A maternally inherited autosomal point mutation in human phospholipase C zeta (PLCζ) leads to male infertility

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Submitted on July 12, 2011; resubmitted on October 7, 2011; accepted on October 12, 2011

BACKGROUND: Male factor and idiopathic infertility contribute significantly to global infertility, with abnormal testicular gene expression considered to be a major cause. Certain types of male infertility are caused by failure of the sperm to activate the oocyte, a process normally regulated by calcium oscillations, thought to be induced by a sperm-specific phospholipase C, PLCζ (PLCζ). Previously, we identified a point mutation in an infertile male resulting in the substitution of histidine for proline at position 398 of the protein sequence (PLCζH398P), leading to abnormal PLCζ function and infertility.

METHODS AND RESULTS: Here, using a combination of direct-sequencing and mini-sequencing of the PLCζ gene from the patient and his family, we report the identification of a second PLCζ mutation in the same patient resulting in a histidine to leucine substitution at position 233 (PLCζH233L), which is predicted to disrupt local protein interactions in a manner similar to PLCζH398P and was shown to exhibit abnormal calcium oscillatory ability following predictive 3D modelling and cRNA injection in mouse oocytes respectively. We show that PLCζH233L and PLCζH398P exist on distinct parental chromosomes, the former inherited from the patient’s mother and the latter from his father. Neither mutation was detected utilizing custom-made single-nucleotide polymorphism assays in 100 fertile males and females, or 8 infertile males with characterized oocyte activation deficiency.

CONCLUSIONS: Collectively, our findings provide further evidence regarding the importance of PLCζ at oocyte activation and forms of male infertility where this is deficient. Additionally, we show that the inheritance patterns underlying male infertility are more complex than previously thought and may involve maternal mechanisms.

Key words: infertility / oocyte activation / sperm / phophospholipase C zeta (PLCζ) / inheritance

Introduction

Oocyte activation, a fundamental step in the process of fertilization involving cortical granule exocytosis, prevention of polyspermy and ultimately the release of the oocyte from meiotic arrest (Whitaker, 2006), involves elevations of intracellular calcium (Ca^{2+}) in response to cellular signalling mediated by inositol 1,4,5-triphosphate (IP3) (Stricker, 1999; Whitaker, 2006; Berridge, 2009). In mammals, this manifests as a series of characteristic Ca^{2+} oscillations which begin soon after gamete fusion and persist beyond the completion of meiosis (Jones, 2005). Mounting evidence suggests that the agent responsible for Ca^{2+} oscillations within activating oocytes is a sperm-specific phospholipase C, phospholipase C zeta (PLCζ), located on chromosome 12 in humans (Cox et al., 2002; Saunders et al., 2002,
Inheritance profiles for mutant PLCζ

Given the fundamental role of PLCζ in activating the oocyte following gamete fusion, PLCζ isoforms exhibiting abnormal expression profiles or defective functional ability, may underlie certain types of human male factor infertility in which oocyte activation is deficient (OAD). Globally, infertility is now estimated to affect one in seven couples (Evans, 2004; Boivin et al., 2007; Ombelet et al., 2008). Intracytoplasmic sperm injection (ICSI; whereby a single sperm is micro-injected directly into the oocyte), is able to rescue fertility in 35–40% of infertility cases (Kashir et al., 2010). However, complete or virtually complete fertilization failure is known to occur in 1–5% of all ICSI cycles (Flaherty et al., 1998; Mahutte and Arici, 2003; Yanagida et al., 2008; Kashir et al., 2010).

The first link between OAD and PLCζ was reported by Yoon et al. (2008), who found that sperm from patients repeatedly failing to activate oocytes via ICSI could not initiate Ca²⁺ oscillations when injected into mouse oocytes, and that this was associated with abnormal expression and localization of PLCζ in sperm. However, this study found no evidence of a genetic condition underlying these observations. Interestingly, the activating ability of such sperm could be rescued upon co-injection with mouse PLCζ mRNA (Yoon et al., 2008), thereby providing significant support for the idea of using PLCζ as a novel therapeutic agent for OAD. Heytens et al. (2009) provided the first genetic link to PLCζ deficiency by identifying a substitution mutation in an infertile male diagnosed with OAD. This mutation occurred within the Y domain of the active site position 398 of the PLCζ open reading frame (ORF), resulting in a histidine to proline substitution (PLCζH398P).

Injection of PLCζH398P cRNA into mouse oocytes resulted in highly abnormal Ca²⁺ transients that were unable to activate oocytes (Heytens et al., 2009). Moreover, a mouse equivalent of PLCζH398P, resulting in a histidine to proline substitution at position 435 of the mouse PLCζ ORF (PLCζH435P), also lacked the ability to induce Ca²⁺ oscillations in oocytes, and was defective in PLC activity (Nomikos et al., 2011). However, a major conundrum with the Heytens et al. (2009) study was that while PLCζH398P was proposed to be heterozygous, it was unclear how it could cause infertility in the patient. Heytens et al. (2009) hypothesized that the infertile phenotype of this patient could have arisen via a dominant negative mechanism. However, the ability of PLCζH398P to act in such a manner was not clearly demonstrated by this study. Subsequently, a study of PLCζH435P showed that the injection of wild-type PLCζ (PLCζWT) elicited a normal Ca²⁺ oscillation profile upon micro-injection in mouse oocytes in the presence of a higher concentration (10 times) of the PLCζH435P mutant (Nomikos et al., 2011), indicating that PLCζH4398P did not act in a dominant negative manner.

Male factor infertility can arise in a number of ways, with genetic causes representing the greatest cause for concern. Moreover, some cases of idiopathic male infertility may represent the results of multiple genetic defects that disrupt spermatogenesis and testicular gene expression (for review, see Kashir et al., 2010), indicating that assisted reproductive technology (ART) interventions such as ICSI could potentially transmit paternal fertility problems from father to son. However, prior study of such genetic causes of infertility has mostly been limited to paternal modes of inheritance, with potential maternal involvement being largely ignored.

Here, in response to concerns about the ability of PLCζH398P to act in a dominant negative fashion, and thus be capable of causing infertility in a heterozygous manner, we carried out a more thorough re-examination of the PLCζ gene from the patient in which the H398P mutation was discovered. We report the identification of a second novel PLCζ mutation in the patient possessing PLCζH398P, and analyse the functional effects of the new mutant form of PLCζ in comparison with PLCζWT and PLCζH4398P. Crucially, we investigate the genetic origin and mode of inheritance of PLCζ mutation across three generations of the patient’s family in an attempt to gain further understanding of how such mutations may exert an effect upon fertility.

Materials and Methods

Sample acquisition and processing

Genomic DNA was extracted from buccal cell samples obtained from nine infertile males whose sperm lacked activation ability, including the patient from whom PLCζH398P had been previously identified (patientH398P, Heytens et al., 2009). All patients had been previously diagnosed with recurrent ICSI failure or abnormal sperm morphology, and were undergoing fertility treatment at the Department of Reproductive Medicine, Ghent University Hospital, Belgium. Patients 1–4, 6 and 7 exhibited globozoospermic sperm morphology, while Patients 5 and 8 exhibited normal sperm morphology. Additional buccal samples were obtained from four family members of patientH398P (mother, father, half brother and daughter conceived by ICSI and assisted oocyte activation methodology). All samples were collected with informed written consent, and with the approval of the Ethical Committee of the Ghent University Hospital (Belgium) and UK ethical boards. Genomic DNA was also extracted, with informed written consent, from the blood of 100 fertile individuals (50 male and 50 female) undergoing pre-implantation genetic diagnosis at Reprogenetics UK, Oxford. All fertile individuals and OAD patients hailed from a range of ethnic backgrounds.

Molecular analysis of the PLCζ gene

Amplicons were generated for patientH398P, four family members of patientH398P and eight further infertile males, by PCR using the High Fidelity Master Kit (Roche Biosciences, UK) and primer sequences/thermal cycling conditions previously described by Heytens et al. (2009). Each exon of the PLCζ gene was amplified from genomic DNA and amplicon nucleotides were sequence determined (Geneservice, UK). Sequenced amplicons were compared with the published PLCζWT ORF sequence (accession number NM_033123). In contrast to the analyses carried out by Heytens et al. (2009), the current study aimed to investigate whether sequence anomalies were homozygous or heterozygous in nature. In order to do this, sequencing peaks were analysed using Chromas Lite freeeware (Technelysium Ltd., Australia).

cRNA synthesis

PLCζWT and PLCζH398P cRNA was synthesized as described previously by Heytens et al. (2009). PLCζH433 was created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) following the manufacturer’s instructions, using a molecular construct containing PLCζWT as the template (forward primer: 5’-TTATCCGAACCTATCAAGATGATGATCAT-3’; reverse primer: 5’-ATGATGCGTCATGCATGATGATGCTATTG-3’). cRNA was stored in single-use aliquots at −80°C until use.
Mouse oocyte collection, culture and micro-injection and Ca\(^{2+}\) imaging

Metaphase II stage oocytes were collected, and cumulus cells removed as previously described (Kurokawa et al., 2005). Denuded oocytes were placed in 50 μl drops of potassium simplex optimized medium (Specialty Media, Phillipsburg, NJ, USA) containing 0.1% polyvinyl alcohol (Sigma, USA) until oocytes were micro-injected with cRNA corresponding to PLC\(_{\text{z}}\) WT or PLC\(_{\text{H233L}}\) (at two separate concentration sets: 344 and 352 ng/μl; 172 and 176 ng/μl, respectively) as described by Kurokawa et al. (2005). Animal handling and procedures were approved by the University of Massachusetts IACUC committee. Intracellular Ca\(^{2+}\) response within injected mouse oocytes was measured as previously detailed (Kurokawa et al., 2005). Ca\(^{2+}\) changes were registered, and filter wheel changes controlled, by SimplePCI software (C-Imaging System, Cranberry Township, PA, USA). Statistical analysis was performed using Graphpad Prism software (GraphPad, USA). Correlations between the amplitude of the first Ca\(^{2+}\) peak, as well as the interval between the first and second Ca\(^{2+}\) peaks following micro-injection of WT and H233L cRNA at both concentrations were determined using the t-test. Data are presented as mean ± SEM and a P ≤ 0.05 was considered statistically significant.

TaqMan single-nucleotide polymorphism genotyping assay

Two genotyping assays employing sequence detection probes were developed using the ‘Custom TaqMan assay design tool’ (Applied Biosystems, UK) and used to screen a population of 100 fertile individuals for the presence of the H233L and H398P mutations, as well as to confirm the inheritance patterns of the H233L and H398P mutations in the patient and his family. The resultant assays were carried out using GTXpress mastermix (Applied Biosystems, UK), with thermal cycling undertaken on a StepOne real-time PCR system (Applied Biosystems, UK). This involved a pre-PCR read at 25°C for 30 s, followed by a DNA polymerase activation step at 95°C for 20 s. This was followed by 40 or 35 cycles (H233L and H398P, respectively) of denaturing and annealing/extension at 95 or 92°C (H233L and H398P, respectively) for 3 s, and 60 or 62°C (H233L and H398P, respectively) for 20 s, at the end of which another read was obtained. A post-PCR read was then obtained at 25°C for 30 s. Results were analysed using StepOne v2.1 software and TaqMan Genotyper software (Applied Biosystems, UK).

Results

Identification of a second novel PLC\(_{\text{z}}\) mutation

In addition to PLC\(_{\text{z}}\) H398P, described previously by Heytens et al. (2009), we identified a base change in the PLC\(_{\text{z}}\) gene from the same infertile patient resulting in the substitution of an adenine (A) nucleotide at position 698 of the ORF sequence to a thymine (T) nucleotide, corresponding to the substitution of a histidine residue for a leucine residue in the catalytic X domain at position 233 (H233L) of the amino acid sequence (Fig. 1A). A 3D model of PLC\(_{\text{z}}\) WT and PLC\(_{\text{H233L}}\) protein (Fig. 1B) was created based upon the known structure of PLCdelta (PLCδ6), which predicted that the PLC\(_{\text{H233L}}\) mutation is likely to reduce the number of neuring leukine acid contacts of this residue, thus disrupting local protein folding.

Micro-injection experiments further demonstrated that 100% of oocytes (16/16) exhibited Ca\(^{2+}\) oscillations in a pattern characteristic of fertilization (>8 spikes per hour), when injected with PLC\(_{\text{z}}\) WT cRNA, and resulted in the activation of 100% (8/8) of injected oocytes (from separate experiments). In contrast, while 67% of total oocytes (12/18) injected with PLC\(_{\text{H233L}}\) cRNA elicited oscillations, these were severely altered in frequency and exhibited reduced amplitude, differing markedly from those normally observed at fertilization, with none of the oocytes injected with PLC\(_{\text{H233L}}\) cRNA (0/8, from separate experiments) activating. While the amplitude of the first Ca\(^{2+}\) peak was significantly reduced (P ≤ 0.05) following injection of H233L cRNA (~0.1 arbitrary units; a.u) compared with oocytes injected with WT cRNA (~0.25 a.u) at concentrations of 352 and 344 ng/μl, respectively, the interval between the first and second Ca\(^{2+}\) peaks following injection of H233L cRNA (~50 min) did not differ significantly from that after injection of WT cRNA (~40 min) at these concentrations (Fig. 2). However, injection of WT and H233L cRNA in mouse oocytes at a reduced concentration (172 and 176 ng/μl, respectively) resulted in a significantly decreased (P ≤ 0.05) amplitude of the first Ca\(^{2+}\) peak following H233L cRNA injection (~0.15 a.u) when compared with WT cRNA injection (~0.3 a.u), as well as a significantly increased (P ≤ 0.05) interval between the first and second Ca\(^{2+}\) peaks (~20 and ~110 min, respectively) following H233L when compared with WT cRNA at these concentrations (Fig. 3).

Genetic modes of inheritance for the H233L and H398P PLC\(_{\text{z}}\) mutations

Mini-sequencing of genomic DNA from the patient revealed that PLC\(_{\text{H233L}}\) and PLC\(_{\text{H398P}}\) were heterozygous in nature (Fig. 4A). The patient’s mother did not possess PLC\(_{\text{H398P}}\), but was heterozygous for PLC\(_{\text{H233L}}\). The father, however, was heterozygous for PLC\(_{\text{H398P}}\). Interestingly, the patient’s daughter did not possess PLC\(_{\text{H398P}}\), but was heterozygous for PLC\(_{\text{H233L}}\). The patient’s half-brother did not possess either mutation (Fig. 4B).

Genetic screening of the prevalence of H233L and H398P in a wider patient population

Single-nucleotide polymorphism (SNP) assays did not detect either PLC\(_{\text{H233L}}\) or PLC\(_{\text{H398P}}\) in 100 fertile individuals (200 chromosomes), or eight OAD patients (six patients with globozoospermia and abnormal sperm morphology and two patients exhibiting normal sperm morphology). SNP assays further confirmed that the patient, his mother and his daughter were all heterozygous for PLC\(_{\text{H233L}}\), and that his half-brother and father do not carry the H233L mutation. However, analyses further confirmed that the patient’s father is heterozygous for the H398P mutation (Fig. 5).

Discussion

Increasing evidence suggests that dysfunctional forms of the oocyte activation factor PLC\(_{\text{z}}\) may underlie certain types of male factor infertility, with accumulating evidence supporting the therapeutic use of PLC\(_{\text{z}}\) to treat cases of OAD (Yoon et al., 2008; Heytens et al., 2009). Taylor et al. (2010) recently showed that the micro-injection of OAD sperm expressing abnormal levels of PLC\(_{\text{z}}\), coincident with a Ca\(^{2+}\) ionophore resulted in high rates of human oocyte activation,
resulting in the birth of a healthy child. Collectively, these studies provide significant support for the clinical application of a recombinant active form of PLCζ as a safer and more endogenous alternative to the artificial activating agents used at present. However, we still know relatively little as to how defective genetic mechanisms associated with PLCζ may underlie some forms of OAD. The discovery of the H398P mutation in PLCζ from a patient with OAD established the first link between PLCζ mutation and human infertility (Heytens et al., 2009). However, the heterozygous nature of this mutation left unresolved the question of how this mutation could result in infertility (Heytens et al., 2009; Nomikos et al., 2011), and the mode of inheritance associated with this mutation remained unknown.

A second novel mutation in PLCζ

Using a modified version of the genetic screening method employed by Heytens et al. (2009), the present study reports the identification and functional characterization of a novel second mutation (PLCζ^{H233L}) from the same patient from whom PLCζ^{H398P} was first identified. Interestingly, while histidine is a polar and hydrophilic amino acid, leucine is a non-polar hydrophobic amino acid. Therefore, the PLCζ^{H233L} mutation is likely to disrupt local interactions within protein folding, in a manner similar to PLCζ^{H398P}. Indeed, 3D modelling predicts that the histidine at position 233 of the PLCζ amino acid sequence may serve a critical role in the maintenance of protein structure by holding the adjacent protein helix in place, and/or stabilising the position of a loop at the bottom of its adjacent helix. However, micro-injection of PLCζ^{H233L} cRNA into mouse oocytes resulted in at least some minor Ca^{2+} transients. Heytens et al. (2009) demonstrated that injection of WT cRNA at a concentration of 100 μg/μl resulted in Ca^{2+} release in a manner similar to that at fertilization. Here, we show that the micro-injection of cRNA corresponding to PLCζ^{WT} at a lower concentration (172 ng/μl) resulted in a similar pattern of Ca^{2+} release, as well as resulting in a high rate of oocyte activation. Micro-injection of cRNA corresponding to PLCζ^{H233L} at a similar concentration (176 ng/μl) resulted in an abnormal Ca^{2+} release profile, with the amplitude of the first Ca^{2+} peak significantly decreased, and the interval between the first and second Ca^{2+} peaks being significantly increased following H233L cRNA injection compared with WT cRNA injection, along with failure to activate oocytes. However, while the injection of H233L cRNA at an increased concentration (352 ng/μl) resulted in no significant difference in the interval between the first and second Ca^{2+} peaks compared with WT cRNA injection at a similar concentration (344 ng/μl), the amplitude of the first Ca^{2+} peak was significantly reduced compared with WT cRNA injected oocytes. Collectively, the observed patterns of

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Figure 1 (A) Schematic representation of PLCζ structure showing the locations of PLCζ^{H233L} and PLCζ^{H398P} within the X and Y domains, respectively. EF, EF hands; X, X-domain; Y, Y-domain; C2, C2 domain. (B) Three-dimensional model of (i) PLCζ^{WT} and (ii) PLCζ^{H233L} protein, focusing upon H233 and the effect of H233L upon local protein interactions. Following mutation, the aromatic residue (Phe^{268}) present in PLCζ^{WT} is not in contact with L233 which only maintains contact to the backbone oxygen of Thr^{267}. 
intracellular $\text{Ca}^{2+}$ release indicated that some degree of functional activity is retained by PLC$\varepsilon^{H233L}$, and that the functional deficit is not as severe as that conferred by PLC$\varepsilon^{H398P}$, or its mouse equivalent H435P which totally abolished the ability of PLC$\varepsilon$ to trigger $\text{Ca}^{2+}$ oscillations in the mouse oocyte and prevented enzyme activity in a PIP2 hydrolysis assay (Nomikos et al., 2011). While H398 is highly conserved among all known PLC$\varepsilon$ isoforms across a range of species (Heytens et al., 2009), H233 is not (data not shown). However, considering the abnormal profile of $\text{Ca}^{2+}$ oscillations evident upon injection of PLC$\varepsilon^{H233L}$ cRNA into mouse oocytes, which were insufficient for activation, it is clearly apparent that PLC$\varepsilon^{H233L}$ may exert reduced functionality, or possibly reduced efficiency in substrate binding, indicating that both mutations contributed towards the patient’s infertility.

The discovery of PLC$\varepsilon^{H233L}$ from the same patient implies that the contribution of mutated PLC$\varepsilon$ protein to the patient’s infertile status is more complex than previously thought. We show that both mutations possess different parental origins, with PLC$\varepsilon^{H398P}$ inherited from the patient’s father, and PLC$\varepsilon^{H233L}$ inherited from the mother. To our knowledge, this represents the first description of an autosomal point mutation to have been passed from a mother to her son, resulting in a deficit of sperm function and male infertility.

**A maternally inherited mutation contributes to male infertility**

While it is difficult to ascertain specific global causative factors for infertility, male factor and idiopathic (unknown) infertility are thought to be major contributors (each occurring in $\sim$28% of cases; Kashir et al., 2010). Accumulating evidence suggests that abnormalities in testicular gene expression may contribute towards idiopathic male infertility.

![Figure 2](image_url)

**Figure 2** $\text{Ca}^{2+}$ traces following micro-injection of cRNA corresponding to PLC$\varepsilon^{WT}$ (A) and PLC$\varepsilon^{H233L}$ (B) at concentrations of 344 and 352 ng/µl, respectively. The histograms represent the average amplitude of the first $\text{Ca}^{2+}$ rise (C) and the average interval between the first and second $\text{Ca}^{2+}$ peaks (D) following micro-injection of WT and H233L cRNA at the aforementioned concentrations. The asterisk (*) on the histogram denotes a statistically significant difference ($P \leq 0.05$). Data are represented as mean ± SEM.
infertility (Kashir et al., 2010). Major known causes of human male infertility include Y chromosome-linked abnormalities and microdeletions which are not associated with a particular syndrome, but lead to impaired spermatogenesis, and mutations in X chromosome-linked genes such as *Akap4*, *Nxf2* and *Tex1* involved in spermatogenesis (Wang and Pan, 2007; Zheng et al., 2010). Due to the single copy, hemizygous nature of these genes, damaging mutations would not be masked by a normal allele (Zheng et al., 2010).

However, screening for X-linked gene mutations in infertile men has yet to reveal any causative factors (Zheng et al., 2010). Furthermore, specific genes have not been identified for a number of autosomal male factor conditions. One such example is globozoospermia, an infertile condition involving round-headed and acrosome-less sperm, estimated to affect \( \sim 0.1\% \) of infertile men (Dam et al., 2007). While numerous studies indicate that globozoospermia may be a multi-factorial genetic syndrome (Kullander and Rausing, 1975; Flörke-Gerloff et al., 1984; Dale et al., 1994; Kilani et al., 2004; Heindryckx et al., 2005), the specific mode of inheritance remains unclear, although a mutation in the SPATA16 gene has been implicated (Dam et al., 2007). One further example is the CFTR gene, thought to be mutated in 60–90% of patients presenting with congenital bilateral absence of the vas deferens, a type of obstructive azoospermia (O’Flynn O’Brien et al., 2010). While this may be considered a maternally inherited autosomal disorder, the problem in this case is abnormal testicular structure instead of a biochemical deficit.

**Figure 3** Ca\(^{2+}\) traces following micro-injection of cRNA corresponding to PLC\(\xi^{WT}\) (A) and PLC\(\xi^{H233L}\) (B) at concentrations of 172 and 176 ng/\(\mu l\), respectively. The histograms represent the average amplitude of the first Ca\(^{2+}\) rise (C) and the average interval between the first and second Ca\(^{2+}\) peaks (D) following micro-injection of WT and H233L cRNA at the aforementioned concentrations. The asterisk (*) on the histograms denotes a statistically significant difference (\(P \leq 0.05\)). Data are represented as mean ± SEM.
Our data suggest that studies investigating the role of genes involved in male infertility should involve not only the father of an infertile male, but also the mother, since it now appears that infertile males may inherit mutated genes from either parent. Indeed, Sazci et al. (2005) described a case in which a son inherited a translocation (t(1;13)(q24;q10)) from his mother, associated with primary infertility in the son, but not the mother. This translocation was associated with a normal phenotype, but was theorized to affect meiosis during spermatogenesis, leading to azoospermia. While causing infertility, this was a chromosomal structural defect, and not an autosomal point-mutation as the one we report herein. Considering that many germ cell-specific genes are also localized at autosomes (Sazci et al., 2005), our study serves to demonstrate that male infertility may be more multi-factorial and complex in nature than previously thought. Indeed, it seems apparent that PLCζ mutations may contribute not only to male infertility but also to male sub-fertility.

Since the patient’s daughter has inherited PLCζH233L, it is possible that if she has male progeny in future, they may also inherit the same mutation and thus experience sub-fertility. Furthermore, our study indicates that the incidence of both PLCζH233L and PLCζH398P is less than 1 in 100 individuals (200 chromosomes) within a general population, and the mutations were not identified in a small group of patients diagnosed with oocyte activation deficiency, suggesting that these particular mutations may be relatively rare, or even unique. However, the small size of the OAD patient population within this study is insufficient to draw specific conclusions regarding

**Figure 4** (A) Mini-sequencing profiles of genomic DNA from the infertile patient exhibiting PLCζ mutation (i) the presence of adenine and thymine peaks indicating that PLCζH233L is heterozygous, and (ii) the presence of adenine and cytosine peaks indicating that PLCζH398P is heterozygous. Green peak: adenine; black peak: cytosine; red peak: thymine. Y-axis: relative fluorescence units; X-axis: amplicon base number. (B) Pedigree showing the inheritance of PLCζH233L and PLCζH398P in the infertile patient and his immediate family.
the prevalence of these two mutations within the general ICSI failed patient population, and future studies should attempt to recruit a much larger cohort of OAD patients.

It is worth noting however, that six of these patients suffered from globozoospermia, a condition linked to a suspected genetic syndrome which results in the formation of sperm with abnormal morphology and without acrosomes (Dam et al., 2007). It is therefore important that future mutation studies should predominantly target males exhibiting oocyte activation failure, without globozoospermia and exhibiting normal sperm morphology. Such studies should attempt to correlate genetic analysis to the specific pattern of PLCζ localization in the sperm head. While Grasa et al. (2008) recently mapped the distribution of PLCζ to acrosomal, equatorial and post-acrosomal regions of fertile human sperm, it remains possible that structural changes of the PLCζ protein, occurring as a result of genetic mechanisms, may result in abnormal expression or localization patterns with associated functional deficit. Indeed, high-resolution immunogold electron microscopy studies have highlighted important roles for the acrosomal membrane and perinuclear acroplaxome in harbouring a large number of structural and functional proteins (Kierszenbaum et al., 2011); mutations of which may lead to defective sperm function and male infertility.

**Mutations in PLCζ may function in a recessive manner**

It is interesting to note that while the patient’s father was heterozygous for PLCζ<sup>H398P</sup>, the patient was conceived naturally without the use of ART. Since PLCζ<sup>H398P</sup> and its mouse equivalent have been demonstrated to abolish the Ca<sup>2+</sup> inducing ability of PLCζ (Heytens et al., 2009; Nomikos et al., 2011), the natural conception of the patient demonstrates that loss of activity mutations in PLCζ need to be present on both maternal and paternal alleles for complete infertility to occur. While speculative at this time, the most likely scenario by which sperm from the patient’s father was able to successfully fertilize oocytes from the mother was if the fertilizing sperm contained normal PLCζ protein in addition to PLCζ<sup>H398P</sup> DNA.

Even though the first point of PLCζ translation during human spermatogenesis has yet to be identified, recent studies indicate that PLCζ is first detectable at the round spermatid stage in hamster (Young et al., 2009) and equine (Bedford-Guas et al., 2011) models. The possible interchange of mRNA transcripts via cytoplasmic bridges in developing spermatids and spermatocytes may have led to the presence of functional WT PLCζ protein within spermatids and spermatocytes containing mutant PLCζ (Dadoune et al., 2004; Heytens et al., 2009). It is also a possibility that PLCζ transcription occurs throughout the meiotic phase of spermatogenesis, potentially resulting in mixed populations of PLCζ transcripts derived from both maternal and paternal alleles (for reviews, see Eddy, 2002; Dadoune et al., 2004; Bettegowda and Wilkinson, 2010).

In summary, we have identified a novel second mutation (PLCζ<sup>H233L</sup>) of the oocyte activation factor PLCζ in an infertile patient, and demonstrate that both PLCζ<sup>H233L</sup> and PLCζ<sup>H398P</sup> mutations are likely to be rare. We propose that these mutations function in a recessive manner. Our findings demonstrate for the first time, that the maternal inheritance of autosomal point mutations in male-specific proteins may result in certain forms of male factor infertility, highlighting the importance of studying maternal inheritance within such conditions. Collectively, our findings not only further support the physiological importance of PLCζ in the oocyte activation process, but also represent a considerable advancement in our understanding of the modes of inheritance of male infertility, and shed significant new light as to how such conditions may arise.

**Authors’ roles**

J.K. and M.K. were responsible for the majority of the experimental work described herein. C.J. was also involved in several aspects of experimental work and data analysis. H.C.L. performed cRNA injections in mouse oocytes and was involved in data analysis along with R.A.F. B.L. was involved in several aspects of experimental work as part of a student research project. R.H. and C.M.D. performed bioinformatic modelling of protein structure. B.H., P.D.S. and D.W. were responsible for sample acquisition from patients and for clinical interpretation. J.K., C.J. and K.C. were responsible for the majority of concept design, analysis and interpretation of data, along with contributions by M.K., D.W. and J.P. All authors were involved in manuscript preparation and K.C. approved the final submitted version.

**Acknowledgements**

The authors thank the patient and his family who kindly consented to participate in this study. The authors also thank Mr Samer AlFarawati and Dr Zhongwei Huang for critical and intellectual discussion, and
Dr Elke Heytens and Miss Claire Young for initial sample acquisition and processing respectively.

**Funding**

The research described herein was funded by project grants from the Royal Society (UK) and the Oxford University Medical Research Fund awarded to K.C. J.K. was funded by a Departmental Studentship (Nuffield Department of Obstetrics and Gynaecology, University of Oxford). D.W. was funded by the NIHR Biomedical Research Centre Programme. Research in R.A.F. lab was supported by a grant from the National Research Initiative Competitive Grant Program from the US Department of Agriculture (2007-35203-17840), and by grant HD051872 from the NIH. P.D.S. is a holder of a fundamental clinical research mandate by the Flemish Foundation of Scientific Research (FWO-Vlaanderen).

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