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Polymorphism of the Testis-Specific Serine/Threonine Kinase 6 (*TSSK6*) Gene in Spermatozoa of Asthenozoospermia Patients at Institut Pasteur of Côte d'Ivoire

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A B S T R A C T

Introduction: Phosphorylation reactions highlight the essential role of protein kinases in sperm motility. *TSSK6* protein is thought to play a crucial role in this process. Indeed, sperm obtained from mice with the *Tssk6*$^{-/-}$ genotype are unable to carry out fertilization and exhibit decreased motility. This leads us to investigate the causes of asthenozoospermia by searching for polymorphisms in the *TSSK6* gene. Therefore, the objective of this study is to identify polymorphisms in the *TSSK6* gene in men affected by asthenozoospermia.

Material & Methods: The methodology involved direct sequencing of spermatozoa DNA. Thirty ejaculates were analyzed, including 20 from asthenozoospermic men and 10 from normozoospermic men. Spermograms were performed according to WHO procedures. DNA extraction was carried out using the phenol/chloroform method followed by conventional PCR. The amplicons were sequenced using the Sanger method, and the sequences were analyzed with BioEdit software. The data were analyzed using Fisher and Mann-Whitney tests.

Results: The results revealed mutations in the *TSSK6* gene in both normospermic and asthenozoospermic ejaculates. The synonymous mutations c.690T>C (p.Tyr230Tyr) and c.372C>A (p.Arg124Arg) were the most frequent, occurring at rates of 50% and 33.33%, respectively. Analysis of the mutations using PolyPhen-2 indicated that all mutations observed in normozoospermic samples are benign and would not affect sperm quality. However, only the mutations described in asthenozoospermic samples are predicted to be damaging to the protein.

Conclusion: In conclusion, mutations in the *TSSK6* gene were observed in infertile men. Deleterious mutations in the *TSSK6* protein are associated with asthenozoospermia.

Keywords: Infertility, Male, Gene, Protein, Mutation, *TSSK6*

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Introduction

Asthenozoospermia is a pathology of sperm quality that reflects impaired spermatozoa motility. It is identified by spermogram in more than half of infertile men (1, 2, 3). The biochemical mechanisms of sperm motility involve protein kinases in a cascade reaction. During capacitation, spermatozoa are hyperactivated and become highly motile (4). This suggests an amplification of the motility mechanism during this maturation stage (5), which is associated with an increase in protein phosphorylation (6). This activation of the signaling mechanism highlights the crucial importance of kinases in spermatozoa motility (7). Among these protein kinases is *TSSK6*, a protein from the testis-specific serine/threonine kinase (TSSK) family, which is predominantly expressed in the testis (8). *TSSK6* is present in both the testis and sperm (9). It participates in the production and functioning of sperm cells, including the phosphorylation of histones and the remodeling of sperm DNA during sperm maturation (9). Additionally, the mutation c.822+126T>G/C observed in the *TSSK6* gene in an infertile population is associated with oligozoospermia and azoospermia (10). Furthermore, sperm cells of mice with the *Tssk6*$^{-/-}$ genotype exhibit increased head abnormalities and reduced motility (9). Thus, the protein *TSSK6* is essential in spermatogenesis and fertilization. Additionally, it has been shown that for its activation, *TSSK6* interacts with Heat Shock Proteins (HSPs) HSP90-1β, HSC70, and HSP70-1 (11, 12), which are involved in protecting cells against apoptosis and oxidative stress (13, 14, 15, 16, 17). Oxidative stress is a major cause of asthenozoospermia, raising the question of *TSSK6*'s involvement in sperm motility. Indeed, mutations in target genes have been reported as risks for male infertility (18, 19, 20, 21). This leads us to investigate the polymorphism of the *TSSK6* gene in infertile patients. Therefore, the objective of this study is to analyze the polymorphism of the *TSSK6* gene in spermatozoa from normospermic and asthenozoospermic ejaculates of patients at Institut Pasteur of Côte d'Ivoire.

Materials and methods

Sperm analysis

Ejaculates were obtained by masturbation after 3 days of sexual abstinence. The patients were received at Institut Pasteur of Côte d'Ivoire for a spermiology test between April 2017 and September 2017. Ejaculates showing only asthenozoospermia or normospermia after analysis were included in this study, comprising 20 asthenozoospermic and 10 normospermic samples.

The spermogram tests were performed according to the standards established by the WHO in 2010, using the SQA-Vision automated system (Medical Electronic System, USA). For the spermocytogram, sperm smears were stained with the RAL555 kit (RAL Diagnostics, France) and examined using a binocular optical microscope with a x100 objective (Magnus-analytics, India). A total of 30 sperm samples were analyzed, including 20 asthenozoospermic and 10 normospermic samples.

This study was approved by the national ethics committee (Reference No.: 036-13/CNESVS) and was conducted according to the Helsinki Declaration. All participants provided written consent.

Molecular analysis

The molecular analysis involved amplifying the *TSSK6* gene and identifying mutations in the amplified fragments. DNA extraction from spermatozoa was performed using the phenol/chloroform method. Conventional PCR was then conducted to amplify the *TSSK6* gene using three primer pairs provided by SIGMA-ALDRICH, based on the work of Su et al., 2010 (10). The primer pair SK6-F1 5'TGAGTCACAAAGCAGGGAGG3' / SK6-R1 5'CGTTGCACACCTCGATGAA3' was used to amplify the 5' UTR-Exon fragment, which is 514 bp in length. The primer pair SK6-F2 5'TCGTCAACAAGTTCCTGCCG3' / SK6-R2

5'TCGAGGCCTTCGGGATAGAG3' was used to amplify an Exon fragment of 550 bp. The third primer pair, SK6-F3 5'GGGTGCATGCCCTTCGACGA3' / SK6-R3 5'GCCAGGTGCGAGGAACAGCG3', was used to amplify the Exon-3' UTR fragment, which is 594 bp in length.

The amplification of the gene took place in a thermocycler (Applied Biosystems™ 9700, USA) following this program: first, predenaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 63.3°C for 30 seconds, and elongation at 72°C for 30 seconds. The reaction concluded with a final elongation at 72°C for 5 minutes.

The reaction medium was prepared using a VWR PCR Kit (Fontenay-sous-Bois, France), which contains Taq Polymerase and all necessary reagents for PCR reactions except DNA and primers, in accordance with the manufacturer's instructions. For the visualization of amplified DNA fragments, electrophoresis was performed with 10 µL amplicon in a 1.5% agarose gel with SYBR Green. The DNA fragments were visualized using a Geldoc image analyzer (Biorad, USA).

The sequencing of the DNA fragments was carried out by the Sanger method using only the forward primer. The generated sequences were analyzed with BioEdit software (22), using as a reference sequence NC_000022.11:19130808-19132623 Homo sapiens chromosome 22, GRCh38.p7 Primary Assembly, obtained from NCBI (January 2, 2018). The study of the effect of mutations on protein function was performed using PolyPhen-2 software (23).

Statistical analysis

The Mann-Whitney test was employed to compare sperm results using the GraphPad Prism software (GraphPad Prism® 6.01). As for the investigation of mutation frequency independence, it was conducted utilizing the Fisher test in R software (1.1.453- © 2009-2018 RStudio, Inc.).

Results

The sperm characteristics are presented in Table 1. Asthenozoospermic ejaculates exhibit lower mobility compared to normospermic samples (p-value = 0.0001). Genetic analysis of sperm unveiled mutations in the analyzed sequence of the *TSSK6* gene (Table 2). In total, 17 types of mutations were detected in 27 ejaculates, representing a mutation frequency of 90%. Among these, there were 9 (90%) normospermic mutants and 18 (90%) asthenozoospermic mutants. These mutations were all substitutions, with mutation frequencies ranging from 3.33% to 50%. The synonymous mutations c.372C>A (p.Arg124Arg) and c.690T>C (p.Tyr230Tyr) had the highest frequencies in this study. The investigation into the independence of mutation frequencies revealed a significant disparity between normospermia and asthenozoospermia (p-value = 0.004), specifically for the mutation c.690T>C (p.Tyr230Tyr). Analysis of the missense mutations identified in normospermic ejaculates by the PolyPhen-2 software indicated that these mutations are benign for the *TSSK6* protein. Conversely, missense mutations such as c.38A>T (p.Lys13Met), c.41T>G (p.Leu14Arg), c.85G>C (p.Ala29Pro), c.782T>G (p.Val261Gly), and c.811G>T (p.Asp271Tyr) observed solely in asthenozoospermic ejaculates were deemed damaging to the protein, along with the nonsense mutation c.703G>T (p.Glu235X). Additionally, synonymous mutations predominated in normospermic ejaculates, while in asthenozoospermic ejaculates, 41.38% were missense and 10.34% were nonsense (Figure 1).

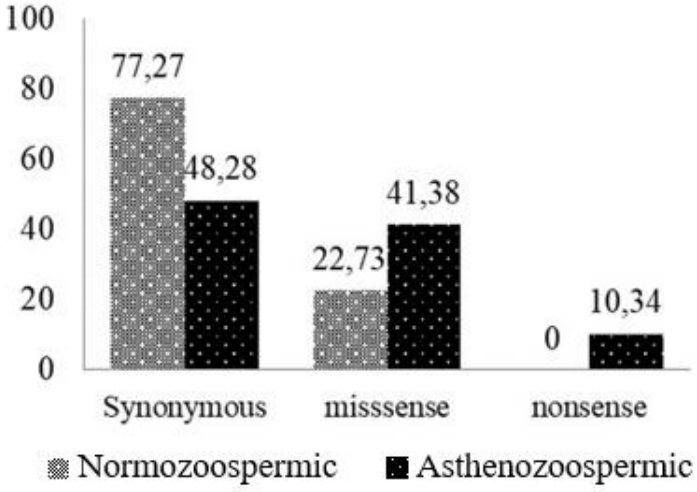

Figure 1. Types of mutations in ejaculates. p-value = 0.065

Table 1. Age and sperm parameters of patients.

	Age (year)	**Ejaculate volume (mL)**	**Spermatozoa count (x10^6 /ejaculate)**	**Spermatozoa vitality (%)**	**Spermatozoa mobility in 1 hour (%)**	**Spermatozoa typical morphology**
Normospermic (N = 10)	37,30±8,83	3,43±1,36	272,2±177,5	80,0±6,98	53,00±10,33	13,0±7,0
Asthenozoospermic (N= 20)	38,45±5,86	3,45±1,30	263,6±217,7	73,0±11,91	31,50±5,87	8,65±3,69
p-Value	0,956	0,974	0,581	0,06	0,0001*	0,07

*: $p < 0.05$

Table 2. Description and analyze of mutations.

Mutations	**References**	**Codons**	**Wild type amino acid**	**Mutant amino acid**	**PolyPhen-2 Prediction**	**Normospermic mutant (%)**	**Asthenozoospermic mutant (%)**	**p-value**
c.38A>T	-	13	Lys	Met	Damaging	0	5	1,000
c.41T>G	-	14	Leu	Arg	Damaging	0	5	1,000
c.85G>C	-	29	Ala	Pro	Damaging	0	5	1,000
c.100T>C	-	34	Tyr	His	Benign	0	10	0,540
c.130G>A	-	44	Asp	Asn	Benign	10	5	1,000
c.139C>A	-	47	Arg	Arg	-	20	0	0,103
c.144G>C	-	48	Ala	Ala	-	10	0	0,333
c.150G>A	rs150432013	50	Pro	Pro	-	0	5	1,000
c.372C>A	-	124	Arg	Arg	-	70	15	0,004*

c.421G>T/C	rs1401212890	141	Val	Leu	Benign	20	5	0,251
c.429G>A	-	143	Leu	Leu	Benign	10	0	0,333
c.439G>A	-	147	Glu	Lys	Benign	20	5	0,251
c.690T>C,	rs7250893	230	Tyr	Tyr	-	60	45	0,699
c.693C>A	-	231	Pro	Pro	-	0	5	1,000
c.703G>T	-	235	Glu	STOP	-	0	15	0,532
c.782T>G	-	261	Val	Gly	Damaging	0	15	0,532
c.811G>T	-	271	Asp	Tyr	Damaging	0	5	1,000

Discussion

The quest to uncover the causes of asthenozoospermia through the proteins involved in sperm metabolism stands as a pivotal aspect in managing male infertility. Indeed, mutations in the genes encoding target proteins have been documented in male infertility cases (18, 19, 20, 21, 24). In this molecular investigation, we identified 17 types of mutations in the sequence of the *TSSK6* gene under scrutiny. Most of these mutations are reported for the first time. Analysis of mutation frequencies suggests that mutations are more prevalent in the DNA of sperm cells compared to lymphocytes. This observation aligns with the findings of Su et al., 2010 (10), in oligozoospermic and azoospermic populations, where a single mutation, c.822+126T>G/C, was identified. This discrepancy could be attributed to the distinctive nature of sperm formation, which involves a heightened risk of errors compared to the process of cell multiplication (25). Specifically, during spermatogenesis, cell multiplication and differentiation occur through a unique process involving double cell division—mitosis followed by meiosis. Mitosis represents the equational division common to other cells, wherein DNA preservation is meticulously maintained through a repair system to mitigate the risk of mutations (26, 27).

During meiosis, a reductional division unique to gametes, replication errors occur, with the most significant being exchanges of chromosome fragments through crossing-over phenomena, which are responsible for genetic variability (17, 28). Consequently, this specific cell division process suggests a higher mutation frequency in gametes compared to somatic cells (29). The elevated number of mutations observed in ejaculates may thus stem from the utilization of sperm DNA (28). Moreover, the spermatozoon, being the male gamete directly implicated in fertilization, assumes a critical role. Consequently, investigating DNA mutations in semen facilitates the assessment of sperm genetic quality and analysis of mutations potentially transmitted to the embryo (30, 31).

The mutations identified were present in both normospermic and asthenozoospermic ejaculates. Normospermic ejaculates were defined based on sperm analysis (1), which indicated normal characteristics. Consequently, the mutations detected in normospermic ejaculates are unlikely to impact sperm quality. Primarily, the mutations within this group consist of synonymous mutations that do not alter the polypeptide sequence of the protein (32). Additionally, for missense mutations, PolyPhen-2 analysis suggests that these mutations would not adversely affect the protein (23). Thus, the mutations observed in normospermic ejaculates are not detrimental to the integrity of the *TSSK6* protein.

Conversely, within the asthenozoospermic group, certain mutations are anticipated to adversely affect the functionality of the *TSSK6* protein (23). These

mutations were exclusively observed in this cohort of pathological ejaculates. Notably, the *TSSK6* protein plays crucial roles in antioxidant control and safeguarding sperm DNA via HSP proteins (11, 12). Moreover, the mechanism underlying sperm mobility hinges on the orchestrated action of kinases in a cascade reaction (4, 5, 6, 7). Given the predominant presence of the protein in the testis and sperm, it underscores the pivotal role of the protein in modulating sperm mobility mechanisms (8). Hence, the activation of the *TSSK6* protein may contribute to a mechanism that involves the activation of proteins engaged in remodeling and condensing sperm DNA, thereby shielding sperm cells from oxidative stress. Consequently, deleterious mutations in the *TSSK6* gene are posited as risk factors for asthenozoospermia (20, 21). Consequently, mutations in the *TSSK6* gene that are detrimental to the protein would likely be linked with male infertility.

Conclusion

This study unveiled mutations in the coding sequence of the *TSSK6* gene in both normospermic and asthenozoospermic ejaculates. The synonymous mutations c.690T>C and c.372C>A were the most prevalent. Notably, all mutations identified in the normospermic group were benign for the *TSSK6* protein. Conversely, mutations observed in asthenozoospermic ejaculates were found to be damaging to the protein. In summary, mutations in the *TSSK6* gene were detected in infertile men, suggesting an association between asthenozoospermia and deleterious mutations in the *TSSK6* gene.

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Conflict of interest

The authors declare that no conflicts of interest exist.

Authors' contributions

K.G.S.N., J.E.H.A., and A.J.D. participated in the study design; K.G.S.N., J.E.H.A., and M.F.N. conducted the experiments; K.G.S.N., J.E.H.A., and F.A.C. contributed to data analysis and interpretation; F.A.C. and A.J.D. supervised the experiments and provided input for manuscript revision. All authors contributed to the final manuscript and approved the submitted version.

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