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Letter to the editor Open Access

Computationally predicted pathogenic *USP9X* mutation identified in infertile men does not affect spermatogenesis in mice

Infertility is a major health issue, affecting approximately 15% of couples of child-bearing age. Although nearly half of idiopathic infertility cases are assumed to have a genetic basis, the underlying causes remain largely unknown in most infertile men. Thus, to shed light on the genetic causes of male infertility, we employed a widely utilized *in silico* method to annotate genetic variants from whole-exome sequencing (WES) data of a consanguineous Pakistani family comprised of two infertile men diagnosed with oligoasthenozoospermia. We identified a novel variant in *USP9X* (c.1920A>C, p.Gln640His), recessively segregated with the infertility phenotype in the recruited family. This variant lies within a highly conserved region of USP9X and was predicted to be deleterious based on five out of seven computational tools. We functionally examined the effects of the variant by generating mice with the same mutation as the patients. Surprisingly, *Usp9x*$^{KI/Y}$ mutant mice showed normal fertility and displayed sperm parameters, testicular and epididymal histology, and meiotic prophase I progression similar to those of their control littermates. These findings indicate that the *USP9X* mutation is not likely to be a pathogenic variant in our patients and *in vivo* functional verification using a mouse model is required to identify potentially pathogenic mutations in infertile patients.

Nearly 15% of couples at the age of reproduction are unable to conceive after a year of unprotected sexual intercourse (Gershoni et al, 2019). Almost 50% of infertility cases are related to male factors, which are mostly accompanied by quantitative defects in semen (Farhi & Ben-Haroush, 2011; Rimoin et al, 2007). It is inferred that mutations in more than 1 000 genes showing germ cell-enriched expression may cause defective spermatogenesis (Djureinovic et al, 2014). Most familial mutations causing infertility have been identified through WES. ANNOVAR has been widely used to identify potential candidate gene mutations and to screen mutations in WES data for various genetic diseases (Wang et al, 2010). *In vivo* functional experiments have also identified a small number of mutations (Guerri et al, 2019; Okutman et al, 2018; Sironen et al, 2020). However, the pathogenicity of most mutations remains uncertain due to a lack of experimental evidence.

Ubiquitin-specific protease 9, X chromosome (*USP9X*) is located on the X chromosome in humans and mammals and encodes a deubiquitylating enzyme that plays functional roles in various biological processes (Stegeman et al, 2013). Conditional disruption of *Usp9x* in germ cells of mice causes apoptosis of early spermatocytes and a severe reduction in spermatozoa in epididymides, and consequently male infertility (Kishi et al, 2017).

Here, we utilized WES and ANNOVAR to identify the underlying genetic cause of male infertility in a consanguineous Pakistani family. We found a missense mutation in *USP9X* (c.1920A>C, p.Gln640His), which was predicted to be a potential pathogenic mutation by *in silico* analyses. Unexpectedly, mice with this mutation were fertile and showed normal spermatogenesis. Therefore, our study highlights the necessity for functional validation of potentially pathogenic mutations identified in infertile patients using mouse models.

A consanguineous Pakistani family comprised of two male patients was enrolled in this study to identify the underlying genetic cause of their male infertility (Figure 1A). Semen analyses revealed that these two patients suffered from oligoasthenozoospermia (Supplementary Table S1). WES was performed on all available family members to decipher the genetic cause of this infertility. Genetic variants were filtered following a series of criteria (Supplementary Figure S1 and Table S2). The WES analysis strategy identified a novel variant in *USP9X* (c.1920A>C, p.Gln640His) as the only

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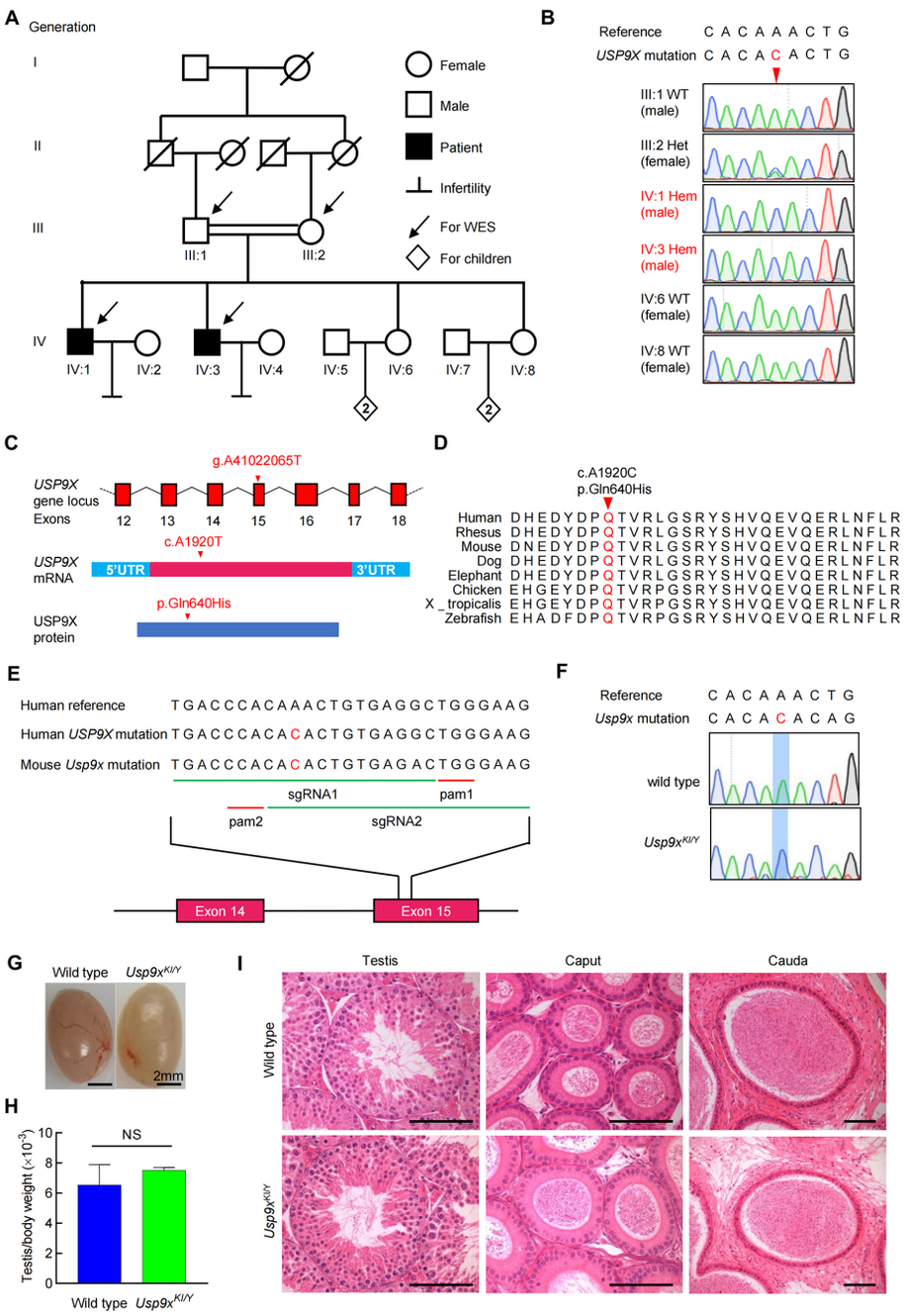

Figure 1 Hemizygous *USP9X* variant identified in a consanguineous infertile family

A: Pedigree of a consanguineous Pakistani family comprised of two male infertile patients. Males are symbolized by squares, females by circles, with slashes indicating deceased. Diamonds indicate offspring, with inside numerals designating number of offspring. Solid squares specify patients and parallel double lines indicate consanguineous marriages. Black arrows represent members selected for WES. B: Chromatograms represent segregation of *USP9X* variant in available members. WT, wild-type; Hem, hemizygous; Het, heterozygous. C: Illustrative representations of *USP9X* gene, RNA, and protein structures showing identified variant at genomic and proteomic levels, respectively. D: Sequence alignment demonstrating conservation of altered amino acid (glutamine) in USP9X, across different organisms. Arrowheads indicate mutation site. E: Schematic of CRISPR/Cas9 strategy utilized for generating *Usp9x*$^{KI/Y}$ mice. The sgRNA-targeting DNA sequence within exon 15 of *Usp9x* gene is displayed. F: Representative chromatogram from *Usp9x*$^{KI/Y}$ mouse confirming genotype. Altered nucleotide is in red and site is in blue in the chromatogram. G: Representative images of testes from adult WT and *Usp9x*$^{KI/Y}$ mice. Scale bars: 2 mm. H: Testis/body weight ratios of 8-week-old WT and *Usp9x*$^{KI/Y}$ mice. Data are mean±standard deviation (*SD*) with at least three mice analyzed per genotype. Student's *t*-test was used for statistical analyses. NS: Not significant. I: H&E staining of testes, and caput and cauda of epididymides from 8-week-old WT and *Usp9x*$^{KI/Y}$ mice. Data are representative images from at least three mice per genotype. Scale bars: 100 μm.

potentially pathogenic variant (Supplementary Table S3). Sanger sequencing confirmed the segregation of the identified mutation (Figure 1B, C). Moreover, phylogenetic examination indicated that the altered amino acid was highly conserved among species (Figure 1D). From SpermatogensisOnline (Zhang et al, 2013), *USP9X* shows a testis-enriched expression pattern. Furthermore, based on previously published human and mouse testicular single-cell RNA sequencing data, *USP9X* shows high expression in spermatogonia and early meiotic cells in both humans and mice (Supplementary Figure S2A, B) (Ernst et al, 2019; Guo et al, 2018). USP9X antibody staining results in human testes from the Human Protein Atlas (https://www.proteinatlas.org) also indicate strong USP9X signals in spermatogonial cells at the base of seminiferous tubules (Supplementary Figure S2C). Thus, based on the above data, we hypothesized that *USP9X* may play a role in spermatogonium development or early meiosis.

Given that the affected amino acid is conserved in humans and mice, a *Usp9x* KI mouse model carrying the equivalent mutation ($Usp9x^{KI/Y}$) to that of our patients was produced through CRISPR/Cas9 genome editing technology (Figure 1E). The genotype of the resulting mutant mice was determined by Sanger sequencing of genomic DNA (Figure 1F; Supplementary Table S5). The *Usp9x* KI mice displayed normal growth and development as well as fertility status similar to their wild-type (WT) littermates (Supplementary Table S4). In addition, no obvious abnormalities in testis size or testis-to-body weight ratios were observed between the *Usp9x* KI mice and WT littermates (Figure 1G, H).

Hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining of testis and epididymis sections revealed intact seminiferous tubule architecture and all types of germ cells, ranging from spermatogonia to mature spermatids, in both KI and WT mice (Figure 1I; Supplementary Figure S3A). The number of $LIN28A^+$ spermatogonia did not differ between the control and KI mice (Supplementary Figure S3B, C). Furthermore, sperm count per epididymis was normal between the *Usp9x* KI mice and their WT littermates (Supplementary Figure S4A). Sperm morphology and abnormalities were examined, but no significant differences were found in sperm morphology between KI and control mice (Supplementary Figure S4B, C). Computer-assisted semen analysis (CASA) was performed to determine the progressive and average velocity of spermatozoa; however, no significant variations were found between the control and KI mice (Supplementary Figure S4D, E). We next stained spermatocyte spreads with antibodies against SYCP3 (an axial element component) and γH2AX (a marker of DNA double-strand breaks (DSBs) and XY body). Meiotic prophase I progression was analyzed, and spermatocytes typical for all prophase I stages were observed in the *Usp9x* KI and WT mice (Supplementary Figure S5A). Furthermore, we counted the cell population of each prophase I stage and found no significant differences in cell population size between the *Usp9x* KI and WT mice (Supplementary Figure S5B).

Usp9x is reported to play an indispensable function in mouse spermatogenesis (Kishi et al, 2017). *Usp9x* was conditionally deleted in the germ cells of male mice through, VASA-Cre, leading to the increased apoptosis of a large number of spermatocytes, a significant reduction of sperm count in male mice, and complete male infertility (Kishi et al, 2017). In our male patients with the *USP9X* missense mutation, sperm count was lower than reference values, similar to the reported conditional knockout mouse phenotype, which supports the idea that the identified variant in *USP9X* is potentially pathogenic and may be the reason for infertility in the recruited family.

Surprisingly, we found that the KI mice displayed similar fertility as WT mice. No significant differences were found between KI and WT mice in all measurements of spermatogenesis. Recent research employed an *in vivo* approach to functionally validate predicted disease-causing single nucleotide polymorphisms (SNPs) in meiosis-specific genes such as *MLH1*, *CDK2*, *SMC1B*, and *TEX15* (Singh & Schimenti, 2015). However, results showed that the *Cdk2* KI mice were infertile, whereas the *Mlh1*, *Smc1b*, and *Tex15* KI mice were completely fertile. This suggests that only the CDK2 variant was correctly predicted by *in silico* tools, further demonstrating the importance of functional validation of variants in KI mouse models. In addition to verification of potentially pathogenic variants, our results and methods will hopefully help clarify the molecular basis of disease-causing mutations. Moreover, as this approach can be applied to any genetic disease, it could be a powerful tool in the field of personalized genomic medicine.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Q.H.S., R.K., and W.L. conceived and designed the experiments. R.K., W.S., M.Z., A.R.J., Q.Z., R.A., and T.A. collected samples. W.L., Y.W.W., H.Z., D.R.Z., X.F.X, A.M. and R.K. performed the experiments. W.L. and Y.W.W. analyzed the data. W.L. and R.K. wrote the paper. H.M., M.Z., and Q.H.S. modified the manuscript. All authors read and approved the final version of the manuscript.

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