

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

Junaid Kashir¹, Michalis Konstantinidis¹, Celine Jones¹,
Bernadette Lemmon¹, Hoi Chang Lee², Rebecca Hamer³,
Bjorn Heindryckx⁴, Charlotte M. Deane³, Petra De Sutter⁴,
Rafael A. Fissore², John Parrington⁵, Dagan Wells¹,
and Kevin Coward^{1,*}

¹Nuffield Department of Obstetrics and Gynaecology, Level 3, Women's Centre, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK ²Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA, USA ³Department of Statistics, University of Oxford, South Parks Road, Oxford OX1 3TG, UK ⁴Department for Reproductive Medicine, Ghent University Hospital, De Pintelaan 185, Ghent 9000, Belgium ⁵Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK

*Correspondence address. E-mail: kevin.coward@obs-gyn.ox.ac.uk

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, PLCzeta (PLC ζ). 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 (PLC ζ ^{H398P}), leading to abnormal PLC ζ function and infertility.

METHODS AND RESULTS: Here, using a combination of direct-sequencing and mini-sequencing of the PLC ζ gene from the patient and his family, we report the identification of a second PLC ζ mutation in the same patient resulting in a histidine to leucine substitution at position 233 (PLC ζ ^{H233L}), which is predicted to disrupt local protein interactions in a manner similar to PLC ζ ^{H398P} and was shown to exhibit abnormal calcium oscillatory ability following predictive 3D modelling and cRNA injection in mouse oocytes respectively. We show that PLC ζ ^{H233L} and PLC ζ ^{H398P} 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 PLC ζ 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 / phospholipase C zeta (PLCzeta) / 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 (Ca²⁺) 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 Ca²⁺ oscillations which begin soon after gamete fusion and persist beyond the completion of meiosis (Jones, 2005). Mounting evidence suggests that the agent responsible for Ca²⁺ oscillations within activating oocytes is a sperm-specific phospholipase C, phospholipase C zeta (PLC ζ), located on chromosome 12 in humans (Cox *et al.*, 2002; Saunders *et al.*, 2002,

2007; Knott *et al.*, 2005; Kouchi *et al.*, 2005; Swann *et al.*, 2006; Partridge *et al.*, 2007; Kashir *et al.*, 2010).

Given the fundamental role of PLC ζ in activating the oocyte following gamete fusion, PLC ζ 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 (Evens, 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 infertile 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 PLC ζ 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 PLC ζ 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 PLC ζ mRNA (Yoon *et al.*, 2008), thereby providing significant support for the idea of using PLC ζ as a novel therapeutic agent for OAD. Heytens *et al.* (2009) provided the first genetic link to PLC ζ deficiency by identifying a substitution mutation in an infertile male diagnosed with OAD. This mutation occurred within the Y domain of the active site at position 398 of the PLC ζ open reading frame (ORF), resulting in a histidine to proline substitution (PLC ζ ^{H398P}).

Injection of PLC ζ ^{H398P} 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 PLC ζ ^{H398P}, resulting in a histidine to proline substitution at position 435 of the mouse PLC ζ ORF (PLC ζ ^{H435P}), 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 PLC ζ ^{H398P} 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 PLC ζ ^{H398P} to act in such a manner was not clearly demonstrated by this study. Subsequently, a study of PLC ζ ^{H435P} showed that the injection of wild-type PLC ζ (PLC ζ ^{WT}) elicited a normal Ca²⁺ oscillation profile upon micro-injection in mouse oocytes in the presence of a higher concentration (10 times) of the PLC ζ H435P mutant (Nomikos *et al.*, 2011), indicating that PLC ζ ^{H398P} 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 PLC ζ ^{H398P} 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 PLC ζ gene from the patient in which the H398P mutation was discovered. We report the identification of a second novel PLC ζ mutation in the patient possessing PLC ζ ^{H398P}, and analyse the functional effects of the new mutant form of PLC ζ in comparison with PLC ζ ^{WT} and PLC ζ ^{H398P}. Crucially, we investigate the genetic origin and mode of inheritance of PLC ζ 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 PLC ζ ^{H398P} had been previously identified (patient^{H398P}; 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 patient^{H398P} (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 PLC ζ gene

Amplicons were generated for patient^{H398P}, four family members of patient^{H398P} 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 PLC ζ gene was amplified from genomic DNA and amplicon nucleotides were sequence determined (Geneservice, UK). Sequenced amplicons were compared with the published PLC ζ ^{WT} 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 free-ware (Technelysium Ltd., Australia).

cRNA synthesis

PLC ζ ^{WT} and PLC ζ ^{H398P} cRNA was synthesized as described previously by Heytens *et al.* (2009). PLC ζ ^{H233L} was created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) following the manufacturer's instructions, using a molecular construct containing PLC ζ ^{WT} as the template (forward primer: 5'-TTATCCAAGCTATACTCAAGTATGCATTAT-3'; reverse primer: 5'-ATGAATGCATACTTGAGTATAGCTTGGATAA-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 injection. Oocytes were micro-injected with cRNA corresponding to $\text{PLC}\zeta^{\text{WT}}$ or $\text{PLC}\zeta^{\text{H233L}}$ (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 \pm SEM and a $P \leq 0.05$ 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 $\text{PLC}\zeta$ mutation

In addition to $\text{PLC}\zeta^{\text{H398P}}$, described previously by Heytens et al. (2009), we identified a base change in the $\text{PLC}\zeta$ 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 $\text{PLC}\zeta^{\text{WT}}$ and $\text{PLC}\zeta^{\text{H233L}}$ protein (Fig. 1B) was created based upon the known structure of $\text{PLC}\delta$ (PLC δ), which predicted that the $\text{PLC}\zeta^{\text{H233L}}$ mutation is likely to reduce the number of neighbouring amino 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 $\text{PLC}\zeta^{\text{WT}}$ 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 $\text{PLC}\zeta^{\text{H233L}}$ 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 $\text{PLC}\zeta^{\text{H233L}}$ cRNA (0/8; from separate experiments) activating. While the amplitude of the first Ca^{2+} peak was significantly reduced ($P \leq 0.05$) 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 ($P \leq 0.05$) 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 ($P \leq 0.05$) 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 $\text{PLC}\zeta$ mutations

Mini-sequencing of genomic DNA from the patient revealed that $\text{PLC}\zeta^{\text{H233L}}$ and $\text{PLC}\zeta^{\text{H398P}}$ were heterozygous in nature (Fig. 4A). The patient's mother did not possess $\text{PLC}\zeta^{\text{H398P}}$, but was heterozygous for $\text{PLC}\zeta^{\text{H233L}}$. The father, however, was heterozygous for $\text{PLC}\zeta^{\text{H398P}}$. Interestingly, the patient's daughter did not possess $\text{PLC}\zeta^{\text{H398P}}$, but was heterozygous for $\text{PLC}\zeta^{\text{H233L}}$. 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 $\text{PLC}\zeta^{\text{H233L}}$ or $\text{PLC}\zeta^{\text{H398P}}$ 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 $\text{PLC}\zeta^{\text{H233L}}$, 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 $\text{PLC}\zeta$ may underlie certain types of male factor infertility, with accumulating evidence supporting the therapeutic use of $\text{PLC}\zeta$ 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 $\text{PLC}\zeta$, coincident with a Ca^{2+} ionophore resulted in high rates of human oocyte activation,

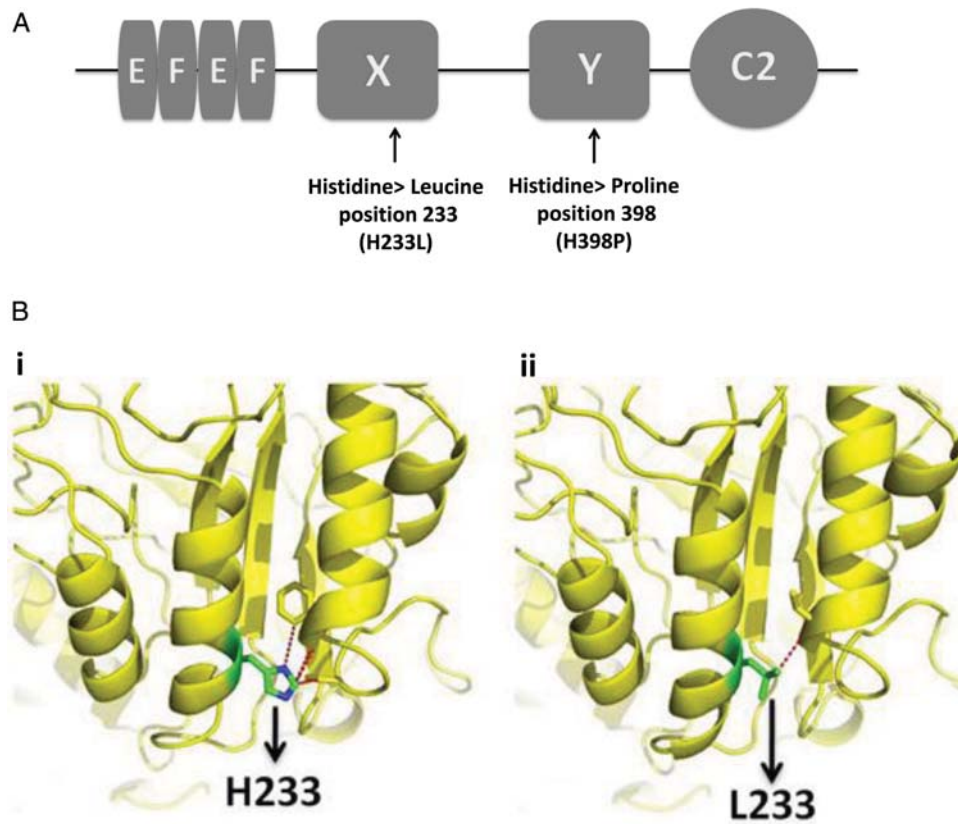


Figure 1 (A) Schematic representation of PLC ζ structure showing the locations of PLC ζ ^{H233L} and PLC ζ ^{H398P} within the X and Y domains, respectively. EF, EF hands; X, X-domain; Y, Y-domain; C2, C2 domain. (B) Three-dimensional model of (i) PLC ζ ^{WT} and (ii) PLC ζ ^{H233L} protein, focusing upon H233 and the effect of H233L upon local protein interactions. Following mutation, the aromatic residue (Phe²⁶⁸) present in PLC ζ ^{WT} is not in contact with L233 which only maintains contact to the backbone oxygen of Thr²⁶⁷.

resulting in the birth of a healthy child. Collectively, these studies provide significant support for the clinical application of a recombinant active form of PLC ζ 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 PLC ζ may underlie some forms of OAD. The discovery of the H398P mutation in PLC ζ from a patient with OAD established the first link between PLC ζ 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 PLC ζ

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 (PLC ζ ^{H233L}) from the same patient from whom PLC ζ ^{H398P} was first identified. Interestingly, while histidine is a polar and hydrophilic amino acid, leucine is a non-polar hydrophobic amino acid. Therefore, the PLC ζ ^{H233L} mutation is likely to disrupt local interactions within protein folding, in a manner similar to PLC ζ ^{H398P}. Indeed, 3D modelling

predicts that the histidine at position 233 of the PLC ζ 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 PLC ζ ^{H233L} cRNA into mouse oocytes resulted in at least some minor Ca²⁺ transients. Heytens *et al.* (2009) demonstrated that injection of WT cRNA at a concentration of 100 $\mu\text{g}/\mu\text{l}$ resulted in Ca²⁺ release in a manner similar to that at fertilization. Here, we show that the micro-injection of cRNA corresponding to PLC ζ ^{WT} at a lower concentration (172 ng/ μl) resulted in a similar pattern of Ca²⁺ release, as well as resulting in a high rate of oocyte activation. Micro-injection of cRNA corresponding to PLC ζ ^{H233L} at a similar concentration (176 ng/ μl) resulted in an abnormal Ca²⁺ release profile, with the amplitude of the first Ca²⁺ peak significantly decreased, and the interval between the first and second Ca²⁺ 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 Ca²⁺ peaks compared with WT cRNA injection at a similar concentration (344 ng/ μl), the amplitude of the first Ca²⁺ peak was significantly reduced compared with WT cRNA injected oocytes. Collectively, the observed patterns of

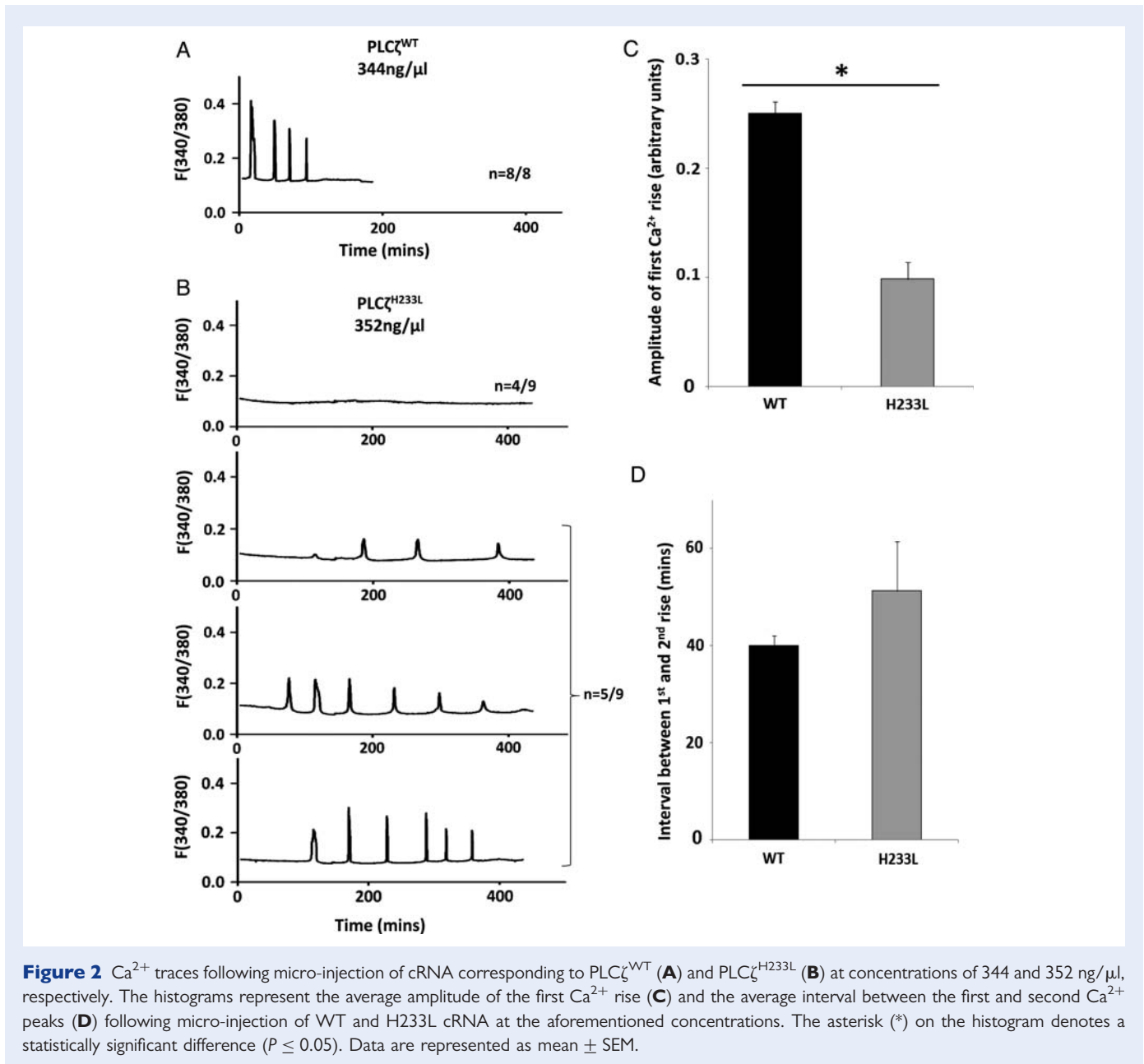


Figure 2 Ca^{2+} traces following micro-injection of cRNA corresponding to PLC ζ^{WT} (A) and PLC ζ^{H233L} (B) at concentrations of 344 and 352 ng/ μ l, respectively. The histograms represent the average amplitude of the first Ca^{2+} rise (C) and the average interval between the first and second Ca^{2+} peaks (D) following micro-injection of WT and H233L cRNA at the aforementioned concentrations. The asterisk (*) on the histogram denotes a statistically significant difference ($P \leq 0.05$). Data are represented as mean \pm SEM.

intracellular Ca^{2+} release indicated that some degree of functional activity is retained by PLC ζ^{H233L} , and that the functional deficit is not as severe as that conferred by PLC ζ^{H398P} , or its mouse equivalent H435P which totally abolished the ability of PLC ζ to trigger 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 ζ isoforms across a range of species (Heytens et al., 2009), H233 is not (data not shown). However, considering the abnormal profile of Ca^{2+} oscillations evident upon injection of PLC ζ^{H233L} cRNA into mouse oocytes, which were insufficient for activation, it is clearly apparent that PLC ζ^{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 ζ^{H233L} from the same patient implies that the contribution of mutated PLC ζ protein to the patient's infertile status is

more complex than previously thought. We show that both mutations possess different parental origins, with PLC ζ^{H398P} inherited from the patient's father, and PLC ζ^{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

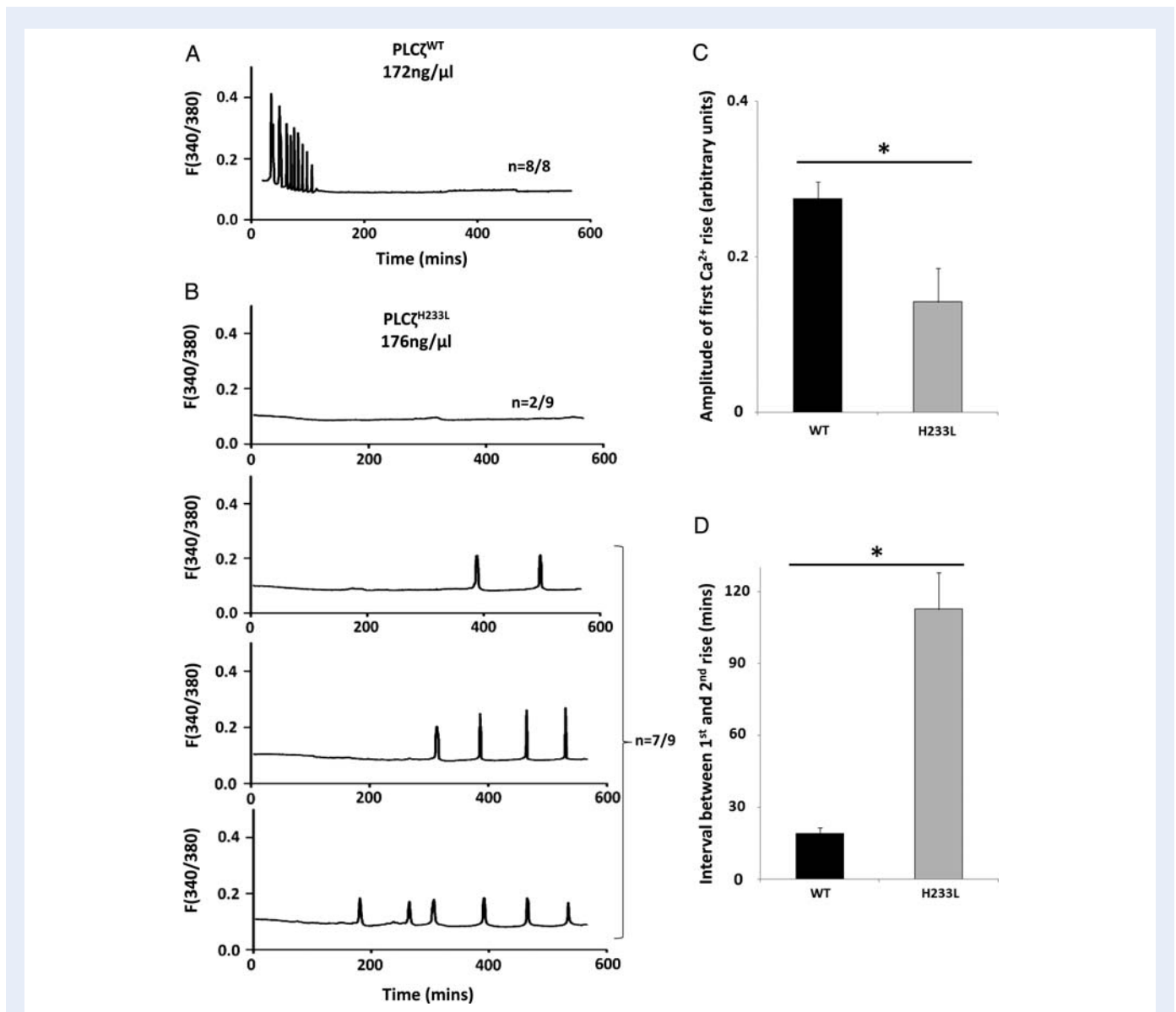


Figure 3 Ca²⁺ traces following micro-injection of cRNA corresponding to PLC ζ ^{WT} (A) and PLC ζ ^{H233L} (B) at concentrations of 172 and 176 ng/μl, respectively. The histograms represent the average amplitude of the first Ca²⁺ rise (C) and the average interval between the first and second Ca²⁺ peaks (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 \pm SEM.

infertility (Kashir *et al.*, 2010). Major known causes of human male infertility include Y chromosome-linked abnormalities and micro-deletions 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 *Tex11* 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 ~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.

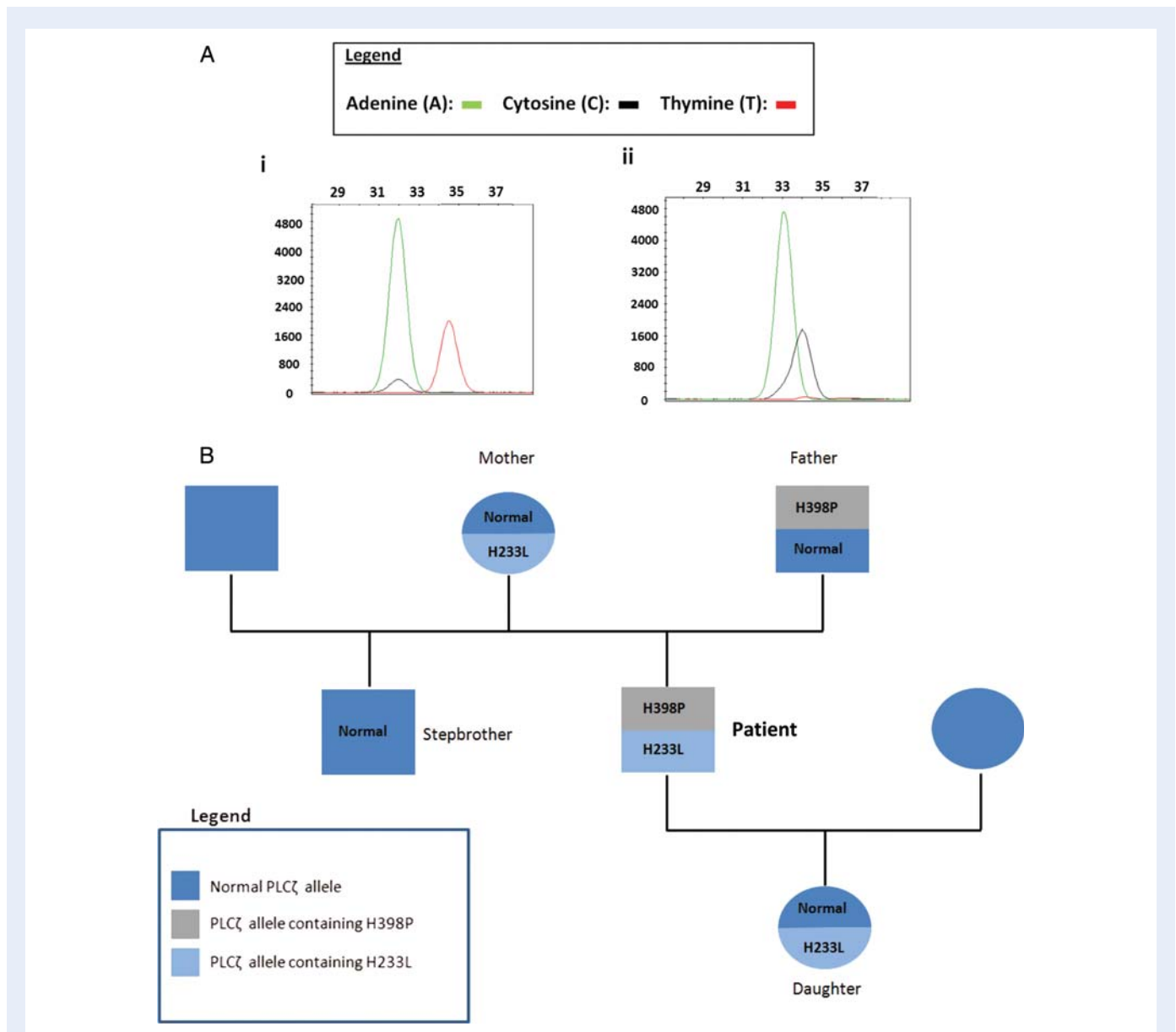


Figure 4 (A) Mini-sequencing profiles of genomic DNA from the infertile patient exhibiting PLC ζ mutation (i) the presence of adenine and thymine peaks indicating that PLC ζ^{H233L} is heterozygous, and (ii) the presence of adenine and cytosine peaks indicating that PLC ζ^{H398P} is heterozygous. Green peak: adenine; black peak: cytosine; red peak: thymine. Y-axis: relative fluorescence units; X-axis: amplicon base number. (B) Pedigree showing the inheritance of PLC ζ^{H233L} and PLC ζ^{H398P} in the infertile patient and his immediate family.

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 PLC ζ mutations may contribute not only to male infertility but also to male sub-fertility.

Since the patient's daughter has inherited PLC ζ^{H233L} , 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 PLC ζ^{H233L} and PLC ζ^{H398P} 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

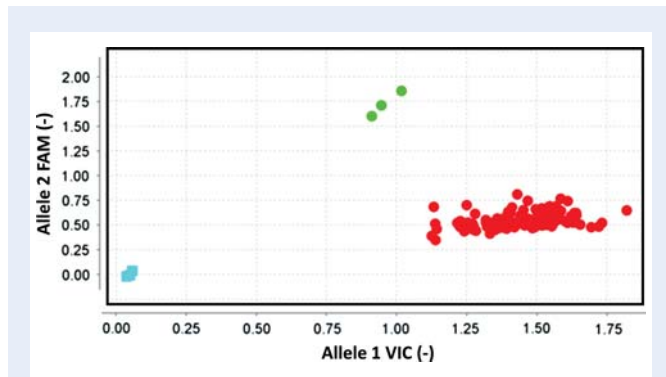


Figure 5 Representative scatter plot indicating the normalized fluorescent intensities of the reporter dyes from each sample, showing screening results for 100 fertile individuals and the patient and his family for PLC $\zeta^{\text{H}233\text{L}}$ and PLC $\zeta^{\text{H}398\text{P}}$. Light blue squares represent negative controls, green dots represent heterozygous individuals (one normal PLC ζ allele, and one mutated PLC ζ allele), while red dots indicated homozygous unaffected individuals (both alleles contain normal PLC ζ). Allele 1 (x-axis) represents the fluorescence intensity of the normal allele labelled with VICTM (red), while allele 2 (y-axis) represents the fluorescence intensity of the mutant allele labelled with carboxyfluorescein (FAM) (blue). A homozygous sample for allele 1 would result in the display of fluorescence intensity for allele 1 (x-axis) and nothing for allele 2 (y-axis). The converse is true for samples that are homozygous for allele 2. A heterozygous sample, resulting in the binding of both probes to the template DNA, results in fluorescence from both reporter dyes (VIC/FAM), which is shown as green on the plot.

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 PLC ζ localization in the sperm head. While Grasa *et al.* (2008) recently mapped the distribution of PLC ζ to acrosomal, equatorial and post-acrosomal regions of fertile human sperm, it remains possible that structural changes of the PLC ζ 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 PLC ζ may function in a recessive manner

It is interesting to note that while the patient's father was heterozygous for PLC $\zeta^{\text{H}398\text{P}}$, the patient was conceived naturally without the

use of ART. Since PLC $\zeta^{\text{H}398\text{P}}$ and its mouse equivalent have been demonstrated to abolish the Ca²⁺ inducing ability of PLC ζ (Heytens *et al.*, 2009; Nomikos *et al.*, 2011), the natural conception of the patient demonstrates that loss of activity mutations in PLC ζ 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 PLC ζ protein in addition to PLC $\zeta^{\text{H}398\text{P}}$ DNA.

Even though the first point of PLC ζ translation during human spermatogenesis has yet to be identified, recent studies indicate that PLC ζ is first detectable at the round spermatid stage in hamster (Young *et al.*, 2009) and equine (Bedford-Guass *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 PLC ζ protein within spermatids and spermatocytes containing mutant PLC ζ (Dadoune *et al.*, 2004; Heytens *et al.*, 2009). It is also a possibility that PLC ζ transcription occurs throughout the meiotic phase of spermatogenesis, potentially resulting in mixed populations of PLC ζ transcripts derived from both maternal and paternal alleles (for reviews, see Eddy, 2002; Dadoune *et al.*, 2004; Bettgowda and Wilkinson, 2010).

In summary, we have identified a novel second mutation (PLC $\zeta^{\text{H}233\text{L}}$) of the oocyte activation factor PLC ζ in an infertile patient, and demonstrate that both PLC $\zeta^{\text{H}233\text{L}}$ and PLC $\zeta^{\text{H}398\text{P}}$ 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 PLC ζ 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).

References

- Bedford-Guaus SJ, McPartlin LA, Xie J, Westmiller SL, Buffone MG, Roberson MS. Molecular cloning and characterization of phospholipase C Zeta in equine sperm and testis reveals species-specific differences in expression of catalytically active protein. *Biol Reprod* 2011;**85**:78–88.
- Berridge MJ. Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta* 2009;**1793**:933–940.
- Bettegowda A, Wilkinson MF. Transcription and post-transcriptional regulation of spermatogenesis. *Philos Trans R Soc Lond B Biol Sci* 2010;**365**:1637–1651.
- Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod* 2007;**22**:1506–1512.
- Cox LJ, Larman MG, Saunders CM, Hashimoto K, Swann K, Lai FA. Sperm phospholipase C zeta from humans and cynomolgus monkeys triggers Ca^{2+} oscillations, activation and development of mouse oocytes. *Reproduction* 2002;**124**:611–623.
- Dadoune JP, Siffroi JP, Alfonsi MF. Transcription in haploid male germ cells. *Int Rev Cytol* 2004;**237**:1–56.
- Dale B, Iaccarino M, Fortunato A, Gragnaniello G, Kyozuka K, Tosti E. A morphological and functional study of fusibility in round-headed spermatozoa in the human. *Fertil Steril* 1994;**61**:336–340.
- Dam AH, Feenstra I, Westphal JR, Ramos L, van Golde RJ, Kremer JA. Globozoospermia revisited. *Hum Reprod Update* 2007;**13**:63–75.
- Eddy EM. Male germ cell gene expression. *Recent Prog Horm Res* 2002;**57**:103–128.
- Evens EM. A global perspective on infertility: an under recognized public health issue. *Carol Pap Int Health* 2004;**18**:1–42.
- Flaherty SP, Payne D, Matthews CD. Fertilization failures and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod* 1998;**13**:155–164.
- Flörke-Gerloff S, Töpfer-Petersen E, Müller-Esterl W, Mansouri A, Schatz R, Schirren C, Schill W, Engel W. Biochemical and genetic investigation of round-headed spermatozoa in infertile men including two brothers and their father. *Andrologia* 1984;**16**:187–202.
- Grasa P, Coward K, Young C, Parrington J. The pattern of localization of the putative oocyte activation factor, phospholipase C ζ , in uncapacitated, capacitate, and ionophore-treated human spermatozoa. *Hum Reprod* 2008;**23**:2513–2522.
- Heindryckx B, Van der Elst J, De Sutter P, Dhont M. Treatment option for sperm- or oocyte-related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI. *Hum Reprod* 2005;**20**:2237–2241.
- Heytens E, Parrington J, Coward K, Young C, Lambrecht S, Yoon SY, Fissore RA, Hamer R, Deane CM, Ruas M et al. Reduced amounts and abnormal forms of phospholipase C zeta in spermatozoa from infertile men. *Hum Reprod* 2009;**24**:2417–2428.
- Jones KT. Mammalian egg activation: from Ca^{2+} spiking to cell cycle progression. *Reproduction* 2005;**130**:813–823.
- Kashir J, Heindryckx B, Jones C, De Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update* 2010;**16**:690–703.
- Kierszenbaum AL, Rivkin E, Tres LL, Yoder BK, Haycraft CJ, Bomens M, Rios RM. GMAP210 and IFT88 are present in the spermatid golgi apparatus and participate in the development of the acrosome-acroplaxome complex, head-tail coupling apparatus and tail. *Dev Dyn* 2011;**240**:723–736.
- Kilani Z, Ismail R, Ghunaim S, Mohamed H, Hughes D, Brewis I, Barratt CL. Evaluation and treatment of familial globozoospermia in five brothers. *Fertil Steril* 2004;**82**:1426–1429.
- Knott JG, Kurokawa M, Fissore RA, Schultz RM, Williams CJ. Transgenic RNA interference reveals role for mouse sperm phospholipase Czeta in triggering Ca^{2+} oscillations during fertilization. *Biol Reprod* 2005;**72**:992–996.
- Kouchi Z, Shikano T, Nakamura Y, Shirakawa H, Fukami K, Miyazaki S. The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase Czeta. *J Biol Chem* 2005;**280**:21015–21021.
- Kullander S, Rausing A. On round-headed human spermatozoa. *Int J Fertil* 1975;**20**:33–40.
- Kurokawa M, Sato K, Wu H, He C, Malcuit C, Black SJ, Fukami K, Fissore RA. Functional, biochemical, and chromatographic characterization of the complete $[Ca^{2+}]_i$ oscillation-inducing activity of porcine sperm. *Dev Biol* 2005;**285**:376–392.
- Mahutte NG, Arici A. Failed fertilization: is it possible? *Curr Opin Obstet Gynecol* 2003;**15**:211–218.
- Nomikos M, Elgmati K, Theodoridou M, Calver BL, Cumbes B, Nounesis G, Swann K, Lai FA. Male infertility-linked point mutation disrupts the Ca^{2+} oscillation-inducing and PIP2 hydrolysis activity of sperm PLC ζ . *Biochem J* 2011;**434**:211–217.
- O'Flynn O'Brien KL, Varghese AC, Agarwal A. The genetic causes of male factor infertility: a review. *Fertil Steril* 2010;**93**:1–12.
- Ombelet W, Cooke I, Dyer S, Serour G, Devroey P. Infertility and the provision of infertility medical services in developing countries. *Hum Reprod Update* 2008;**14**:605–621.
- Parrington J, Davis LC, Galione A, Wessel G. Flipping the switch: how a sperm activates the egg at fertilization. *Dev Dyn* 2007;**236**:2027–2038.
- Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. PLC zeta: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Development* 2002;**129**:3533–3544.
- Saunders CM, Swann K, Lai FA. PLC zeta, a sperm-specific PLC and its potential role in fertilization. *Biochem Soc Symp* 2007;**74**:23–36.
- Sazci A, Ercelen N, Ergul E, Akpınar G. Male factor infertility associated with a familial translocation t(1;13)(q24;q10). *Fertil Steril* 2005;**83**:1548–1550.
- Stricker SA. Comparative biology of calcium signalling during fertilisation and egg activation in mammals. *Dev Biol* 1999;**211**:157–176.
- Swann K, Saunders CM, Rogers NT, Lai FA. PLCzeta (zeta): a sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals. *Semin Cell Dev Biol* 2006;**17**:264–273.

- Taylor SL, Yoon SY, Morshedi MS, Lacey DR, Jellerette T, Fissore RA, Oehninger S. Complete globozoospermia associated with PLCzeta deficiency treated with calcium ionophore and ICSI results in pregnancy. *Reprod Biomed Online* 2010;**20**:559–564.
- Wang PJ, Pan J. The role of spermatogonially expressed germ cell-specific genes in mammalian meiosis. *Chromosome Res* 2007;**15**:623–632.
- Whitaker M. Calcium at fertilization and in early development. *Physiol Rev* 2006;**86**:25–88.
- Yanagida K, Fujikura Y, Katayose H. The present status of artificial oocyte activation in assisted reproductive technology. *Reprod Med and Biology* 2008;**7**:133–142.
- Yoon SY, Jellerette T, Salicioni AM, Lee HC, Yoo MS, Coward K, Parrington J, Grow D, Cibelli JB, Visconti PE et al. Human sperm devoid of PLC, zeta I fail to induce Ca²⁺ release and are unable to initiate the first step of embryo development. *J Clin Invest* 2008;**118**:3671–3681.
- Young C, Grasa P, Coward K, Davis LC, Parrington J. Phospholipase C zeta undergoes dynamic changes in its pattern of localization in sperm during capacitation and the acrosome reaction. *Fertil Steril* 2009;**91**:2230–2242.
- Zheng K, Yang F, Wang PJ. Regulation of male fertility by X-linked genes. *J Androl* 2010;**31**:79–85.