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Original Research Article

Polymorphism of the testis-specific serine/threonine kinase 2 gene and risk of asthenozoospermia in Côte d'Ivoire

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ABSTRACT

Background: The testis-specific serine/threonine protein kinase (TSSK2) is an indispensable protein responsible for the mobility of spermatozoa expressed specifically in the germ cells during spermatogenesis and present in the mature spermatozoa. Its gene mutation could constitute a risk of infertility. The aim of this study is to investigate the polymorphism of this *TSSK2* gene in men with asthenozoospermia.
Methods: The ejaculates were obtained from patients attending the reproductive biology unit of Institut Pasteur of Côte d'Ivoire for their spermiological evaluations. The semen analyses are performed with the automatic sperm analyzer SQA-Vision. 30 sperms, including 20 asthenozoosperms and 10 normosperms, were selected from their spermiological results and the spermatozoa DNA was extracted by the phenol/chloroform method. Direct Sequencing of the spermatozoa DNA fragments was done using the Sanger method. The frequencies of mutation were analysis with the Fisher and Mann-Whitney tests.
Results: It was revealed 17 mutations in 22 ejaculates. The frequent mutations are c.839C>T (T280M), c.816G>C (L372L), c.1026G>A (R342R), c.785A>C (H262P) and c.80A>G (K27R) with respectively frequencies of 50.0%, 26.67%, 16.67%, 13.33% and 10.0%. The analysis of these mutations indicated a significant difference in the frequency of occurrence of mutations between normosperms and asthenozoosperms (p-value = 0.01).
Conclusions: This study shows that mutations in the *TSSK2* gene are more common in asthenozoosperm ejaculates than normosperm ejaculates. This fact suggests the probable association of mutations in the *TSSK2* gene with asthenozoospermia.

Keywords: Asthenozoospermia, Human spermatozoa, Gene mutation, Male infertility, *TSSK2* polymorphism.

INTRODUCTION

Asthenozoospermia is one of the most common ejaculate pathologies in male infertility.[1-4] This pathology, which is characterized by a lack of motility of spermatozoa in the ejaculate, can be caused by several factors, including genetic mutations.[5] In fact, asthenozoospermia is associated with mutations observed in certain genes that

are involved in spermatogenesis and sperm metabolism.[6-8] The mobility of the spermatozooa is ensured by a set of biochemical mechanisms with the presence of important phosphorylation reactions.[9]

Furthermore, phosphorylation defects of specific proteins in the sperm tail in response to capacitation have been described in asthenozoosperm patients and are associated strictly with reduced mobility compared to fertile groups.[10,11] Therefore, TSSK2, a serine / threonine kinase protein that is found specifically in the testicles at the germ cells during spermatogenesis and in mature spermatozoa, is one of the proteins involved in this infertility.[12,13]

Previous studies have shown that it plays an important role in the functioning of the spermatozoon. Indeed, it has been reported that mutations in this *TSSK2* gene could lead to morphological and functional abnormalities in spermatozoa.[14,15] In addition, the study conducted by Zhang et al.[16] revealed that mutations c.80A>G (rs3747052) and c.774C>T (rs1052756) of the *TSSK2* gene could be associated with infertility in men. In addition the study conducted by Xu et al.[17] and Shetty et al.[13] sho,wed that the inhibition of the activity of the TSSK2 protein for its substrate TSKS could lead to infertility. Indeed, it is involved in the phosphorylation of serine-288 TSKS substrate and SPAG16 protein actively involved in the formation and functioning of the flagellum, essential element of sperm motility.[18,19] Its involvement in the cytoskeleton and flagella axoneme was also noted.[20] As a result, mutations in the *TSSK2* gene could be a risk factor for asthenozoospermia and therefore male infertility. Thus, the *TSSK2* gene is very important in the search for the causes of male infertility.

The objective of this study is to investigate the polymorphism of this gene in an asthenozoospermia population in Côte d'Ivoire.

METHODS

After sperm assessment, the extraction of sperm DNA was done subsequently by using the phenol / chloroform method. DNA pellet obtained was used to amplify the gene fragment. As for the amplification of the *TSSK2* gene DNA sequences, a conventional PCR was performed in a thermocycler (Applied Biosystems^{Tm} 9700, USA) using primers according to the work of Zhang et al.[16] The PCR have been performed with VWR PCR Kit (Fontenay-sous-Bois, France) containing Taq Polymerase and all reagents for PCR reactions, except DNA and primers, according to the manufacturer's instructions.

The primers supplied by Sigma-Aldrich (Isère, France), SK2-F1/SK2-R1 5' CAATGCTGAGTGTTCCACC CCTG 3' / 5' CTGATGTCGGAGTCGTCATAGGG 3' allowed to amplify the fragment of 810 bp of the 5'UTR + exon region and SK2-F2/SK2-R2 5' ATGCCCTATGACGACTCCGA 3' / 5' TGCTGCTA AGTGGAAGAAAG 3' for the fragment of 615 bp of the exon + 3'UTR region of the *TSSK2* gene. The regions of the amplified gene and their sizes were determined by performing sequence alignment with the products of a PCR simulation using the Serial Cloner software 2.6.1. Thus, the amplification of the gene was performed according to the established program as follows: First, a pre-denaturation was carried out at 94°C for 5 minutes. Then, the reaction was followed by 35 reaction cycles comprising for each cycle a denaturation at 94°C for 30 seconds, the hybridization at 60°C and 55°C for the SK2-F1/SK2-R1 and SK2- F2/SK2-R2 respectively, for 30 seconds and an elongation at 72°C for 60 seconds. Finally, the reaction was terminated by further elongation at 72°C for 10 minutes.

In order to reveal the amplified DNA fragments, an electrophoretic study was conducted. Indeed, 10 µL of each PCR products were migrated in a 1.5% agarose gel with SYBR green and the visualization of the DNA fragments was done using an image analyzer Geldoc (Biorad, USA). Sequencing of the DNA fragments was done using the Sanger method. The sequences generated were read with the software BioEdit Sequence Alignment Editor 7.2.6[21] using the sequence NC_000022.11: 19130808-19132623 Homo sapiens chromosome 22, GRCh38.p12 (GCF_000001405.38) Primary Assembly obtained online on NCBI the January 2, 2019 (https://www.ncbi.nlm.nih.gov/gene/23617).

Statistical analysis

The results obtained from the analyses were analyzed by the R software (1.1.453- © 2009-2018 RStudio, Inc.). The Fisher test was used to study the independence of the frequencies of the mutations between normosperms and asthenozoosperms. The Mann-Whitney test (GraphPad Prism® 6.01) was used to compare the averages of the spermiological parameters of the ejaculates and the proportions of the mutations between the two types of population. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Sperm mobility

The analysis of the ejaculates received gave the spermiological results which showed that 20 patients (66.67 %) had spermatozoa whose mobility is less than 40 %. Statistical analysis of spermiological parameters showed that there is no significant difference between normosperms and asthenozoosperms with the exception of mobility. Indeed, the total mobility of normozoosperm and asthenozoosperm ejaculates were respectively 53.00±10.33 and 31.50±5.87 (P = 0.0001) (Table 1). The table indicates the means with standard deviation of sperm characteristics. Statistical test used is Mann-Whitney.

Table 1: Spermiological parameters of patient ejaculates,

	Ejaculate volume (mL)	Spermatozoa count (x10^6/ejaculate)	Spermatozoa vitality (%)	Spermatozoa mobility in 1 hour (%)	Spermatozoa typical morphology (%)
Normospermia (N = 10)	3.43±1.36	272.2±177.5	80.0±6.98	53.00±10.33	13.0±7.0
Asthenozoospermia (N = 20)	3.45±1.30	263.6±217.7	73.0±11.91	31.50±5.87	8.65±3.69
p-Value	0.974	0.581	0.06	0.0001*	0.07
* Significant difference between the spermatozoa mobility.					

Table 2: Description of *TSSK2* gene mutation

Mutation	Reference	Mutation category	Codon	Amino acid wild type/ mutant	Mutation type
c.80A>G	rs3747052	Transition	27	Lys/Arg	Missense
c.221A>G	rs76318912	Transition	74	Lys/Arg	Missense
c.279C>G		Transversion	93	Gly/Gly	Synonymous
c.313C>A		Transversion	105	Gln/Lys	Missense
c.340C>A		Transversion	114	Arg/Ser	Missense
c.415T>A		Transversion	139	Cys/Ser	Missense
c.420G>C		Transversion	140	Glu/Asp	Missense
c.701A>T		Transversion	234	Asp/Val	Missense
c.775C>A		Transversion	259	Gln/Lys	Missense
c.785A>C		Transversion	262	His/Pro	Missense
c.814C>T		Transition	272	Leu/Phe	Missense
c.816G>C		Transversion	272	Leu/Leu	Synonymous
c.826A>T		Transversion	276	Lys/X	Nonsense
c.839C>T	rs1052763	Transition	280	Thr/Met	Missense
c.882C>T	rs759143051	Transition	294	Arg/Arg	Synonymous
c.892A>T		Transversion	298	Arg/X	Nonsense
c.1026G>A	rs1052773	Transition	342	Arg/Arg	Synonymous

Table 3: Frequencies of TSSK2 gene mutation

Mutation	Total mutants	Frequencies in total effective (%)	Normosperm		Asthenozoosperm	
			Effectives of mutants	Frequencies of mutation (%)	Effectives of mutants	Frequencies of mutation (%)
c.80A>G	3	10.00	0	0	3	15
c.221A>G	1	3.33	0	0	1	5
c.279C>G	1	3.33	0	0	1	5
c.313C>A	1	3.33	1	10	0	0
c.340C>A	1	3.33	1	10	0	0
c.415T>A	1	3.33	0	0	1	5
c.420G>C	1	3.33	0	0	1	5
c.701A>T	1	3.33	0	0	1	5
c.775C>A	1	3.33	0	0	1	5
c.785A>C	4	13.33	0	0	4	20
c.814C>T	1	3.33	0	0	1	5
c.816G>C	8	26.67	2	20	6	30
c.826A>T	1	3.33	0	0	1	5
c.839C>T	15	50.00	3	30	12	60
c.882C>T	1	3.33	0	0	1	5
c.892A>T	1	3.33	0	0	1	5
c.1026G>A	5	16.67	1	10	4	20
Mann-Whitney test p-value				0.01*		

Fisher test for the independence of the frequencies of the mutations between normosperms and asthenozoosperms did not show any significative difference for all mutations (P > 0.05).

TSSK2 gene mutation

As for the results of the genetic analysis of the sperm DNA, they revealed mutations in the coding sequence of the *TSSK2* gene. We found 17 mutations. They are all substitutions distributed as follows: 31.91% of synonymous mutations, 63.83% of missense mutation and 4.26% of nonsense mutations (Table 2).

In general, the frequencies of the mutations vary between 3.33 and 50%. Mutations with high frequencies are c.839C>T (T280M), c.816G>C (L372L), c.1026G>A (R342R), c.785A>C (H262P) and c.80A>G (K27R) with the frequencies of 50.0%, 26.67%, 16.67%, 13.33% and 10.0% respectively (Table 3).

These mutations are found in 22 ejaculates that is at 73.33% of the population studied. In normosperms, mutations are present in 60 % of the population with 40% single mutant (Figure 1). Only 5 types of mutations have been found. They are the mutations c.839C>T (T280M), c.816G>C (L372L), c.1026G>A (R342R), with the frequencies 30%, 20%, 10% respectively. The c.340C>A (R114S) and c.313C>A (Q105K) mutations were found only in normosperms with a frequency of 10% (Table 3). As for asthenozoosperms, 15 mutations were found in 80 % of ejaculates with ejaculates having triple and more mutations (Figure 1). The c.839C>T (T280M), c.816G>C (L372L) and c.1026G>A (R342R) mutations were found in 60%, 30% and 20%, respectively, in these pathological ejaculates. It is in these ejaculates that the c.785A>C (H262P) and c.80A>G (K27R) mutations were observed at frequencies of 20 % and 15%.

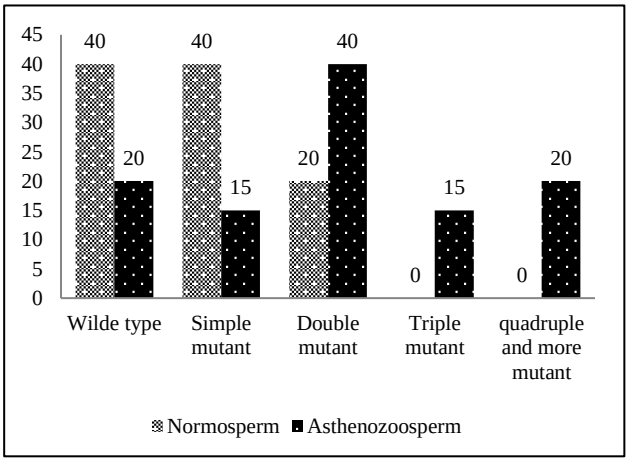

Figure 1: Proportion of ejaculates according the numbers of mutations. Fisher test no significant (P = 0.252).

The Fisher tests showed no significant difference in all mutations between normosperms and asthenozoosperms. While, the Mann-Whitney test showed a significant difference between the averages of the proportions of the mutations of the two types of population with a p-value of 0.01.

DISCUSSION

Asthenozoospermia is one of the most common pathologies in men infertility.[3,4] The spermiological analysis of asthenozoosperm ejaculates showed that they are characterized by low mobility of spermatozoa.[22] This anomaly may be associated with other pathologies (oligo-teratozoospermia) encountered in the case of infertility with associated anomalies. The spermogram carried out on the ejaculates showed indeed a low mobility in the group of asthenozoosperm ejaculates compared to normosperms. In these ejaculates, the mobilities observed, are all less than 40%. Which characterize the pathology asthenozoospermia.

Regarding the analysis of the sequence encoding the *TSSK2* gene, mutations in the normospermic and asthenozoospermic ejaculates of the patients received at the Institut Pasteur of Côte d'Ivoire have been highlighted. Among these mutations, there is the substitution K27R (c.80A>G) which has been described by Zhang et al, in a population of azoosperm and severe oligozoosperm, as a polymorphism of the *TSSK2* gene associated with male infertility.[16] In fact, the amino acids of positions 18 to 26 involved in the nucleotide-phosphate binding of the protein constitute the ATP-binding protein site. This position is very close to this substitution and could disturb the operation of this fixation site. This could therefore be a risk factor in male infertility.[16] In our study, this mutation was found only in asthenozoosperms with a proportion of 15% suggesting its probable association with asthenozoospermia. However, the Fisher test gives a non-significant difference between normal sperms and asthenozoosperms. It is the same for the synonymous mutation c.1026G>A (R342R) whose frequency was 10% in normosperms against 20% in asthenozoosperms.

In addition, the c.839C>T mutation resulting in the substitution of threonine (T-280), an alcohol-functional amino acid, with methionine, a sulfur amino acid, observed in this study would also be implicated in the male infertility.[14] The influence of the mutation on the protein function could be explained by the fact that mutation is located in the C-terminal domain which plays a role in regulating the activity of the TSSK2 protein.[14] This mutation was found in 50% of ejaculates analyzed. It is therefore considered a very important mutation in this study. It is twice as important in asthenozoosperm ejaculates as in normosperm ejaculates. This suggests its probable association with asthenozoospermia. In addition, 60% of these pathological ejaculates carry this mutation. Thus, the mutation c.839C>T (T280M) could

be one of the characteristics of asthenozoosperm ejaculates. It is therefore responsible for the reduced mobility of spermatozoa.

Moreover, among the mutations described frequently, two seem to be new. It is the missense mutation c.785A> C which causes the substitution of histidine at position 262 (H-262) by proline (H262P). It has been described only in pathological ejaculates with a frequency of 20 %. As for the synonym mutation c.816G>C (L272L), it is present in both types of ejaculates. In addition to the c.340C>A (R114S) and c.313C>A (Q105K) mutations, all other mutations are found in asthenozoosperms. The analysis of the mutation showed that 80% of asthenozoosperms have the mutated gene with more than four mutations in the ejaculates. Moreover, the proportions of the types of ejaculates based on the presence or absence of mutation showed that ejaculates with more mutations are all asthenozoosperms. This suggests that the more mutations the *TSSK2* gene has, the higher the risk of being asthenozoosperm.[6,16] In addition, the Mann-Whitney test indicated a significant difference between the number of mutants in normosperms and asthenozoosperms with a p-value of 0.01. This shows that mutations in the *TSSK2* gene of spermatozoa would be more prevalent in asthénozoospermes.[16] Indeed, the high presence of mutations in the gene could lead to damage to the quality of the resulting protein TSSK2 and thus prevent its proper functioning.[5,8] Thus, this could cause damage to the sperm motility mechanism that causes asthénozoospermie.[17,19,23] Mutations in the *TSSK2* gene would then be higher in pathological ejaculates and constitutes a risk factor in male infertility.

CONCLUSION

This study shows that mutations in the *TSSK2* gene are more common in asthenozoosperm ejaculates than normosperm ejaculates. This fact suggests the probable association of mutations in the *TSSK2* gene with this pathology. The important role played by TSSK2 protein in the production and functioning of the spermatozoon led us to consider that, mutations in this gene could be a risk factor for male infertility. It would be interesting to extend this study to a bigger sampling to better appreciate the implication of the mutations of this gene in this pathology.

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