

Role of testis-specific serine kinase 1B in undiagnosed male infertility

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Abstract. Male infertility is a global problem affecting a considerable part of the male population. Current guidelines and practices aimed at diagnosing the cause of this problem still have low diagnostic yield. As novel candidate genes for infertility emerge, their functional role needs to be investigated in patient populations. The present study aimed to investigate testis-specific serine kinase 1B (*TSSK1B*), which was discovered in a previously diagnosed patient. Sanger sequencing of the coding regions and exon borders of *TSSK1B* was performed in a cohort of 100 male Bulgarian patients with unresolved infertility causes. Missense mutations were discovered in 10% of patients and were associated with clinical data on sperm dysmorphology. Two previously unreported mutations were discovered, p.3D>N and p.52F>L. All mutations were scored via *in silico* predictors and protein modelling using AlphaFold2. The present findings indicated an association between *TSSK1B* mutations and asthenoteratozoospermia, with further missense mutations in patients with azoospermia and teratozoospermia. Mutations in *TSSK1B* may be a cause of undiagnosed cases of male infertility and should be considered when molecular diagnostics are warranted.

Introduction

Male infertility is a complex, often multifactorial pathological condition affecting ~7% of the global male population (1). While the etiology of infertility is wide-ranging, up to

15% of males with infertility have an underlying causative genetic defect (1-3). Mouse knockout models have identified >400 genes, including *ATM*, *SYCP1-3*, *SYCE1* and *CADM*, leading to monogenic infertility in men (4). Studies on the molecular mechanisms of cell-cell interactions have provided a better understanding of the causes of fertility issues due to fertilization defects (5,6). While there are established guidelines and practices for diagnostic karyotyping, azoospermia factor (AZF) deletion screening and cystic fibrosis transmembrane conductance regulator testing, the overall diagnostic yield is as low as 4% (7). However, rapid advancements in genomic medicine achieved by next-generation sequencing (NGS) technologies lack of results standardization, creating a knowledge gap in the clinical area of male infertility (8).

A literature review on monogenic forms of male infertility identified 78 genes associated with male infertility in 1,337 publications in 2019 (9), with this number rising by 33% to 104 genes by 2022 (10). In the era of genomics and personalized medicine, this number is low compared with other clinical fields, such as intellectual disability (8). To improve biological understanding as well as diagnostic yield and clinical relevance of genetic testing, further genes leading to male infertility must be identified.

Our previous study outlined a possible cause for asthenoteratozoospermia in a patient harboring a deletion of ~8-Mb in the 5q22.2q23.1 locus, including the testis-specific serine kinase 1B (*TSSK1B*) gene (11). Genes belonging to kinase and phosphatase families are responsible for activation and deactivation of intra- and extracellular transduction via phosphorylation and dephosphorylation (12). These processes are key for correct regulation and metabolic processes of the cell that are mediated by different receptors and enzymes (13). TSSKs are part of the AMP-activated protein kinase family (14). They comprise six genes, which are almost exclusively present in the testes (>1,000-fold concentration compared with other organs). These genes serve a role in spermatogenesis and are responsible for correct morphogenesis and differentiation following meiosis when spermatid elongation occurs. This has been demonstrated using recombinant mouse models, where

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sterile phenotypes have been observed in double *Tssk1* and *Tssk2* knockout (KO) and *Tssk6* KO mice (15). A sub-fertile phenotype with reduced *TSSK1* and *TSSK2* levels has also been observed in a *Tssk4* KO model (16,17). These results highlight the importance of TSSKs in research, as they can be targeted by both drugs and inhibitors, suggesting that TSSKs may not only cause infertility, but can also provide a route for the development of male non-hormonal contraceptive drugs. Originally, *TSSK* genes were found in mouse tissue using degenerate oligonucleotide primers while searching for novel kinases, which led to the discovery of *TSSK1*. Subsequently, *TSSK2* was identified via low stringency screening due to its close proximity and genetic linkage to *TSSK1* (18,19). Following the discovery of these genes, yeast two-hybrid and co-immunoprecipitation experiments were performed to detect proteins that may interact with these kinases. A novel 65 kDa protein was identified, namely testis-specific kinase substrate (TSKS), that interacts with both *TSSK1* and *TSSK2* (19).

According to studies conducted on a diverse array of species, it was proposed that the TSSK family originated with *Tssk5* in amphibians ~380 million years ago (MYA). Following the Paleocene-Eocene radiation period, a novel gene appeared in primates and humans, known as *Tssk1b* (Fig. 1). Moreover, in most species *Tssk1/2* are linked on one chromosome and their activity is hypothesized to be parallel, as their combined absence has been shown to cause infertility in KO mice (17). However, with the appearance of *Tssk1b* (duplicated on another chromosome and not linked to *Tssk2*), *Tssk1* was inactivated through negative selection and mutation to become a pseudogene known as *Tssk1* (Fig. 1). Even though the genes diverge from one another, they exhibit sequence conservation and similarity. The N-terminal domain is represented by the 1-272 amino acid sequence, which bears the kinase domain, whereas the C-terminal is present in the 273-end sequence. In humans, N-terminal similarity between *Tssk1b* and *Tssk2* is 82.0%, which is similar to other mammalian species, while C-terminal similarity between the two genes is notably lower at 14.7%. This may be due to the regulatory function of the C-terminal domain, which is specific for each kinase. This could also mean that each kinase serves specific functions, in addition to exhibiting overlapping effects. With regards to evolutionary conservation, the C-terminal of *Tssk1* shows 65.7% mean sequence conservation compared with the C-terminal of *Tssk2*, which is 87.6%. When considering retrogenes and duplications, newer genes seem to have the ability to evolve faster, meaning that their conservation rate will be lower. Based on these observations on conservation rates, it is hypothesized that *Tssk2* was the first gene to appear on the chromosome following the retroposition of *Tssk1* (20). Compared with non-mammalian species, the C-terminal conservation of human *Tssk1b* is lower (61.4%) than that of *Tssk2* (86.8%), whereas N-terminal conservation is similar (92.5 and 94.9%, respectively) (20). This suggests that the two domains are affected by different selective evolutionary pressures.

Spermatogenesis is divided into three main phases: Mitotic division, which generates a pool of spermatocytes; meiosis to generate haploid spermatids and spermiogenesis, in which spermatids differentiate into spermatozoa (21). Nayak *et al* (22) tested mouse tissue obtained from maturing seminiferous tubules at different periods after birth. The procedure included immuno-fluorescent staining to visualize

difference in expression of both *Tssk1* and *Tssk2*. *Tssk1* expression was first observed at low levels in week 3, concurrent with the appearance of round spermatids, predominantly in cells in meiotic metaphase. By contrast, *Tssk2* was detected in cells undergoing spermiogenesis at weeks 4-5 and was not detected in metaphase dividing cells (22). This highlights the importance of *TSSK1* (the first gene to be expressed) in both early sperm development and mature sperm formation (22).

In spermatogenesis, following meiosis, a cloud-like organelle (nuage) appears in the round spermatid known as chromatoid body and is localized around the haploid nucleus (23). The chromatoid body is the RNA-controlling center responsible for organization and regulation of mRNA and RNA pathways associated with the haploid genome of the spermatid (Fig. 2A) (24). Its function is rather short-term and during the transition from round to elongated spermatid, the chromatoid body loses certain functions and enzymes (such as murine P-element induced wimpy testis family member proteins) to split into two well-defined structures, namely satellite and ring (Fig. 2B) (25). It has been observed that *Tssk1* and *Tssk2*, as well as TSKS, accumulate in both the ring around the flagellum and in the satellite in the cytoplasm. Experiments in both wild-type and double *Tssk1,2* KO mice revealed that KO results in disturbed mitochondrial sheath formation, rendering the sperm cells non-functional (17).

The sheath-forming complex includes not only *TSSK1B*, but also testis-specific phosphatase, *Ppp1cc2*, which serves a key role in completion of spermatogenesis in mice (26). Pull-down assays using GST-Ppp1cc2 expressed and purified from bacteria as bait against protein lysates from mouse testis tissue have been performed to determine the interaction between Ppp1cc2 and other proteins (27). An indirect interaction of Ppp1cc2 with *Tssk1* was shown to be mediated through TSKS via RVxF motif (at amino acid position 51-55), which interacts with both proteins to form a complex. To test the activity of Ppp1cc, deletion via mutagenesis was performed in male mice; this resulted in germ cell reduction primarily occurring during spermatid elongation (post-meiosis) phase at the time of chromatoid body dissociation, causing the spermatogenic cycle to halt (28). Thus, the testicular kinase/phosphatase complex is key for formation of the mitochondrial sheath during spermatogenesis.

Considering the literature and our previous experience regarding the role of the *TSSK1B* gene in a patient with asthenoteratozoospermia, the aim of the present study was to perform *TSSK1B* genetic screening on a selected group of mutation-negative male patients with infertility. The study aimed to assess overall genomic variability within *TSSK1B* compared with previously reported variants in population databases, investigate the association between missense mutations in *TSSK1B* and clinical phenotype with regards to semen quality and to assess findings using *in silico* predictors and novel protein folding algorithms.

Materials and methods

Patients. The examined patient cohort comprised 100 Bulgarian male patient DNA samples obtained in a period of 3 years (January 2019 to December 2021) from the genetic biobank of Genetic Medico-Diagnostic Laboratory

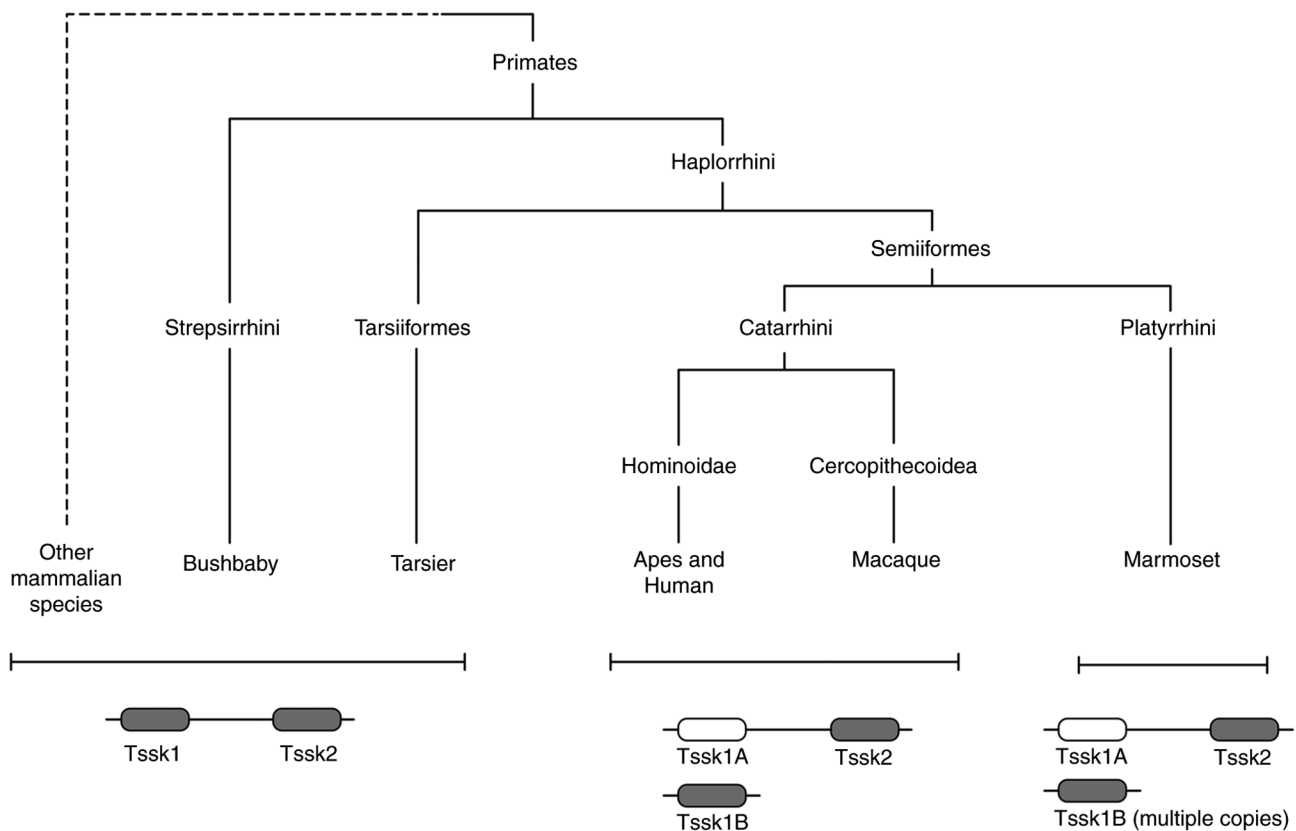


Figure 1. Tree diagram of the evolutionary origin of *Tssk1B*. Dashed lines represent multiple branches not shown here. Solid lines represent direct descendants. White, pseudogene; gray, normally expressed genes. Strepsirrhini, Tarsiiformes and other mammalian species appearing before 100 million years ago possess *Tssk1* and *Tssk2* genes located on the same chromosome, separated by a sequence of ~3 kb. Semiiformes have a *Tssk1B* gene translocated on another chromosome, and in the case of Platyrrhini multiple copies of the gene. In Semiiformes *Tssk1A* becomes a pseudogene due to acquisition of *Tssk1B*. Tssk, testis-specific serine kinase.

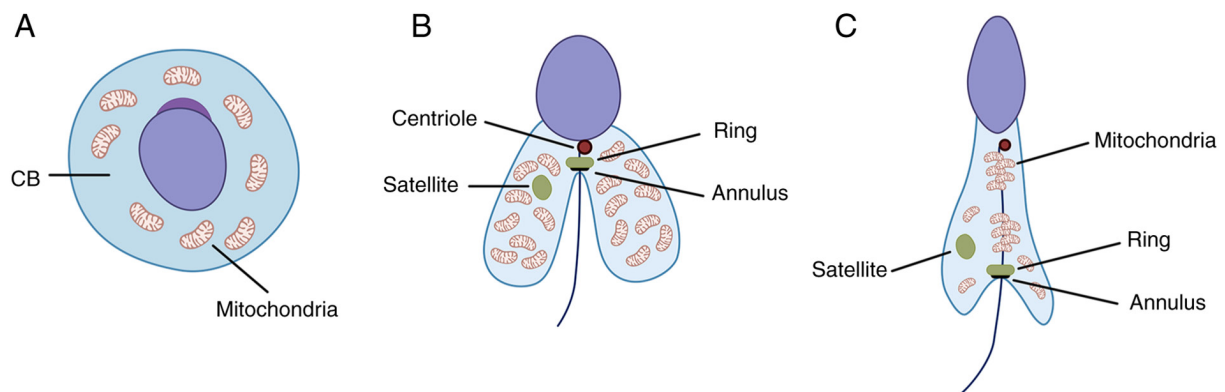


Figure 2. Illustrative representation of spermatid elongation and chromatoid body segregation. (A) Round spermatid carrying a chromatoid body containing primarily MIWI proteins, located around the nucleus. (B) Early stage of spermatid elongation, where CB loses MIWI proteins but acquires *Tssk1*, 2 and *TSSK* and segregates into a satellite and ring. (C) During spermatid elongation the ring shrinks and moves downstream of the spermatid tail along with the annulus. Above the ring, mitochondria interact with the axoneme to form a mitochondrial sheath. Later, the centriole degrades and cytoplasmic volume decreases. Tssk, testis-specific serine kinase; CB, chromatoid body; MIWI, murine P-element Induced wimpy testis family member proteins.

‘Genica’ (Sofia, Bulgaria). Patients included in the study (age, 18-53; mean age, 34 years) had a positive history of sperm dysmorphology according to European Society of Human Reproduction and Embryology/Nordic Association for Andrology criteria using Sperm Class Analyzer, a system of quantitative and qualitative analysis of human sperm parameters (29). Included patients were selected based on negative results from Y-chromosome microdeletion testing.

Ethical approval was obtained from the ethical board of Medical University (Sofia Bulgaria). For all subjects, written informed consent was provided.

PCR and Sanger sequencing. Molecular testing for microdeletions in the AZF region were performed as described in the guidelines and standards of the European Academy of Andrology (30).

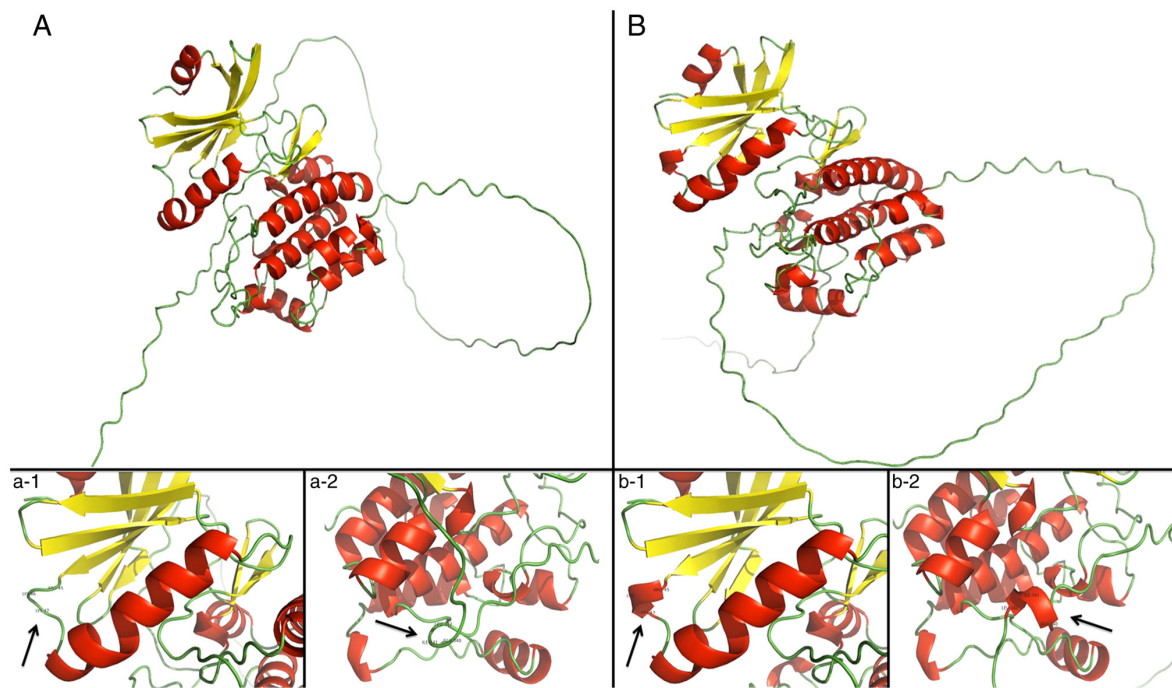


Figure 3. Alignment of the canonical TSSK1B protein and models created with AlphaFold2. Arrows indicate folding alterations. (A) canonical TSSK1B protein, where A1 and A2 show the normal folding. (B) Protein model of the p.66M>V mutation, showing two small helix motifs not found in the canonical TSSK1B protein (B1 and B2). Tssk, testis-specific serine kinase.

Genomic DNA was isolated from peripheral blood using the QIAamp® DNA Blood Mini Kit (Qiagen GmbH) following the manufacturer's recommendations. DNA was eluted in approximately 200 μ l of buffer AE. Amplification was performed using forward (5'-CTAGGAGGCAGGAAC AGCAG-3') and reverse (5'-ACTGCCTTCCTTCTCTGG CT-3') primers. Thermocycling conditions were as follows: 5 min denaturation at 95°C, 35 cycles of 95°C for 45 sec, 60°C for 45 sec, 72°C for 90 sec and final extension for 5 min at 72°C to obtain a 1,327 bp product. PCR products were verified by 3% agarose gel electrophoresis and visualized with ethidium bromide. Sanger sequencing of the *TSSK1B* gene was performed using BigDye® Terminator cycle sequencing kit v.3.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI 3130 sequencer.

Protein model. Protein models for the detected *TSSK1B* missense mutations were simulated using AlphaFold2 v2.1.0 (29) for each altered TSSK1b protein sequence specified by a missense mutation in the patient group (Fig. 3). *In silico* predictions were performed using Ensembl Variant Effect Predictor (31) with pathogenicity scores (from both SIFT and PolyPhen) classified according to the standards of the American College of Medical Genetics (32).

Statistical analysis. Allele frequencies of each variant found in the cohort were calculated and compared with those in the gnomAD v2.1.1 database (<https://gnomad.broadinstitute.org/>).

Results

Variant discovery in *TSSK1*. Screening of the *TSSK1B* gene within the patient group revealed 11 nucleotide

variations, with certain patients presenting with up to four variants (three synonymous, one missense), five of which were protein-altering missense mutations and six of which were synonymous mutations (Table I). Two missense mutations, p.3D>N and p.52F>L, are novel, without previous reports in GnomAD (33). The other missense mutations, p.66M>V, p.237R>C and p.293G>E, occur with a higher allele frequency in the study cohort compared with global databases (GnomAD v2.1.1).

Correlations with sperm dysmorphology phenotypes. Available clinical data were analyzed to demonstrate a potential link between the mutations detected in the patient cohort and clinical phenotype (Table I). The mutations p.3D>N and p.52F>L were discovered in patients with azoospermia. The p.66M>V and p.237R>C mutations resulted in asthenoteratozoospermia, the same phenotype detected in our initial *TSSK1B* case report (11). The missense variant p.293G>E was seen in patients with azoospermia and teratozoospermia. Certain patients harbored two or three synonymous variants in addition to a protein-altering mutation.

Structure alterations caused by missense variants. Alignments to canonical TSSK1B protein revealed potential misfolding of the protein in four of the models (p.3D>N, p.66M>V, p.237R>C and p.293G>E). This occurred within the kinase domain of the protein, between positions 45-47, creating a small Arg-Lys-Lys helix immediately after a predicted β -sheet structure. Additionally, the mutation p.66M>V created a small helix motif of Glu-Ile-Leu at position 340-342. From *in silico* prediction methods for mutations p.3D>N, p.66M>V and p.237R>C, pathogenic scoring from Sorting Intolerant from Tolerant (SIFT) and PolyPhen was observed.

Table I. Tssk, testis-specific serine kinase 1B variants in the patient cohort, with scores from SIFT, PolyPhen and scoring according to the ACMG guidelines.

| CDS position | Protein position | Variant | Amino acids | SIFT | PolyPhen | ACMG | Cohort | gnomAD | Clinical phenotype | Incidence rate, % |
|--------------|------------------|------------|-------------|---------------------------------|---------------------------|---------------|--------|------------------------|--------------------------|--------------------------|
| 7 | 3 | Missense | D/N | Deleterious (0.00) | Possibly damaging (0.566) | VUS | 0.010 | - | N/A | 2.56 (N/A) |
| 156 | 52 | Missense | F/L | Deleterious (0.00) | Probably damaging (0.925) | VUS | 0.005 | - | Azoospermia | 3.84 (azoospermia) |
| 196 | 66 | Missense | M/V | Tolerated (0.31) | Benign (0.000) | Likely benign | 0.005 | 7.37x10 ⁻⁴ | Azoospermia | 3.80 |
| 438 | 146 | Synonymous | K | - | - | - | 0.005 | 3.98x10 ⁻⁶ | - | - |
| 510 | 170 | Synonymous | A | - | - | - | 0.055 | 9.94x10 ⁻² | - | - |
| 522 | 174 | Synonymous | T | - | - | - | 0.045 | 6.92x10 ⁻² | - | - |
| 540 | 180 | Synonymous | A | - | - | - | 0.055 | 6.9x10 ⁻² | - | - |
| 709 | 237 | Missense | R/C | Deleterious (0.01) | Possibly damaging (0.849) | Likely benign | 0.005 | 2.86x10 ⁻⁴ | Asthenoteratozoo spermia | 14.28 |
| 878 | 293 | Missense | G/E | Tolerated low confidence (0.89) | Benign (0.021) | Benign | 0.030 | 03.14x10 ⁻² | N/A | 7.69 (N/A) |
| 978 | 326 | Synonymous | T | - | - | - | 0.005 | - | Azoospermia | 3.84 (azoospermia) |
| 996 | 332 | Synonymous | A | - | - | - | 0.015 | 3.46x10 ⁻² | Teratozoospermia | 50.00 (teratozoospermia) |

VUS, variant of uncertain significance; CDS, CoDing Sequence position; SIFT, Sorting Intolerant From Tolerant; ACMG, American College of Medical Genetics; N/A, not applicable.

Discussion

Our previous report presented the first known case of a *TSSK1B* deletion associated with a clinical phenotype of asthenoteratozoospermia and male infertility (11). Since then, the TSSK family has emerged as a potential cause for male infertility and may be a target for the development of novel male contraceptive solutions through targeted inhibition of *TSSK1B* (15). By performing comprehensive targeted patient screening to investigate the role of *TSSK1B* in male infertility, the present study discovered both known and novel missense variants that should be evaluated *in vivo* to determine the extent of their clinical manifestation. Mutations discovered toward the C-terminus of *TSSK1B*, namely p.3D>N and p.52F>L, did not exhibit the same phenotype as in our initial report (asthenoteratozoospermia), yet yielded pathogenic scores via *in silico* prediction methods. Of note, p.66M>V and p.237R>C mutations were associated with the clinical phenotype discovered in our initial study. Finally, the p.293G>E mutation manifested as two clinical phenotypes, azoospermia and teratozoospermia. While classification via ACMG guidelines shows either that mutations are of unknown significance or possibly benign, this may be due to >0.01 population frequency in reference databases (34). Infertility-causing mutations might not be functionally investigated because of their high representation in a population. Such sequence alterations may not directly affect the quality of life of the individual; hence they are treated as benign.

While *in silico* predictors such as PolyPhen and SIFT (31) have been used to determine the potential outcome of a missense mutation, the next step in the assessment of novel pathological variants is to build and compare protein models. Although AlphaFold2 does not provide a solution to the protein folding problem (34), it has an accuracy level comparable to that of X-ray crystallography (35). This novel artificial intelligence program achieved a milestone level of accuracy in the biannual Critical Assessment of Protein Structure Prediction experiment in 2020 and is used to predict protein structures for databases such as the European Bioinformatics Institute. While it is still not validated for clinical use, the results produced are notable, with a margin of error of only 1.6 angstroms when simulating the folding of the protein (36). Using this method, altered amino acid sequence caused by the missense mutations identified in our patient cohort were simulated; AlphaFold2 predicted a small helical motif in four of the altered sequences. This fold occurred seemingly with no regard to the location of the change yet it was predicted to affect the kinase domain of TSSK1B. As previous studies show, damaging or removing one of the copies of this gene can cause infertility (14,17), which indicates that small structure alterations lead to a pathological changes.

To support the present findings, the evolutionary background of TSSKs and integration of the Ppp1cc2-Tsk1 complex were investigated, which suggested that this gene is key for sperm maturity. *TSSK1B* was the first of the family to evolve in humans and the complex with Ppp1cc2 is essential for proceeding with the spermatid elongation stage and completing spermatogenesis. Furthermore, *TSSK1B* is the earliest kinase of this family to be expressed, at 3 weeks opposed to other

TSSKs expressed at 3.5 weeks (22,37), which further outlines its importance in spermatogenesis.

In the present cohort, 11 of 100 patients carried a missense mutation in *TSSK1B*; although this frequency is biased by the cohort size and patient selection, an 11% carrier rate warrants further study. Not all mutations are equal: *In silico* predictors showed that 3 of the 5 protein-altering variants have scores that are interpreted as damaging. As the N-terminus of *TSSK1* is highly conserved, this suggests a potential damaging outcome caused by the mutations reported in the present study. Further functional studies are required to verify the pathological consequences of these alterations.

Multiple studies have highlighted the need for novel male contraceptives (38,39). Current methods are limited to condoms and vasectomy, compared with the variety of available female contraceptives; the limitations of these methods are high failure rate of condoms and irreversibility of surgical vasectomy (39). While trials of hormonal methods are successful, they come with side effects, such as weight gain, acne, mood changes and changes in libido (39,40). Previous studies demonstrate promising results in targeting TSSKs using kinase inhibitors and the potential application of TSSK allosteric inhibitors (15,41), thereby emphasizing not only the important role of this family of kinases in spermatogenesis, but also how they can be targeted pharmaceutically.

The only putative pathological manifestation of *TSSK1B* has been studied in mice, where haploinsufficiency results in decreased sperm maturation and offspring carrying exclusively the wild-type *TSSK1B* allele (14). The present patient cohort was referred for a number of clinical phenotypes of infertility and some individuals carried a heterozygous variant of *TSSK1B*. Functional study investigating *TSSK1B* function in human spermatozooids is warranted. Second, through the advent of NGS, carrier screening can be performed in cases with previously unidentified genetic causes. Lastly, research into this gene may lead to novel male contraceptive solutions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TK and IT confirm the authenticity of all the raw data. TK and IT conceptualized the study. TK, VZ and DAS designed the study. KD, DM and ST performed the experiments and wrote the manuscript. IT, VZ and DAS performed the statistical analysis. TK, VZ and DAS supervised the study. KD visualized data. TK, IT, VZ and DAS reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted according to the guidelines of the Declaration of Helsinki. The study was approved by the Ethics Committee of Sofia Medical University. All patients provided signed informed consent forms for participation in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article.

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