLCZ1* mutations in males lead to normal sperm motility and morphology but cause poor fertilization or failure of

Table 3: Clinical outcomes of the patients with phospholipase C zeta 1 mutations after artificial oocyte activation treatment

Case	*AOA treatment cycle*	*Total oocytes (n)*	*MII oocytes (n)*	*2PN, n/total (%)*	*Available embryos (n)*	*Transferred embryo (n)*	*Gestational sac (n)*	*Live birth (n)*	*Body weight (g)*	*Body length (cm)*	*Sex*
Family 1 II-1	1	13	12	11/12 (91.7)	11	2	2	2	2160 and 1900	45 and 44	Male and male
Family 1 II-2	1	10	9	7/9 (77.8)	7	2	2	2	3050 and 2800	47 and 46	Male and male
Family 2 II-1	1	10	6	4/6 (66.7)	2	2	2	Pregnancy	-	-	-

AOA: artificial oocyte activation; 2PN: two pronuclei; -: no value

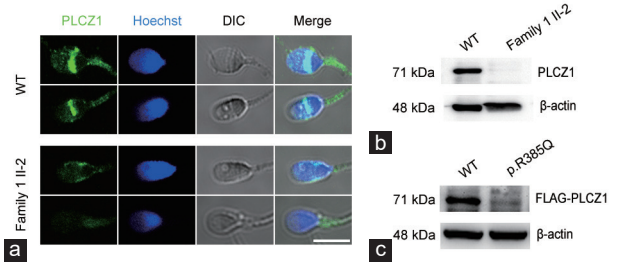

Figure 2: Expression and localization of PLCZ1. (**a**) Confocal immunofluorescence images revealed that reduced protein level of PLCZ1 in spermatozoa from patient II-2 of family 1. Single-sperm immunofluorescence analysis for PLCZ1 (red) and Hoechst (blue) was performed in *PLCZ1*-mutated and normal spermatozoa. Scale bar = 5 μm. (**b**) The PLCZ1 protein was significantly reduced in sperm from patient II-2 of family 1, who was affected by p.R385Q and R412Efs*15 mutations. β-actin was used as a loading control. (**c**) Western blotting analysis of FLAG expression levels in transfected HEK293T cells. WT: wild-type; PLCZ1: phospholipase C zeta 1; DIC: differential interference contras.

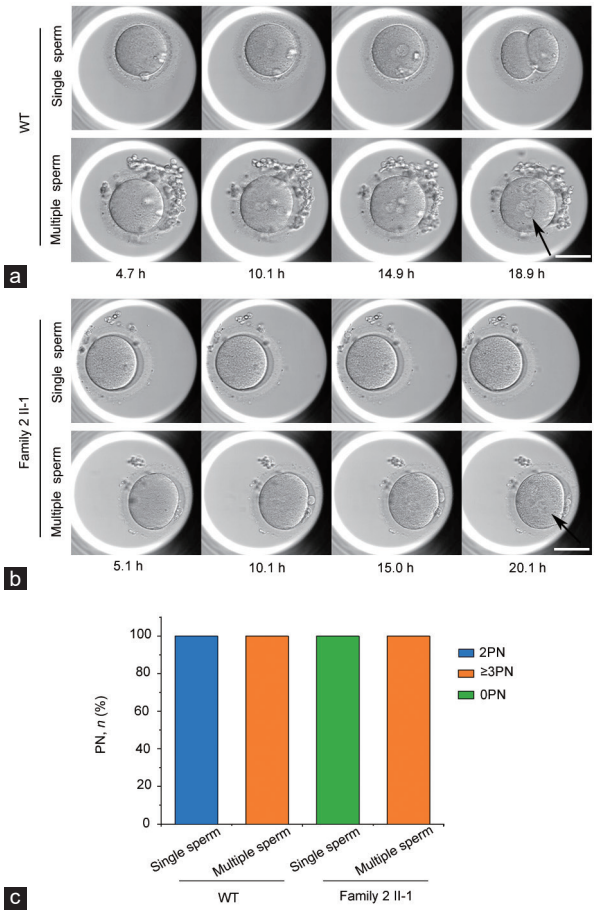

Figure 3: Fertilization status following injection of single or multiple *PLCZ1*-mutated sperm from family 2 II-1. (**a**) Time-lapse images of oocytes at 4.7 h, 10.1 h, 14.9 h, and 18.9 h after ICSI of WT sperm. Scale bar = 100 μm. The arrow indicates pronuclei. (**b**) Time-lapse images of oocytes at 5.1 h, 10.1 h, 15.0 h, and 20.1 h after ICSI of *PLCZ1*-mutated sperm. Scale bar = 100 μm. The arrow indicates pronuclei. (**c**) Percentages of 0PN, ≥3PN, and 2PN oocytes. WT: wild-type; *PLCZ1*: phospholipase C zeta 1; PN: pronuclear.

ICSI.[9–11] Recently, Peng *et al.*[25] reported that mutations in *PLCZ1* induce male infertility associated with polyspermy. Polyspermy is the fertilization of an oocyte by more than one sperm, which causes embryonic arrest. In humans, two mechanisms (the "oocyte membrane block" and the "zona pellucida block") have been proposed to explain polyspermy; both involve Ca^{2+} oscillations.[26–30] Normally, once a sperm enters an oocyte, PLCZ1 immediately triggers Ca^{2+} oscillations to block polyspermy.[2,31,32] *PLCZ1*-deficient sperm reduces cortical granule release and slows or eliminates membrane blocking, resulting in polyspermy.[18] In our study, the expression of PLCZ1 protein was attenuated in patient II-2 of family 1 with compound heterozygous p.R385Q and p.R412Efs*15 mutations. This individual had undergone one failed IVF attempt characterized by polyspermy, which is consistent with the previous report.[25] Thus, it was suggested that the abnormal localization and expression of PLCZ1 protein in sperm might be associated with polyspermy.

Males with pathogenic *PLCZ1* mutations typically experience TFF after ICSI; however, in two previous studies, 2PN zygotes successfully formed after treatment with Ca^{2+} ionophores.[33,34] However, the three infertile males in the present study all experienced polyspermy after IVF. We, thus, hypothesized that the phenotypic difference between ICSI and IVF might be associated with the intracellular Ca^{2+} level induced during fertilization. When a single *PLCZ1*-mutated sperm is injected into an oocyte, the level of released Ca^{2+} may not attain the threshold for PN formation, which thus fails. During an IVF cycle, the Ca^{2+} oscillations induced by a single *PLCZ1*-mutated sperm do not activate PN formation or block polyspermy, allowing multiple sperm to enter. Nozawa *et al.*[18] proposed that delaying the plasma membrane block of polyspermy would lead to multiple sperm entering the oocyte in mice. Furthermore, we speculated that the Ca^{2+} oscillations induced by multiple *PLCZ1*-mutated sperm exceeded the threshold for PN formation and were associated with multiple PN formation after IVF. To validate this, multiple sperm from patient II-1 of family 2 were injected into human IVM-MII oocytes. As expected, injection of multiple *PLCZ1*-mutated sperm triggered oocyte activation and PN formation. However, PN formation was lacking when multiple *PLCZ1*-mutated sperm were injected into mice, suggesting an interspecies difference.[35,36] *Plcz1*-knockout male mice were subfertile rather than completely infertile,[17,18] suggesting that other sperm factors or a redundant pathway may rescue the absence of *Plcz1* in mouse oocytes.

AOA is commonly used to treat patients with *PLCZ1* mutations who experience TFF or fertilization failure after ICSI.[33,34,37–39] In the present study, three couples obtained viable embryos and became pregnant following AOA treatment during their ICSI cycles. Therefore, ICSI-AOA treatment should be commenced as soon as possible for patients with biallelic mutations in *PLCZ1*, which would reduce

treatment duration. After the first IVF treatment, a genetic test should be offered to couples with polyspermy.

Our study had certain limitations. We analyzed only three male patients who suffered from polyspermy after IVF. More patients are required to study the genetic causes of polyspermy. Second, the MII donor oocytes used in the multiple sperm injection experiments were derived via *in vitro* maturation.

In conclusion, we report polyspermy after IVF treatment in humans with *PLCZ1* mutations. A novel missense mutation, c.1154C>T, p.R385Q, was identified in *PLCZ1*. We believe that our findings will aid genetic diagnoses after abnormal fertilization and identify appropriate therapeutic measures for patients with sperm-derived polyspermy.

AUTHOR CONTRIBUTIONS

KYT, WWL, and JYL mainly contributed to the study design, data analysis, and manuscript writing. DYL, YZX, and KC collated the patients' samples. CL, LWC, and LWS performed the mouse experiments. JYL and KYT conducted the manuscript writing with the help from all authors. JYL and GNH conceived the study and supervised the study progress. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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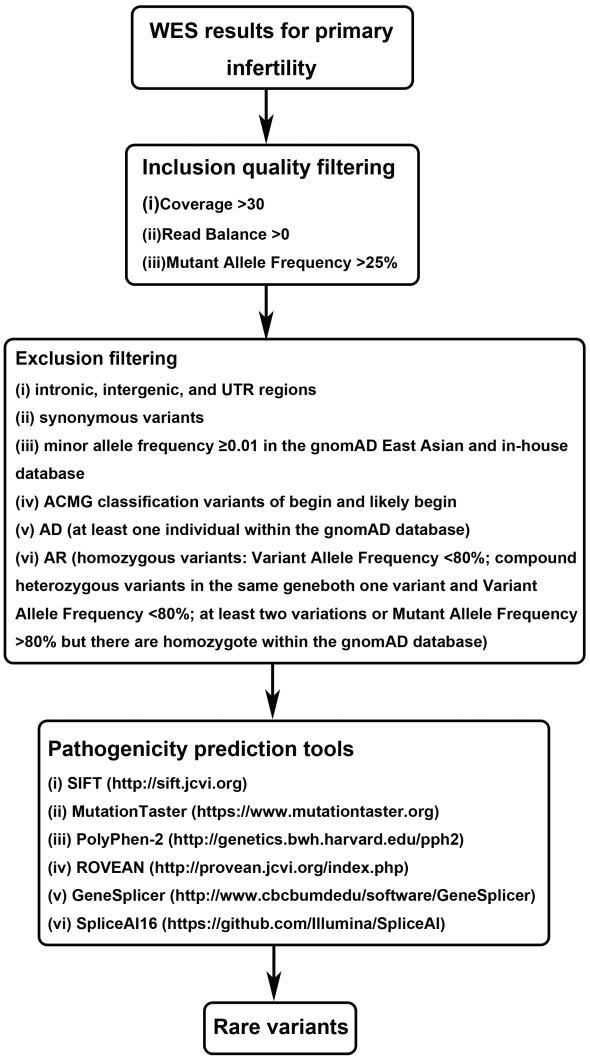
Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

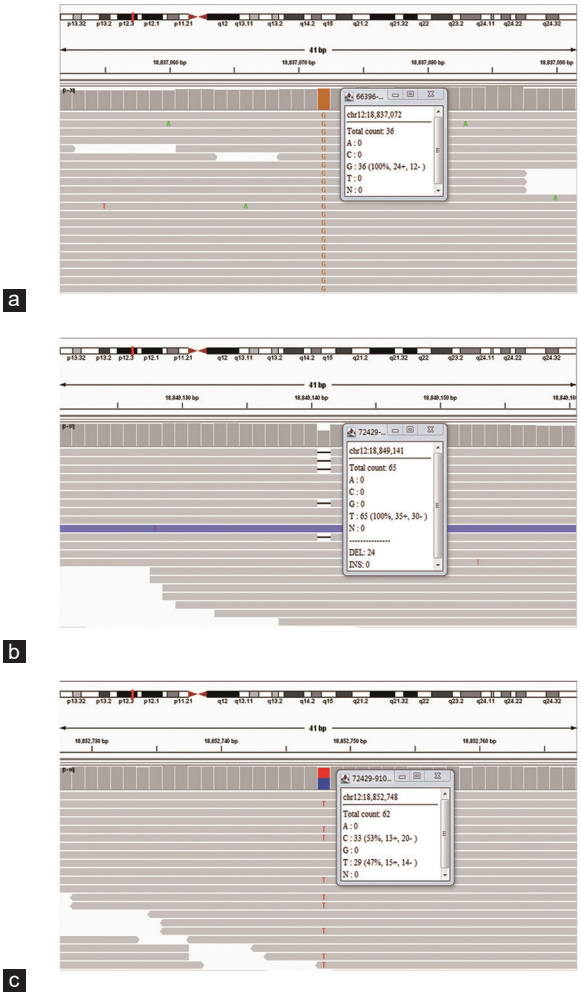
REFERENCES

1 Kline D, Kline JT. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev Biol* 1992; 149: 80–9.
2 Balakier H, Dziak E, Sojecki A, Librach C, Michalak M, *et al*. Calcium-binding proteins and calcium-release channels in human maturing oocytes, pronuclear zygotes and early preimplantation embryos. *Hum Reprod* 2002; 17: 2938–47.
3 Tsaadon L, Kaplan-Kraicer R, Shalgi R. Myristoylated alanine-rich C kinase substrate, but not Ca^{2+}/calmodulin-dependent protein kinase II, is the mediator in cortical granules exocytosis. *Reproduction* 2008; 135: 613–24.
4 Ducibella T, Fissore R. The roles of Ca^{2+}, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. *Dev Biol* 2008; 315: 257–79.
5 Stricker SA. Comparative biology of calcium signalling during fertilisation and egg activation in mammals. *Dev Biol* 1999; 211: 157–76.
6 Berridge MJ. Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta* 2009; 1793: 933–40.
7 Amdani SN, Jones C, Coward K. Phospholipase C zeta (PLCζ): oocyte activation and clinical links to male factor infertility. *Adv Biol Regul* 2013; 53: 292–308.
8 Kashir J, Konstantinidis M, Jones C, Lemmon B, Lee HC, *et al*. A maternally inherited autosomal point mutation in human phospholipase C zeta (PLCζ) leads to male infertility. *Hum Reprod* 2012; 27: 222–31.
9 Torra-Massana M, Cornet-Bartolomé D, Barragán M, Durban M, Ferrer-Vaquer A, *et al*. Novel phospholipase C zeta 1 mutations associated with fertilization failures after ICSI. *Hum Reprod* 2019; 34: 1494–504.
10 Mu J, Zhang Z, Wu L, Fu J, Chen B, *et al*. The identification of novel mutations in *PLCZ1* responsible for human fertilization failure and a therapeutic intervention by artificial oocyte activation. *Mol Hum Reprod* 2020; 26: 80–7.
11 Wang FS, Zhang JJ, Kong S, Li CJ, Zhang ZG, *et al*. A homozygous nonsense mutation of *PLCZ1* cause male infertility with oocyte activation deficiency. *J Assist Reprod Genet* 2020; 37: 821–8.
12 Yuan P, Zheng L, Liang H, Lin Q, Ou S, *et al*. Novel mutations in the *PLCZ1* gene associated with human low or failed fertilization. *Mol Genet Genomic Med* 2020; 8: e1470.
13 Dai J, Dai C, Guo J, Zheng W, Zhang T, *et al*. Novel homozygous variations in *PLCZ1* lead to poor or failed fertilization characterized by abnormal localization patterns of PLCζ in sperm. *Clin Genet* 2020; 97: 347–51.
14 Yuan P, Yang C, Ren Y, Yan J, Nie Y, *et al*. A novel homozygous mutation of phospholipase C zeta leading to defective human oocyte activation and fertilization failure. *Hum Reprod* 2020; 35: 977–85.
15 Escoffier J, Lee HC, Yassine S, Zouari R, Martinez G, *et al*. Homozygous mutation of *PLCZ1* leads to defective human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. *Hum Mol Genet* 2016; 25: 878–91.
16 Cardona Barberán A, Boel A, Vanden Meerschaut F, Stoop D, Heindryckx B. Diagnosis and treatment of male infertility-related fertilization failure. *J Clin Med* 2020; 9: 3899.
17 Hachem A, Godwin J, Ruas M, Lee HC, Ferrer Buitrago M, *et al*. PLCζ is the physiological trigger of the Ca^{2+} oscillations that induce embryogenesis in mammals but conception can occur in its absence. *Development* 2017; 144: 2914–24.
18 Nozawa K, Satouh Y, Fujimoto T, Oji A, Ikawa M. Sperm-borne phospholipase C zeta-1 ensures monospermic fertilization in mice. *Sci Rep* 2018; 8: 1315.
19 Essen LO, Perisic O, Katan M, Wu Y, Roberts MF, *et al*. Structural mapping of the catalytic mechanism for a mammalian phosphoinositide-specific phospholipase C. *Biochemistry* 1997; 36: 1704–18.
20 Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online* 2011; 22: 632–46.
21 Rybouchkin A, Dozortsev D, de Sutter P, Qian C, Dhont M. Intracytoplasmic injection of human spermatozoa into mouse oocytes: a useful model to investigate the oocyte-activating capacity and the karyotype of human spermatozoa. *Hum Reprod* 1995; 10: 1130–5.
22 Yazawa H, Yanagida K, Sato A. Human round spermatids from azoospermic men exhibit oocyte-activation and Ca^{2+} oscillation-inducing activities. *Zygote* 2007; 15: 337–46.
23 Araki Y, Yoshizawa M, Abe H, Murase Y, Araki Y. Use of mouse oocytes to evaluate the ability of human sperm to activate oocytes after failure of activation by intracytoplasmic sperm injection. *Zygote* 2004; 12: 111–6.
24 Heindryckx B, De Gheselle S, Gerris J, Dhont M, De Sutter P. Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. *Reprod Biomed Online* 2008; 17: 662–8.
25 Peng Y, Lin Y, Deng K, Shen J, Cui Y, *et al*. Mutations in *PLCZ1* induce male infertility associated with polyspermy and fertilization failure. *J Assist Reprod Genet* 2023; 40: 53–64.
26 Wolf DP. The block to sperm penetration in zona-free mouse eggs. *Dev Biol* 1978; 64: 1–10.
27 Tahara M, Tasaka K, Masumoto N, Mammoto A, Ikebuchi Y, *et al*. Dynamics of cortical granule exocytosis at fertilization in living mouse eggs. *Am J Physiol* 1996; 270: C1354–61.
28 Matson S, Markoulaki S, Ducibella T. Antagonists of myosin light chain kinase and of myosin II inhibit specific events of egg activation in fertilized mouse eggs. *Biol Reprod* 2006; 74: 169–76.
29 Mei W, Lee KW, Marlow FL, Miller AL, Mullins MC. hnRNP I is required to generate the Ca^{2+} signal that causes egg activation in zebrafish. *Development* 2009; 136: 3007–17.
30 Fuentes R, Tajer B, Kobayashi M, Pelliccia JL, Langdon Y, *et al*. The maternal coordinate system: molecular-genetics of embryonic axis formation and patterning in the zebrafish. *Curr Top Dev Biol* 2020; 140: 341–89.
31 Zafar MI, Lu S, Li H. Sperm-oocyte interplay: an overview of spermatozoon's role in oocyte activation and current perspectives in diagnosis and fertility treatment. *Cell Biosci* 2021; 11: 4.
32 Kuroda K, Ito M, Shikano T, Awaji T, Yoda A, *et al*. The role of X/Y linker region and N-terminal EF-hand domain in nuclear translocation and Ca^{2+} oscillation-inducing activities of phospholipase C zeta, a mammalian oocyte-activating factor. *J Biol Chem* 2006; 281: 27794–805.
33 Kashir J, Ganesh D, Jones C, Coward K. Oocyte activation deficiency and assisted oocyte activation: mechanisms, obstacles and prospects for clinical application. *Hum Reprod Open* 2022; 2022: hoac003.
34 Meng X, Melo P, Jones C, Ross C, Mounce G, *et al*. Use of phospholipase C zeta analysis to identify candidates for artificial oocyte activation: a case series of clinical pregnancies and a proposed algorithm for patient management. *Fertil Steril* 2020; 114: 163–74.
35 Wachten D, Jikeli JF, Kaupp UB. Sperm sensory signaling. *Cold Spring Harb Perspect Biol* 2017; 9: a028225.
36 Zhao ZH, Meng TG, Li A, Schatten H, Wang ZB, *et al*. RNA-Seq transcriptome reveals different molecular responses during human and mouse oocyte maturation and fertilization. *BMC Genomics* 2020; 21: 475.
37 Cardona-Barberán A, Boel A, Vanden-Meerschaut F, Stoop D, Heindryckx B. SPERM FACTORS AND EGG ACTIVATION: fertilization failure after human ICSI and the clinical potential of *PLCZ1*. *Reproduction* 2022; 164: F39–51.

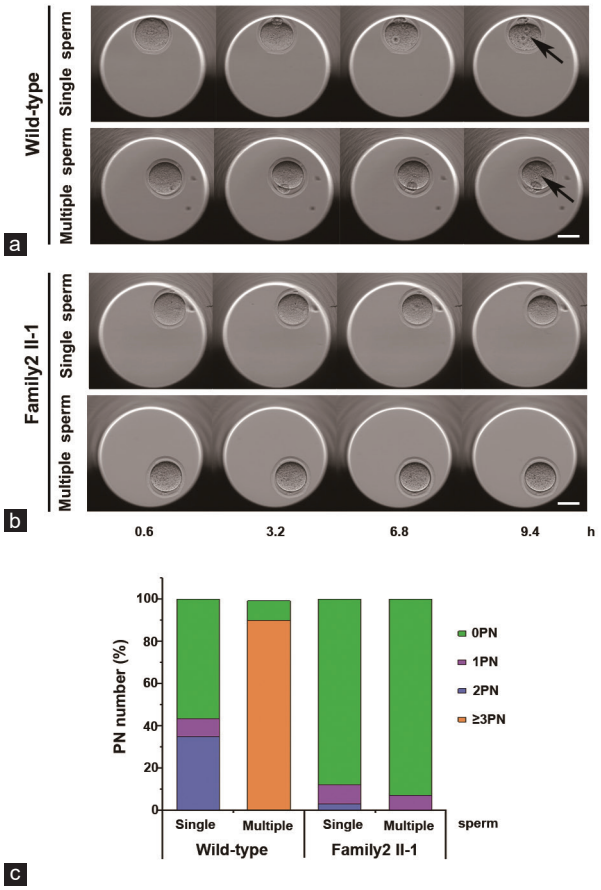
38 Lam KK, Wong JY, Cheung TM, Li RH, Ng EH, *et al*. A retrospective analysis of artificial oocyte activation in patients with low or no fertilisation in intracytoplasmic sperm injection cycles. *J Obstet Gynaecol* 2022; 42: 648–53.
39 Zhao S, Cui Y, Guo S, Liu B, Bian Y, *et al*. Novel variants in *ACTL7A* and *PLCZ1* are associated with male infertility and total fertilization failure. *Clin Genet* 2023; 103: 603–8.



Supplementary Figure 1: The filter criteria for rare variants. Rare variant candidates were selected using ACMG guidelines. Variant Allele Frequency: the percentage of sequence reads with the variant; In-house database: WES databases from 200 unrelated volunteers of Chinese population with normal fertility (at least one child); UTR: untranslated region; ACMG: American College of Medical Genetics and Genomics.



Supplementary Figure 2: The IGV illustration of three variants in *PLCZ1*. IGV screenshots depicting of (**a**) the c.1733T>C loci from patient II-1 of family 2, (**b**) the c.1234del and (**c**) c.1154C>T loci from patient II-2 of family 1. IGV: Integrative Genomics Viewer; *PLCZ1*: phospholipase C zeta 1.



Supplementary Figure 3: Fertilization status after single or multiple injection of wild-type and *PLCZ1*-mutated sperm in mice. (**a**) Time-lapse images of oocytes after ICSI of wild-type sperm. Arrows: pronuclei. Scale bar = 50 μm. (**b**) Time-lapse images of oocytes after ICSI of *PLCZ1*-mutated sperm. Scale bar = 50 μm. (**c**) Percentages of oocytes with different PN numbers. AOA: artificial oocyte activation; ICSI: intracytoplasmic sperm injection; *PLCZ1*: phospholipase C zeta 1.

Supplementary Table 1: Clinical characteristics of three infertility couples

Characteristic	*Family 1 II-1*	*Family 1 II-2*	*Family 2 II-1*
Female factor			
Age (year)	32	27	32
BMI (kg m^{-2})	23.3	27.8	22.2
E_2 (pg ml^{-1})	19.1	26.6	25.3
Progesterone (ng ml^{-1})	0.3	0.3	0.2
FSH (IU l^{-1})	4.9	4.6	3.7
LH (IU l^{-1})	1.8	1.6	2.4
AMH (ng ml^{-1})	4.6	10.1	8.0
Male factor			
Age (year)	39	33	30
Semen volume (ml)	3.7	3.6	4.0
Sperm concentration ($\times 10^6$ ml^{-1})	68	49	59
Total sperm ($\times 10^6$)	251.6	176.4	236
PR (%)	45	51	38
Total motility (PR + NP; %)	52	64	45
Normal sperm morphology (%)	5	5	4

Semen volume (lower reference limit: 1.5 ml), sperm concentration (lower reference limit: 15×10^6 ml^{-1}), progressive motility rate (PR; lower reference limit: 32%), normal sperm morphology (lower reference limit: 4%), according to the World Health Organization, 2010. BMI: body mass index; AMH: anti-Müllerian hormone; FSH: follicle-stimulating hormone; LH: luteinizing hormone; PR: progressive motility; NP: non-progressive motility

Supplementary Table 2: The list of rare variants obtained after filtering

Family	*Case*	*Chr*	*Chr position*	*Gene*	*OMIM link*	*Trascript (hg19)*	*Variant (HGVS)*	*Protein (HGVS)*	*Function*	*Zygosity*	*Freq_ gnomAD_eas*	*ACMG*	*Mutation Taster*	*PolyPhen-2*
1	II-2	1	55247076	*TTC22*	NA	NM_001114108	c.1550T>C	p.L517P	Missense	Het	NA	VUS	D	Probably D
		1	185985213	*HMCN1*	608548	NM_031935	c.5033A>G	p.D1678G	Missense	Het	NA	VUS	D	Probably D
		2	179355523	*PLEKHA3*	607774	NM_019091	c.295A>G	p.R99G	Missense	Het	0.00006	VUS	D	Possible D
		2	196749499	*DNAH7*	610061	NM_018897	c.5573G>T	p.G1858V	Missense	Het	NA	VUS	D	Probably D
		3	52830825	*ITIH3*	146650	NM_002217	c.352G>A	p.A118T	Missense	Het	NA	VUS	D	Probably D
		3	58552370	*FAM107A*	608295	NM_001076778	c.380T>G	p.V127G	Missense	Het	NA	VUS	D	Probably D
		3	122632087	*SEMA5B*	609298	NM_001031702	c.2465G>C	p.R822T	Missense	Het	NA	VUS	D	Probably D
		3	124906146	*SLC12A8*	611316	NM_024628	c.325A>G	p.M109V	Missense	Het	NA	VUS	D	Probably D
		4	26487490	*CCKAR*	118444	NM_000730	c.395T>C	p.L132P	Missense	Het	NA	VUS	D	Probably D
		4	54244013	*FIP1L1*	607686	NM_030917	c.8C>T	p.A3V	Missense	Het	NA	VUS	D	Probably D
		4	101109111	*DDIT4L*	607730	NM_145244	c.305G>T	p.G102V	Missense	Het	NA	VUS	D	Probably D
		4	108566106	*PAPSS1*	603262	NM_005443	c.1358C>T	p.P453L	Missense	Het	NA	VUS	D	Probably D
		5	75581670	*SV2C*	610291	NM_014979	c.1112G>A	p.R371Q	Missense	Het	NA	VUS	D	Probably D
		5	76028616	*F2R*	187930	NM_001992	c.566C>G	p.S189C	Missense	Het	NA	VUS	D	Probably D
		5	93807324	*C5orf36*	NA	NM_001145678	c.1568T>C	p.V523A	Missense	Het	NA	VUS	D	Probably D
		6	7542311	*DSP*	125647	NM_004415	c.163G>T	p.G55C	Missense	Het	0.00008	VUS	D	Probably D
		7	103194278	*RELN*	600514	NM_005045	c.5798G>T	p.G1933V	Missense	Het	NA	VUS	D	Probably D
		8	139890218	*COL22A1*	610026	NM_152888	c.433G>A	p.V145M	Missense	Het	NA	VUS	D	Probably D
		9	18892418	*ADAMTSL1*	609198	NM_001040272	c.4675C>G	p.R1559G	Missense	Het	NA	VUS	D	Probably D
		9	138710442	*CAMSAP1*	613774	NM_015447	c.3980G>A	p.R1327Q	Missense	Het	NA	VUS	D	Probably D
		10	5468674	*NET1*	606450	NM_001047160	c.185A>T	p.D62V	Missense	Het	NA	VUS	D	Probably D
		10	98820500	*SLIT1*	603742	NM_003061	c.838G>A	p.G280S	Missense	Het	NA	VUS	D	Probably D
		11	4107730	*STIM1*	605921	NM_003156	c.1498C>T	p.R500W	Missense	Het	0.00005	VUS	D	Probably D
		12	18849141	*PLCZ1*	608075	NM_033123	c.1234delA[a]	p.R412Efs	Frameshift	Het	0.00015	p	NA	NA
		12	18852748	*PLCZ1*	608075	NM_033123	c.1154G>A[a]	p.R385Q	Missense	Het	0.00005	VUS	D	Possible D
		12	110819589	*ANAPC7*	606949	NM_016238	c.1202G>A	p.R401Q	Missense	Het	NA	VUS	D	Probably D
		15	43874092	*PPIP5K1*	610979	NM_001130858	c.736A>T	p.I246F	Missense	Het	NA	VUS	D	Probably D
		15	65856574	*HACD3*	615940	NM_016395	c.554A>G	p.H185R	Missense	Het	NA	VUS	D	Probably D
		16	2633480	*PDPK1*	605213	NM_002613	c.1019C>T	p.P340L	Missense	Het	NA	VUS	D	Possible D
		17	28747973	*CPD*	603102	NM_001304	c.1109G>A	p.R370H	Missense	Het	NA	VUS	D	Probably D
		17	39914757	*JUP*	173325	NM_002230	c.1667T>A	p.M556K	Missense	Het	NA	VUS	D	Probably D
		17	48674223	*CACNA1G*	604065	NM_018896	c.3197C>T	p.S1066L	Missense	Het	NA	VUS	D	Probably D
		17	71419659	*SDK2*	607217	NM_001144952	c.1763A>G	p.Q588R	Missense	Het	NA	VUS	D	Possible D
		18	12496079	*SPIRE1*	609216	NM_001128626	c.995G>C	p.R332P	Missense	Het	NA	VUS	D	Probably D
		18	29339961	*SLC25A52*	616153	NM_001034172	c.664G>A	p.G222S	Missense	Het	NA	VUS	D	Probably D
		21	35147312	*ITSN1*	602442	NM_003024	c.1496T>C	p.I499T	Missense	Het	NA	VUS	D	Probably D

Contd...

Supplementary Table 2: Contd...

Family	*Case*	*Chr*	*Chr position*	*Gene*	*OMIM link*	*Trascript (hg19)*	*Variant (HGVS)*	*Protein (HGVS)*	*Function*	*Zygosity*	*Freq_ gnomAD_eas*	*ACMG*	*Mutation Taster*	*PolyPhen-2*
	Female partner of II-2	1	9671838	*TMEM201*	NA	NM_001130924	c.1793G>T	p.S598I	Missense	Het	NA	VUS	D	Possible D
		1	23779233	*ASAP3*	616594	NM_017707	c.380C>G	p.P127R	Missense	Het	NA	VUS	D	Probably D
		1	32936914	*ZBTB8B*	NA	NM_001145720	c.689A>C	p.K230T	Missense	Het	NA	VUS	D	Probably D
		1	51822445	*EPS15*	600051	NM_001981	c.2618G>A	p.R873Q	Missense	Het	NA	VUS	D	Probably D
		1	112270042	*C1orf183*	NA	NM_019099	c.442C>T	p.R148W	Missense	Het	NA	VUS	D	Probably D
		2	8870866	*KIDINS220*	615759	NM_020738	c.5300G>A	p.R1767K	Missense	Het	NA	VUS	D	Probably D
		2	39053749	*DHX57*	NA	NM_198963	c.2722A>G	p.K908E	Missense	Het	NA	VUS	D	Probably D
		2	136261974	*ZRANB3*	615655	NM_032143	c.86delT	p.L29Cfs	Frameshift	Het	NA	LP	NA	NA
		2	231973992	*HTR2B*	601122	NM_000867	c.685C>A	p.P229T	Missense	Het	NA	VUS	D	Probably D
		3	62180805	*PTPRG*	176886	NM_002841	c.1288G>A	p.D430N	Missense	Het	NA	VUS	D	Probably D
		3	128853717	*ISY1*	612764	NM_020701	c.499G>C	p.D167H	Missense	Het	NA	VUS	D	Probably D
		5	11346566	*CTNND2*	604275	NM_001332	c.1546T>A	p.Y516N	Missense	Het	NA	VUS	D	Probably D
		5	176943376	*DDX41*	608170	NM_016222	c.211C>T	p.R71W	Missense	Het	NA	VUS	D	Possible D
		6	136472371	*PDE7B*	604645	NM_018945	c.456G>T	p.M152I	Missense	Het	0.00005	VUS	D	Possible D
		7	73152050	*ABHD11*	NA	NM_001145364	c.304G>A	p.A102T	Missense	Het	NA	VUS	D	Probably D
		7	100173514	*LRCH4*	NA	NM_181581	c.854T>C	p.M285T	Missense	Het	NA	VUS	D	Probably D
		7	107217905	*DUS4L*	NA	NM_002319	c.1756C>T	p.H586Y	Missense	Het	NA	VUS	D	Probably D
		7	128491586	*FLNC*	102565	NM_001458	c.5746G>A	p.V1916M	Missense	Het	0.00005	VUS	D	Probably D
		10	128810626	*DOCK1*	601403	NM_001380	c.1081C>T	p.H361Y	Missense	Het	NA	VUS	D	B
		12	54405086	*HOXC8*	142970	NM_022658	c.650G>A	p.R217Q	Missense	Het	NA	VUS	D	Probably D
		13	35685056	*NBEA*	604889	NM_015678	c.1943A>T	p.K648I	Missense	Het	NA	VUS	D	Probably D
		14	50911851	*MAP4K5*	604923	NM_006575	c.1234_1247delGCATCAACCATAAA	p.A412Tfs	Frameshift	Het	NA	LP	NA	NA
		17	2599730	*CLUH*	616184	NM_015229	c.2171C>T	p.P724L	Missense	Het	NA	VUS	D	Probably D
		17	56332270	*LPO*	150205	NM_001160102	c.955C>G	p.P319A	Missense	Het	NA	VUS	D	Possible D
		18	29432443	*TRAPPC8*	614136	NM_014939	c.3527A>G	p.Y1176C	Missense	Hom	0.00143	VUS	D	Probably D
		19	1112909	*SBNO2*	615729	NM_014963	c.2287G>A	p.G763R	Missense	Het	NA	VUS	D	Possible D
		19	5032957	*KDM4B*	609765	NM_015015	c.56G>A	p.R19H	Missense	Het	0.00005	VUS	D	Possible D
		19	10559781	*PDE4A*	600126	NM_001111307	c.575G>A	p.R192H	Missense	Het	NA	VUS	D	Probably D
		19	23159174	*ZNF728*	NA	XM_001726961	c.964_965delAA	p.N322Pfs	Frameshift	Het	NA	LP	NA	NA
		19	56170630	*U2AF2*	191318	NM_007279	c.104G>A	p.R35Q	Missense	Het	NA	VUS	D	Possible D
		20	3785573	*CDC25B*	116949	NM_021873	c.1708C>T	p.R570W	Missense	Het	NA	VUS	D	Probably D
		22	50170762	*BRD1*	604589	NM_014577	c.2648G>A	p.R883Q	Missense	Het	NA	VUS	D	Probably D
2	II-1	1	27874095	*AHDC1*	615790	NM_001029882.3	c.4532C>T	p.T1511M	Missense	Het	NA	VUS	D	Probably D
		1	196876494	*CFHR4*	605337	NM_001201550.2	c.667_676delACGTCCTTCC	p.T223Rfs*27	Frameshift	Het	NA	VUS	NA	NA
		1	202287641	*LGR6*	606653	NM_001017403.1	c.2210T>G	p.V737G	Missense	Het	NA	VUS	D	Probably D
		1	223933038	*CAPN2*	114230	NM_001748.4	c.457G>A	p.V153M	Missense	Het	NA	VUS	D	Probably D

Contd...

Supplementary Table 2: Contd...

Family	*Case*	*Chr*	*Chr position*	*Gene*	*OMIM link*	*Trascript (hg19)*	*Variant (HGVS)*	*Protein (HGVS)*	*Function*	*Zygosity*	*Freq_ gnomAD_eas*	*ACMG*	*Mutation Taster*	*PolyPhen-2*
		2	38818676	*HNRNPLL*	611208	NM_138394.3	c.304A>T	p.I102L	Missense	Het	NA	VUS	D	Possible D
		2	231740366	*ITM2C*	609554	NM_030926.5	c.293T>C	p.V98A	Missense	Het	NA	VUS	D	Probably D
		3	47716999	*SMARCC1*	601732	NM_003074.3	c.1805G>A	p.R602H	Missense	Het	NA	VUS	D	Probably D
		3	51624464	*RAD54L2*	NA	NM_015106.3	c.28G>A	p.D10N	Missense	Het	NA	VUS	D	Probably D
		3	130284245	*COL6A6*	616613	NM_001102608.2	c.1069C>T	p.R357W	Missense	Het	NA	VUS	D	Probably D
		6	24423229	*MRS2*	NA	NM_020662.3	c.1172G>A	p.R391H	Missense	Het	0.00006	VUS	D	Probably D
		6	148840976	*SASH1*	607955	NM_015278.4	c.1156C>T	p.R386C	Missense	Het	0.00006	VUS	D	Probably D
		7	100470247	*TRIP6*	602933	NM_003302.2	c.1180A>G	p.K394E	Missense	Het	NA	VUS	D	Probably D
		7	120979364	*WNT16*	606267	NM_057168.1	c.1063T>C	p.C355R	Missense	Het	NA	VUS	D	Probably D
		8	2967804	*CSMD1*	608397	NM_033225.5	c.6484T>A	p.F2162I	Missense	Het	NA	VUS	D	Probably D
		9	37541216	*FBXO10*	609092	NM_012166.2	c.550T>G	p.F184V	Missense	Het	NA	VUS	D	Probably D
		9	114151915	*KIAA0368*	616694	NM_001080398.1	c.3902G>A	p.R1301H	Missense	Hom	0.00006	VUS	D	Probably D
		9	130928646	*CIZ1*	611420	NM_012127.2	c.2527C>T	p.P843S	Missense	Het	0.00009	VUS	D	Probably D
		10	116044685	*VWA2*	618281	NM_001272046.1	c.953A>G	p.Y318C	Missense	Het	NA	VUS	D	Probably D
		10	135193787	*PAOX*	615853	NM_152911.3	c.466G>A	p.G156R	Missense	Het	NA	VUS	D	Probably D
		12	18837072	*PLCZ1*	608075	NM_033123.3	c.1733T>C[a]	p.M578T	Missense	Hom	0.00025	VUS	D	Probably D
		12	81610754	*ACSS3*	614356	NM_024560.3	c.1429A>G	p.K477E	Missense	Het	NA	VUS	D	Probably D
		15	82512539	*EFL1*	617538	NM_024580.5	c.1324C>T	p.R442C	Missense	Hom	0.0001	VUS	D	Probably D
		16	4934787	*PPL*	602871	NM_002705.4	c.3869A>T	p.E1290V	Missense	Het	NA	VUS	D	Probably D
		16	16130354	*ABCC1*	158343	NM_004996.3	c.703C>A	p.P235T	Missense	Het	NA	VUS	D	Probably D
		16	28738511	*EIF3C*	603916	NM_003752.4	c.1763T>C	p.L588P	Missense	Het	NA	VUS	D	Probably D
		16	58030933	*ZNF319*	NA	NM_020807.2	c.1237G>A	p.E413K	Missense	Het	NA	VUS	D	Probably D
		18	39570433	*PIK3C3*	602609	NM_002647.3	c.629G>A	p.R210Q	Missense	Het	NA	VUS	D	Possible D
		19	17417067	*MRPL34*	611840	NM_023937.3	c.158A>G	p.Y53C	Missense	Het	NA	VUS	D	Probably D
		19	19680364	*PBX4*	608127	NM_025245.2	c.662C>T	p.A221V	Missense	Het	0.00006	VUS	D	Probably D
		20	2840991	*VPS16*	608550	NM_022575.3	c.348delG	p.R117Dfs*94	Frameshift	Het	NA	VUS	NA	NA
		21	43412886	*ZBTB21*	616485	NM_001098402.1	c.1319C>T	p.P440L	Missense	Het	NA	VUS	D	Probably D
	Female partner of II-1	1	10386212	*KIF1B*	605995	NM_001365951.3	c.2719C>T	p.R907C	Missense	Het	NA	VUS	D	Probably D
		1	17256492	*CROCC*	615776	NM_014675.5	c.503G>A	p.R168Q	Missense	Het	NA	VUS	D	Probably D
		2	37088355	*STRN*	614765	NM_003162.4	c.1589A>G	p.Q530R	Missense	Het	NA	VUS	D	Possible D
		2	179213982	*OSBPL6*	606734	NM_032523.4	c.1019G>A	p.R340H	Missense	Het	NA	VUS	D	Probably D
		3	50369504	*RASSF1*	605082	NM_007182.5	c.439A>G	p.N147D	Missense	Het	NA	VUS	D	Probably D
		4	57356515	*SRP72*	602122	NM_006947.4	c.1337A>G	p.H446R	Missense	Het	NA	VUS	D	Probably D
		4	175649846	*GLRA3*	600421	NM_006529.4	c.271T>G	p.Y91D	Missense	Het	NA	VUS	D	Probably D
		6	12161933	*HIVEP1*	194540	NM_002114.4	c.6749C>T	p.P2250L	Missense	Het	NA	VUS	D	Probably D

Contd...

Supplementary Table 2: Contd...

Family	*Case*	*Chr*	*Chr position*	*Gene*	*OMIM link*	*Trascript (hg19)*	*Variant (HGVS)*	*Protein (HGVS)*	*Function*	*Zygosity*	*Freq_ gnomAD_eas*	*ACMG*	*Mutation Taster*	*PolyPhen-2*
		6	90604205	*GJA10*	611924	NM_032602.2	c.18A>T	p.L6F	Missense	Het	0.00005	VUS	D	Possible D
		6	108070944	*SCML4*	NA	NM_198081.5	c.230C>A	p.S77Y	Missense	Het	NA	VUS	D	Probably D
		6	116289885	*FRK*	606573	NM_002031.3	c.484G>A	p.V162I	Missense	Het	NA	VUS	D	Possible D
		7	6542736	*GRID2IP*	610639	NM_001145118.2	c.2966T>A	p.L989H	Missense	Het	NA	VUS	D	Probably D
		7	12375832	*VWDE*	NA	NM_001135924.3	c.4589G>A	p.G1530D	Missense	Het	NA	VUS	P	Probably D
		7	72718232	*NSUN5*	615732	NM_148956.4	c.929G>A	p.G310D	Missense	Het	NA	VUS	D	Probably D
		8	39607244	*ADAM2*	601533	NM_001464.5	c.1817G>A	p.C606Y	Missense	Het	0.00005	VUS	P	Probably D
		8	59498238	*NSMAF*	603043	NM_003580.4	c.2632delA	p.I878Sfs*12	Frameshift	Het	NA	VUS	NA	NA
		9	21333840	*KLHL9*	611201	NM_018847.4	c.1019A>G	p.H340R	Missense	Het	NA	VUS	D	Probably D
		10	82298182	*SH2D4B*	NA	NM_001388272.1	c.95G>A	p.R32Q	Missense	Het	NA	VUS	D	Probably D
		10	92509295	*HTR7*	182137	NM_019859.4	c.596T>C	p.M199T	Missense	Het	NA	VUS	D	Probably D
		11	5011893	*MMP26*	605470	NM_021801.5	c.387dupC	p.I130Hfs*30	Frameshift	Het	NA	VUS	NA	NA
		11	129990697	*APLP2*	104776	NM_001142276.2	c.500A>T	p.H167L	Missense	Het	0.00005	VUS	D	Possible D
		12	6729674	*LPAR5*	606926	NM_020400.6	c.741C>G	p.F247L	Missense	Het	0.00007	VUS	D	Probably D
		12	123892095	*KMT5A*	607240	NM_020382.7	c.904T>C	p.C302R	Missense	Het	NA	VUS	D	B
		12	123892186	*KMT5A*	607240	NM_020382.7	c.995T>C	p.L332P	Missense	Het	NA	VUS	D	Probably D
		15	43856336	*PPIP5K1*	610979	NM_001394395.1	c.3200G>C	p.R1067P	Missense	Het	NA	VUS	D	Probably D
		17	8387536	*MYH10*	160776	NM_001256012.3	c.5095A>G	p.R1699G	Missense	Het	0.00005	VUS	D	Probably D
		17	28791683	*CPD*	603102	NM_001304.5	c.3994G>T	p.D1332Y	Missense	Het	NA	VUS	D	Probably D
		17	74735066	*MFSD11*	NA	NM_001242532.5	c.143G>T	p.G48V	Missense	Het	NA	VUS	D	Probably D
		17	77984526	*TBC1D16*	616637	NM_019020.4	c.212A>G	p.E71G	Missense	Het	NA	VUS	D	Probably D
		19	16263852	*HSH2D*	608349	NM_001382417.1	c.216NA1G>A	NA	Splice	Het	NA	VUS	NA	NA
		19	19378863	*TM6SF2*	606563	NM_001001524.3	c.643C>T	p.R215C	Missense	Het	NA	VUS	D	Probably D
		19	54930465	*TTYH1*	605784	NM_020659.4	c.290C>T	p.A97V	Missense	Het	NA	VUS	D	Probably D
		22	18185048	*BCL2L13*	NA	NM_015367.4	c.496G>A	p.E166K	Missense	Het	NA	VUS	D	Probably D

[a]Candidate *PLCZ1* variants. HGVS: Human Genome Variation Society (http://www.hgvs.org); OMIM: Online Mendelian Inheritance in Man; ACMG: American College of Medical Genetics and Genomics; VUS: variant of unknown significance; p: pathogenic; LP: likely pathogenic; Het: heterozygote; Hom: homozygote; D: damaging; P: polymorphism; B: benign; NA: not available