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A homozygous nonsense mutation of *PLCZ1* cause male infertility with oocyte activation deficiency

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Abstract

Purpose To identify the pathogenic *PLCZ1* mutation involved in male infertility and fertilization failure.

Methods All coding regions of *PLCZ1* were sequenced by Sanger sequencing. The expression and localization of PLCZ1 in sperm was determined by Western blotting and immunofluorescence. To promote the fertilization rate, the infertile man with *PLCZ1* mutation was treated with intracytoplasmic sperm injection (ICSI) accompanied by assisted oocyte activation (AOA) in the following cycle.

Result We identified a novel homozygous *PLCZ1* nonsense mutation, c.588C>A (p.Cys196Ter) in an infertile man from a consanguineous family. No PLCZ1 protein was detected by Western blotting and immunofluorescence in ejaculated sperm from the patient. The treatment of ICSI + AOA avoided fertilization failure but did not result in pregnancy in the following cycle.

Conclusion Our study confirmed the essential role of PLCZ1 in fertilization and male fertility, which indicated the potential prognostic value of testing for *PLCZ1* mutations in primary infertile men with sperm-derived fertilization failure.

Keywords Male infertility · Oocyte activation · Sperm · Fertilization failure · PLCZ1

Introduction

Intracytoplasmic sperm injection (ICSI) is widely used in severe conditions responsible for male infertility (e.g., azoospermia, oligozoospermia, asthenozoospermia, teratozoospermia) and in patients with fertilization failure or low fertilization rates after conventional in vitro fertilization (IVF) [1]. Complete fertilization failure after ICSI has been found in 1–3% of the patients receiving the treatment, though ICSI commonly achieves high fertilization rates in most cases [2]. The failure is mostly caused by a deficiency of oocyte activation [2]. Oocyte activation is driven by the sperm oocyte-activating factor (SOAF) released by spermatozoon at the time of gamete fusion, which results in a sequence of events triggered by the release of intracellular calcium in the oocyte cytoplasm [3].

The phospholipase C zeta 1 (PLCZ1) protein has been considered as the strongest one among several candidates for the SOAF, such as the WW domain-binding protein 2 (WBP2) N-terminal like (WBP2NL or the postacrosomal sheath WW domain-protein, PAWP) [4], WBP2 [5], and the phospholipase C zeta 1 (sperm PLCZ1 or PLCζ) [6]. Compound heterozygous or homozygous missense *PLCZ1* mutations have been reported in two unrelated infertile men with complete fertilization failure after ICSI. Moreover, the injection of recombinant *PLCZ1* RNA or PLCZ1 protein into mice oocytes can rescue the oocyte activation ability of *PLCZ1*-deficient sperm [7–9]. Using CRISPR-Cas9 knockout procedure, it was recently reported that the sperm of the *PLCζ*-null mice

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failed to trigger Ca2+ oscillations after ICSI [10]. Taken together, these studies suggest that *PLCZ1* may be essential for oocyte activation and male fertility.

In this report, we identified a homozygous *PLCZ1* nonsense mutation c.588C>A (p.Cys196Ter) in a primary infertile male. The patient had a previous failed procedure of in vitro fertilization (IVF) due to non-fertilization and sperm from the patient showed a complete fertilization failure after one ICSI procedure. Therefore, the patient could be regarded as a human *PLCZ1* 'knockout' model. *PLCZ1*mutations should be involved in genetic screening for infertile couples with previous complete fertilization failure after ICSI.

Materials and methods

Clinical samples

Four unrelated primary infertile couples with complete fertilization failure after one cycle of ICSI were recruited from Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical Univsesity, Hefei, China between January 2013 and December 2018, who previously had an unsuccessful IVF cycle due to a completely failed fertilization. All men and their partners had a normal karyotype. One healthy fertile man who had a normal karyotype, normal semen parameters according to the World Health Organization guidelines [11], and a successful reproductive history for the last 2 years served as a normal control. Genomic DNA samples were prepared from all men and their available family members. Written informed consent was obtained from each participant. The biomedical research ethics committee of Anhui Medical University approved this study.

Semen analysis and sperm morphology

Semen analysis for each individual was carried out at least twice in the same laboratory of Reproductive Medicine Center in our hospital according to published guidelines [11]. Sperm morphology was assessed using the Papanicolaou staining and transmission electron microscopy.

Sanger sequencing of *PLCZ1* and *PAWP*

Genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The coding regions of the *PLCZ1* gene were amplified as a previously described method [7]. The primers used to amplify the coding regions of *PAWP* were designed as shown in Table 1. PCR products of *PLCZ1* and *PAWP* were sequenced on an ABI 3100 DNA analyzer (Applied Biosystem, Foster City, CA, USA).

Table 1 Genomic PCR primers used to amplify *PAWP* exons for Sanger sequencing

Exon	F/R[a]	Primer sequence (5′ to 3′)	PCR Size (bp)
1	F	GGCCACTCACCTATCACTCA	387
	R	GAGCCGCCATGTCTATCTCA	
2	F	TATTGTGTGCCGTCTGTTCC	282
	R	TTTCTTCCACCACCCTTCAG	
3/4	F	ACTGAAGGGTGGTGGAAGAA	826
	R	CCACAAGCCTTAAAACCCCA	
5	F	GTAAGGTGGTGATTAAATAC TCTG	388
	R	TCACTTGCTGACTTTGCCA	
6	F	TCTTGCGTCAGTTTGCTTCA	662
	R	CCCATAAGATTGCCCTTCCAAA	

F represents forward primers and R represents reverse primers

Immunofluorescence assay

The immunofluorescence assay was performed as previously described methods [12, 13], using anti-PLCζ rabbit polyclonal antibodies (Covalab, Villeurbanne, France) as Grasa et al. used [14]. Anti-WBP2NL rabbit polyclonal antibodies (Proteintech, Chicago, IL, USA) and Purified Mouse Anti Human CD46 antibodies (BD Biosciences, Pharmingen™, USA) were also used.

Western blotting analysis

Western blotting was performed as previously described methods [12, 13], using rabbit polyclonal antibodies against PLCζ, and WBP2NL, and anti-α-tubulin antibodies for the loading control (Sigma Aldrich, St. Louis, MO, USA).

Outcome of ICSI

Motile spermatozoa with normal morphology were chosen for ICSI, which was carried out as previously described procedure [15]. AOA procedure was also performed as a previously described method [16]. Briefly, the cleavage medium (COOK Medical, Brisbane, Australia) containing 10 μmol/L calcium ionophore (A23187) (Sigma Aldrich, St. Louis, MO, USA) was prepared before ICSI. Thirty minutes after ICSI, the oocytes were activated and cultured in the prepared cleavage medium for 10 min. These inseminated oocytes were washed three times with fresh cleavage medium without calcium ionophore and then were cultured in the medium; fertilization assessment was performed 17± 1 h post-insemination.

Table 2 Semen parameters of male partners from four unrelated primary infertile couple with complete fertilization failure after one ICSI cycle following a failed previous IVF due to non-fertilization

Patients	Patient 1	Patient 2	Patient 3	Patient 4
Sperm volume (ml)	5.6	3.2	2.8	4.2
Nb spz ($\times 10^6$)	113	75.3	63.8	90.3
Round cells ($\times 10^6$)	2.5	1.9	3.2	2.8
Motility A + B, 1 h (%)	40.6	48.2	50.2	60.3
Vitality(%)	53.8	60.3	62.3	72.0
Normal spermatozoa(%)	97	96	98	97

Results

Semen analysis and sperm morphology

We performed IVF for four infertile couples, but this procedure failed for all of them. Their semen parameters were normal, as shown in Table 2. To increase the fertilization rate, ICSI was performed for these patients, but they still had total fertilization failure after the treatment. Figure 1 shows the normal morphology of their sperm.

Identification of the *PLCZ1* mutation c. 588C>A (p.Cys196Ter)

To further analyze the cause of fertilization failure, we first amplified and sequenced the coding regions of the *PLCZ1* gene using the previously designed primers [7, 17]. A novel homozygous nonsense mutation in *PLCZ1*, c.588C>A (p.Cys196Ter) (NM_033123.3) was identified in one man from a consanguineous family (Fig. 2a). Segregation analysis showed that his parents had the heterozygous mutation (Fig. 2b). The c.588C>A variant was absent from the exome aggregation consortium (ExAC) database and genome aggregation database (gnomAD). The nonsense mutation c.588C>A (p.Cys196Ter) was predicted to produce a loss-of-function effect of the gene by introducing a premature termination codon (PTC) in *PLCZ1* mRNA. To obtain the mutation frequency among infertile men, we sequenced the *PLCZ1* gene in three additional unrelated men having no history of consanguinity, but no mutations in *PLCZ1* were identified. We then sequenced the coding regions of the *PAWP* gene and found no mutations in the above-mentioned four men.

As shown in Fig. 2d, the PLCZ1 protein contained four EF-hand domains (EF1-EF4) located in the N-terminal region, an X catalytic domain, an XY-linker, a Y catalytic domain, and a

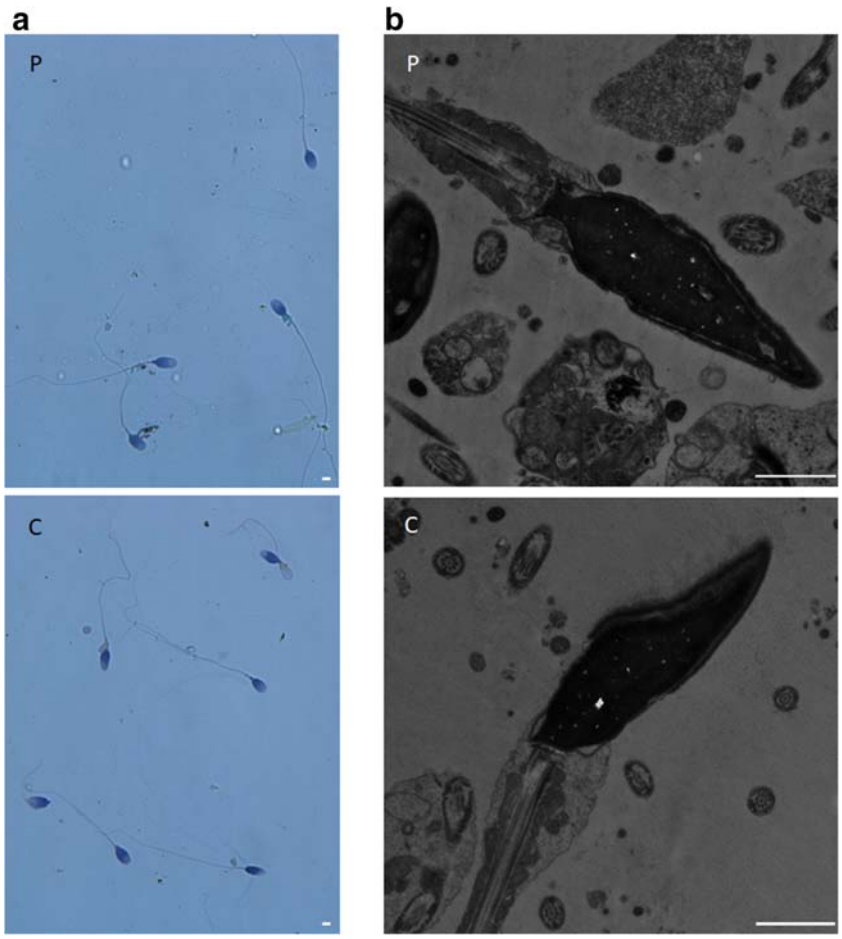

Fig. 1 **a** Photomicrographs of ejaculated semen smear stained by Papanicolaou from the patient (P) and the control (C). **b** Transmission electron micrographs of the spermatozoa from the patient and the control (C). Bar = 2 μm

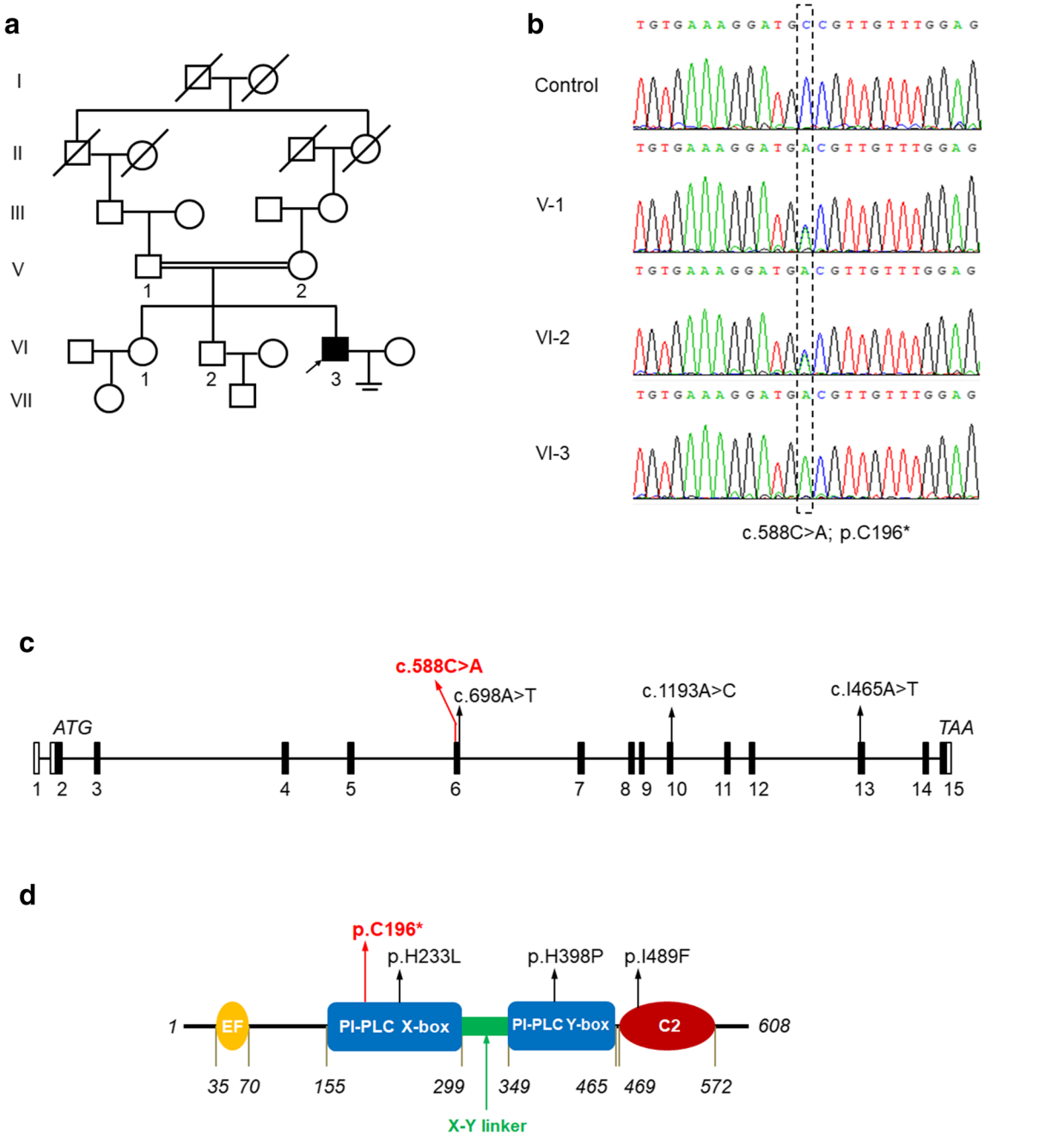

Fig. 2 **a** Pedigree of the patient with *PLCZ1* mutation is shown. **b** The father (V-1) and unaffected brother (VI-2) have a heterozygous mutation in *PLCZ1*, and the patient has a homozygous mutation in *PLCZ1*. **c** Coding exons are indicated in a black box, untranslated region and non-coding exon 1 in a clear box, intron in a line; and the localization of the reported missense mutations and the newly identified nonsense mutation in this study are indicated by black lines and a red line, respectively. **d** The domain architecture of PLCZ1 and the location of the *PLCZ1* variants, p.C196* and p.H233L in PI-PLC X-box, p.H398P in PI-PLC Y-box, p.I489F in C2

C2 domain in the C-terminal region. X and Y catalytic domains are vital for its catalytic activity of PLCZ1, while EF domains, XY-linker, and C2 domain regulate the activity of the protein and act in targeting the intracellular membrane. The nonsense mutation located in the X catalytic domain of PLCZ1 was predicted to introduce a PTC in the PLCZ1 mRNA, which would cause the PLCZ1 protein to be largely shortened, possibly resulting in loss of function.

Expression and localization of PLCζ in *PLCZ1*-mutated sperm

To determine whether PLCζ was expressed in the *PLCZ1*-mutated patient's sperm, Western blotting was performed with an anti-PLCζ rabbit polyclonal antibody (Covalab, Villeurbanne, France). A specific band at the native molecular weight (NM) of 70 kDa appeared in the control sperm, but

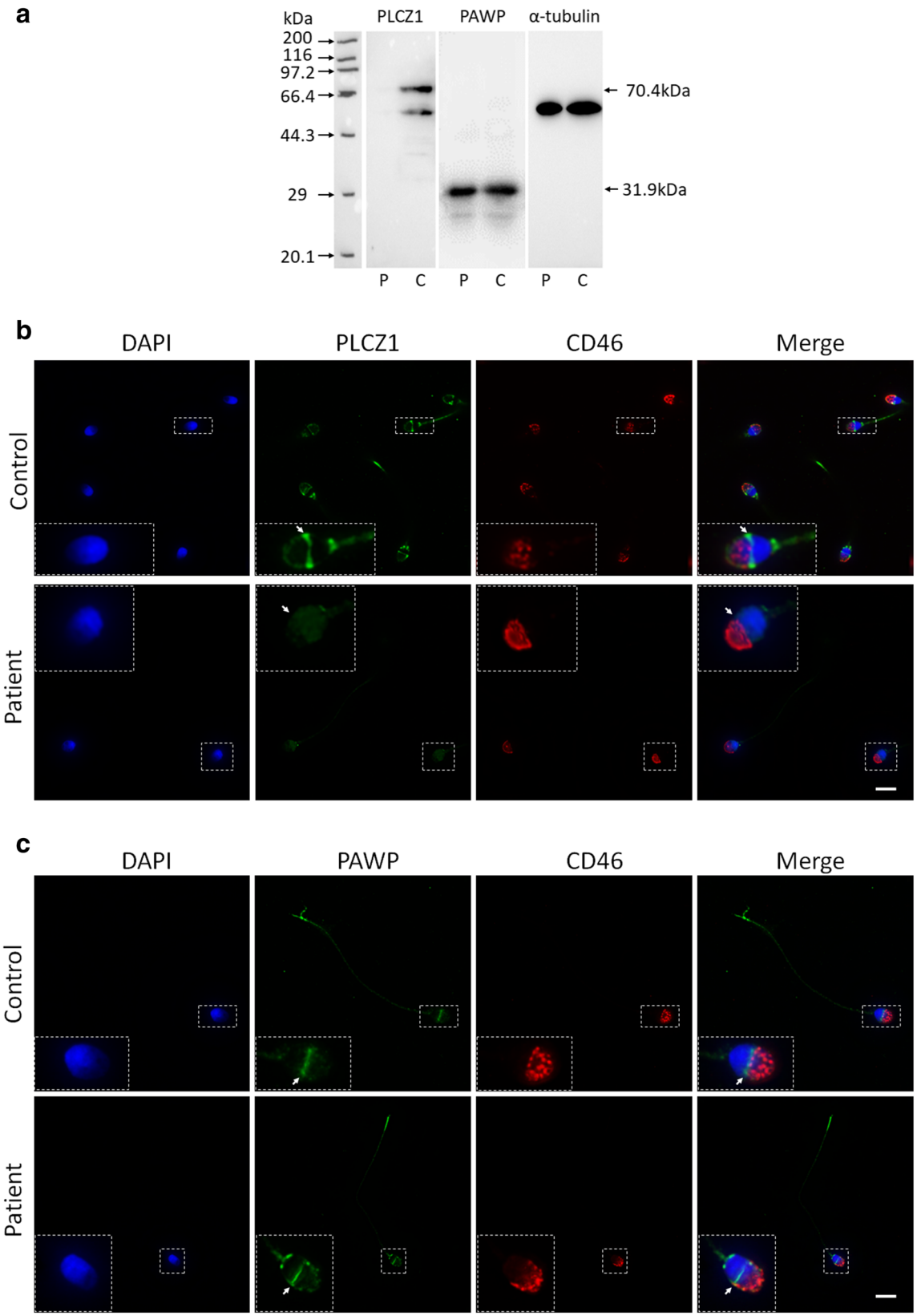

Fig. 3 **a** PLCZ1, PAWP and α-tubulin levels were tested by Western blotting on ejaculated sperm from the patient (P) and a control (C). Molecular weights shown in the left lane were determined according to the protein molecular weight marker (Takara, broad range, 3452Q). **b**, **c** The expression and localization of PLCZ1 and PAWP in sperm by immunofluorescence from the patient and a control

Table 3 Outcomes of a previous cycle of in-vitro fertilization (IVF) before the treatment of ICSI in patients

Patients (sex, age)	P1 (M, 34; F, 29 years)	P2 (M, 27y; F, 27 years)	P3 (M, 28y; F, 27 years)	P4 (M, 36y; F, 29 years)
Stimulation protocol	Long	Long	Long	Long
No. of oocytes retrieved	16	11	13	17
No. of fertilized oocytes	0	0	0	0
No. of D3 embryos	0	0	0	0
No. of D5 embryos	–	–		

P patient, *M* male, *F* female, *Long* long protocol

was not present in the *PLCZ1*-mutated patient's sperm (Fig. 3a). The PLCZ1 band at ~ 50 kDa in the control sperm may be a proteolytic fragment or a truncated endogenous form of PLCZ1, as detected in a number of previous studies [17–19], which was absent in the sperm with a homozygous *PLCZ1* mutation.

To access the subcellular localization of PLCζ in the sperm head, we simultaneously marked the nuclei of sperm with 4′,6-diamidino-2-phenylindole (DAPI) and the acrosome with CD46 antibodies. The results showed that PLCζ was predominantly localized in the equatorial region and slightly localized in the postacrosomal region in the control sperm. PLCζ staining disappeared in the equatorial region but a very weak and disseminated staining appeared in the postacrosomal region in the *PLCZ1*-mutated sperm (Fig. 3b). This weak staining may be nonspecific binding of the rabbit polyclonal antibodies in this region [20].

Since no mutation was found in the *PAWP* gene, as a control, we analyzed the localization and expression of PAWP by immunofluorescence and Western blotting using anti-PAWP antibodies in the above-mentioned sperm. Both the localization and expression of PAWP were found to be similar in *PLCZ1*-mutated and control sperms (Fig. 3c).

ICSI + AOA outcomes

Since the patients had fertilization failure with IVF and the first ICSI cycle (Table 3) and the *PLCZ1* c.588C>A mutation was detected in patient 1, to avoid repeating fertilization failure, patient 1 was advised to choose assisted oocyte activation in the following ICSI cycle to avoid repeating fertilization failure. Table 4 shows that the patient continued to have a low fertilization rate and a poor embryo development. In contrast, the other three patients with no *PLCZ1* or *PAWP* mutations also accepted the option of ICSI + AOA treatment in the subsequent cycle, they had a normal fertilization rate and obtained well-developed embryos that were successfully implanted in their female partners (Table 4).

Discussion

Here, we report the first homozygous nonsense mutation c.588C>A (p.Cys196Ter) in *PLCZ1* in one infertile man from a consanguineous family with complete fertilization failure in an ICSI cycle after a failed previous cycle of IVF due to non-fertilization. Previous studies have reported a compound

Table 4 Results of intracytoplasmic sperm injection (ICSI) in patients who had experienced a failed previous IVF due to non-fertilization

Patients (sex, age)	P1 (M, 34; F, 29 years)			P2 (M, 27 years; F, 27 years)		P3 (M, 28 years; F, 27 years)	P4 (M, 36 years; F, 29 years)		
No of cycles	1st	2nd	3rd	1st	2nd	1st	2nd	1st	2nd
Stimulation protocol	Long	Long	Mild	Long	Mild	Long	Long	Long	Mild
No. of oocytes retrieved	13	9	2	15	8	10	19	23	15
No. of MII oocytes	10	8	2	15	8	10	8	21	13
With AOA or not (Y/N)	N	Y	Y	N	Y	N	Y	N	Y
No. of fertilized oocytes	0	3	2	0	7		4	–	5
No. of D3 embryos	–	3	2	–	5	–	5	–	1
No. of D5 embryos		2 (4BC, 3BC)	0		0		2 (4BB, 4BB)		1 (4BB)
No. of D6 embryos		0	0		2 (4BB, 4BB)		1 (4BB)		0
Embryos transferred		2 (4BC, 3BC)			1 (4BB)		2 (4BB, 4BB)		1 (4BB)
Pregnancy		–			+		+		+
Live birth					Not yet		Not yet		Not yet

P patient, *M* male, *F* female, *Long* long protocol, *Mild* mild stimulation protocol

heterozygous missense mutations (p.H233L and p. H398P) and a homozygous missense mutation (I489F) in *PLCZ1*, in one infertile man of an European origin and two infertile brothers of a African origin, respectively [7–9].

The novel nonsense mutation in *PLCZ1* identified in this study further expanded the mutation spectrum of *PLCZ1*. The PTC-containing mRNA has been predicted to be degraded by a nonsense-mediated mRNA decay (NMD) [21] with no protein production, which was consistent with the absence of protein in patient's sperm with the *PLCZ1* mutation.

Recently, *Plcz1*-knockout mice generated using CRISPR-Cas9 system showed that the loss of Plcz1 did not affect spermatogenesis and sperm quality parameters [10]. While sperm failed to trigger calcium oscillations after microinjection into the oocytes, some of these oocytes may be fertilized through an alternative route of activation and may be developed to the blastocyst stage in vitro. *Plcz1*-knockout male mice are only subfertile and natural mating of the *Plcz1*-knockout males with wild-type females can generate their pups in vivo, with a severely reduced number of pups per litter on average [10]. Like previous reported cases, the infertile man with the homozygous *PLCZ1* mutation also had normal semen parameters but had complete fertilization failure after ICSI treatment [7–9, 22, 23]. This discrepancy between human and mice suggested that there may be redundant pathways or synergistic substances to rescue the sperm oocyte-activating defect in mouse oocytes, but no such mechanisms exists for oocyte activation in the human oocytes [10]. Noteworthy, the fertilization rate was improved by subsequent treatment of ICSI + AOA, although the transferred embryos were not of top quality.

It has been reported that mutations in the *TLE6* (MIM: 612399) gene [24] or the *WEE2* (MIM: 614084) gene [25–28] can be responsible for fertilization failure and female infertility. However, in our study, the first patient referred to us was from a consanguineous family; therefore, male factor for fertilization failure was preferentially considered. First, we screened this patient for *PLCZ1* mutations and found a novel homozygous nonsense mutation. Then, we screened for the gene mutations in three additional unrelated patients with fertilization failure after ICSI but found no mutations. In addition, no mutations in *PAWP* were identified in these patients.

The fact that three patients without *PLCZ1* mutations continued to have a fertilization failure after ICSI suggests that there may be some other unknown genes for the SOAF released by sperm. We have excluded the possibility of *PAWP* involved in the fertilization failure. Another possibility is female factor for fertilization failure. However, in this study and in all these patients, fertilization rate was markedly improved through ICSI + AOA treatment in the subsequent cycles. It is less possible that female factors (e.g., *WEE2* mutations) may have contributed to a fertilization failure in these patients; as the *WEE2*-mutated oocytes cannot be fertilized even after ICSI + AOA treatment with normal sperms [25–28]. Thus, it seems that the exact cause of fertilization failure may be very complicated after ICSI treatment alone in patients without *PLCZ1* mutations. In further studies, more efforts should be taken to explore the molecular mechanism of fertilization, in sperm, oocytes, and the reciprocity between them.

It remains controversial whether the variations in protein levels or the subcellular localization patterns of PLCZ1 affects the fertilization rates after ICSI treatment. Some studies have shown low expression levels of PLCZ1, as determined by Western blotting and immunofluorescence, in patients with repeated failed fertilization results after ICSI [23, 29]. But other studies have shown that PLCZ1 levels and distribution do not correlate with fertilization rates after ICSI [20, 30, 31]. There is a lack of studies on large cohorts of ICSI fertilization failure patients. Since there were only three patients with ICSI fertilization failure without *PLCZ1* mutations in our cohort, more individuals should be recruited for future studies.

In conclusion, the homozygous nonsense mutation c.588C>A (p.Cys196Ter) in *PLCZ1* may be regarded as a unique human *PLCZ1* 'knockout' model similar to the *Plcz1*-knockout male mice with the disruption of *Plcz1*. The loss of PLCZ1 function causes complete fertilization failure after ICSI and male infertility in the Asian populations. This study further expanded the spectrum of *PLCZ1* mutation. ICSI + AOA treatment is an optional treatment for these primary infertile men with sperm-derived fertilization failure.

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Compliance with ethical standards

This study was approved by the ethics committee. Written informed consent was obtained from each participant.

Competing interests The authors declare that they have no competing interests.

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