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(RESEARCH ARTICLE)

Evaluation of protamine 2 genes in spermatozoa of teratospermic and oligospermic men in Nigeria

Emmanuel Sunday Oni [1,*], Ojo Moses Oke [1], Josephine Kpalap [2], Samuel Kehinde Wojuade [4], Adewale Oke [3] and Emmanuel Ayomide Oni [1]

[1] *Department of Medical Laboratory Science, Joseph Ayo Babalola University, Ikeji-Arakeji, Osun State, Nigeria.*
[2] *Department of Medical Laboratory Science, Rivers State University Port-Harcourt, Nigeria.*
[3] *Department of Medical Laboratory Science, McPherson University, Lagos Ibadan Express way, Ibadan, Nigeria.*
[4] *Abims Fertility and Andrology Clinic 25, Olowu Street, Off Awolowo way, Ikeja, Lagos.*

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Abstract

There have been several reports of single nucleotide polymorphisms (SNPs) linked to different kinds of male infertility. The aim of the study was to evaluate single nucleotide polymorphisms in protamine 2 gene (PRM2) in spermatozoa of teratospermic and oligospermic infertile men in Nigeria. At some fertility clinics in Lagos, Nigeria, twenty-two (22) teratospermic and thirty-five (35) oligospermic infertile men as well as thirty (35) normospermic fertile men (control) who volunteered and gave consent were recruited for the cross-section study after meeting the inclusion criteria and confirmation of their fertility statuses by the use of computer-Assisted Sperm Analyzer (CASA). Semen was collected from the participants under the WHO guideline for semen collection and processing. Spermatozoa's DNA was extracted with the use of Proteinase K Storage Buffer. Nanodrop 1000 spectrophotometer was used to quantify the isolated genomic DNA. PRM II F: 5-AGGGCCCTGCTAGTTGTGA-3' and PRM II R: 3'- CAGATCTTGTGGGCTTCTCG -5, were used as primers. Sequencing of sperm DNA was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. Agarose gel electrophoresis was used to show the amplified PRM2. In the study, 7 SNPs in the teratospermic infertile men, 8 SNPs in the oligospermic infertile men and 7 SNPs in the normospermic fertile men were discovered. The SNPs between the teratospermic infertile men and the oligospermic infertile men are significantly different. We propose that considerably larger genome-wide investigations are required to confidently validate these SNPs and find new SNPs linked with male infertility.

Keywords: Protamine 2 gene; Single nucleotide polymorphisms; Teratospermic; Oligospermic; Infertile

1. Introduction

Male infertility is defined as a man's failure to impregnate a fertile woman after minimum of twelve months of regular and unprotected sexual intercourse. (Oni *et al*., 2023). Infertility affects approximately 13% to 15% of all couples globally (Esteves *et al*., 2011). Male infertility is most caused by deficiencies in the sperm, which is used as a surrogate measure of male fecundity (WHO, 2010). Many men with male infertility have oligospermia, and over 80% of infertile men have an increase in spermatozoa with abnormal morphology (teratospermia) (Anawalt, 2024).

Teratospermia is a condition in which the sperms in a man's ejaculate are defective or not well formed, while oligospermia is a male fertility issue characterized by a low sperm count (Hollan, 2018). A healthy sperm count is frequently required for fertility.

* Corresponding author: Oni Emmanuel Sunday

The World Health Organization (WHO) classifies sperm counts at or above 15 million sperm per milliliter (mL) of semen as average. Anything below that is considered low and is diagnosed as oligospermia. Severe oligospermia is diagnosed when sperm counts fall between 0 and 5 million sperm/mL (Hollan, 2018). Evaluation is the starting point for treatment of infertility as it may suggest specific causes and appropriate treatment modalities (Makar & Toth, 2002).

Genetic factors may be involved in idiopathic male infertility, which accounts for 40% of cases, even though the related genes remain unknown (WHO, 2010). Protamine and DNA were isolated and discovered from the sperm more than a century ago by Friedrich Miescher. They are the most abundant sperm nuclear proteins in many species and act by packaging the paternal genome (Aoki & Carrell, 2003). It has been shown that protamines mutation and polymorphism occur in infertile men (Jiang *et al.,* 2015)

Some alterations in protamines like protamine gene mutation, abnormal transcription regulation, expression deregulation and unsatisfactory post-translational processing can lead to male infertility. In addition, protamine mutations account for abnormal spermatogenesis which results to sperm DNA break and chromatin damages (Ferling *et al*., 2006)

Despite substantial knowledge available on the fundamental aspects of the transcptional mechanisms, so far there have been relatively few studies assessing the potential involvement of changes in protamine gene transcription factors in human male infertility (Krausc & Sassone-Corsi, 2005). Because of the extensive evidence for deregulation of protamine expression in male infertility this issue would deserved further attention.

To the best of our knowledge, there are insufficient data on the study of protamine 2 gene with respect to teratospermia and oligospermia infertility in Nigeria. Thus, the aim of the present study was to evaluate single nucleotide polymorphisms in protamine 2 gene in spermatozoa of teratospermic and oligospermic infertile men in Nigeria.

2. Material and methods

2.1. Study Area

The study was conducted in Abims fertility clinics in Lagos Metropolis, Lagos State, Nigeria.

2.2. Scope of Experimental Design

Ninety-two (92) Nigerian men (aged 30-59 years) who visited Fertility Clinics in Lagos State at the time of assessment were selected as fertile and infertile subjects. The clinical procedures for screening semen subjects were performed at the fertility clinic and included a complete personal and familiar medical history to rule out heritable conditions, a physical examination, and a minimum of two semen analyses performed in accordance with World Health Organization guidelines (WHO, 2010), with the exception of motility assessments, which were performed at room temperature. Spermiograms contained volume, pH, sperm concentration, a four-category motility rating, vitality, and morphology.

Duplicate aliquots of ≥200 cells were used to evaluate motility and sperm count, with controls in place to ensure acceptable discrepancies between duplicates. Sperm concentration was carried out on diluted, immobilized samples utilizing haemocytometer chambers. To obtain objective measures of sperm kinematics, fresh ejaculates underwent computer-assisted sperm analysis (CASA) using a Hamilton-Thorn 2030 system (software version 6.4) (WHO, 2010). Serological tests for HIV I and II, hepatitis B and C, CMV, and syphilis were performed at baseline and at the conclusion of the contributions. Semen parameters from each individual donation were examined to compare semen quality to a baseline assessment.

The collection techniques were consistent across all subjects. According to the World Health Organization Laboratory Manual for the Examination and Processing of Human Semen, the selection criteria were total motility of less than 32% (WHO, 2010).

2.3. Volunteer group

Ninety-two (92) subjects were recruited for the study.

2.4. Control group

This group consisted of thirty-five (35) male fertile subjects within the age range of 30-59 years.

2.5. Experimental group

This group consisted of twenty two (22) teratospermic infertile men and thirty five (35) oligospermic infertile men within the age range of 30-59 years.

2.6. Inclusion Criteria

According to World Health Association guidelines, the inclusion criteria were based on the sperm total motility of less than 32% (WHO, 2010) for infertile males.

2.7. Exclusion Criteria

Varicocele, cryptorchidism, iatrogenic infertility, testis trauma, prior genital infections, exposure to chemotherapy or radiation, Klingelherr's syndrome, cystic fibrosis, addiction to alcohol or cigarettes, and exposure to the environment, such as driving, baking, mining, or working in chemical plants, were among the criteria that excluded male subjects from the study.

2.8. Semen collection and processing

Semen was extracted by ejaculation and placed into sterile plastic containers following a three-day period without sexual activity. The samples were left on the bench at 25 °C room temperature and given 40 minutes to liquefy. The specimens of liquid semen were centrifuged for 15 minutes at 4500 rpm. After that, each seminal sample of supernatant was cautiously aliquoted into Eppendorf tubes. After that, the seminal plasma was frozen at -20°C for molecular examination.

2.9. Confirmation of Subject Status

The CASA machine was capable of fully detecting motile sperms. During the operation, at least 109 motile spermatozoa are tracked. The CASA machine was linked to computer software, allowing for data organizing and statistical analysis.

2.10. Evaluation of Semen Motility by Computer Assisted Sperm Analyzer (CASA)

The CASA machine was capable of fully detecting motile sperms. During the operation, at least 109 motile spermatozoa are tracked. The CASA machine was linked to computer software, allowing for data organizing and statistical analysis.

2.11. DNA Extraction from Semen

Proteinase K Storage Buffer was used to extract the DNA from the sperm.

2.12. Sperm DNA Quantification

An instrument called the Nanodrop 1000 spectrophotometer was used to quantify the isolated genomic DNA. By double clicking the Nanodrop icon, the equipment's software may be accessed. Using DNA elution buffer, the apparatus was blanked after being initiated with 2 µL of sterile, nuclease-free water. After loading two microliters of the extracted DNA onto the lower pedestal, the higher pedestal was lowered so that it made contact with the extracted DNA on the lower plaque. Selecting the "measure" button allowed us to determine the concentration of DNA.

2.13. Amplification of Protamine 2 Gene

Using the primers PRM II F: 5-AGGGCCCTGCTAGTTGTGA-3' and PRM II R: 3'- CAGATCTTGTGGGCTTCTCG -5', the protamine II genes from the semen samples were amplified for 35 cycles at a final volume of 25 microliters in an ABI 9700 Applied Biosystems thermal cycler. The PCR mix included: The X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95 ºC for 5 minutes; denaturation, 94 ºC for 30 seconds; annealing, 61 ºC for 30 seconds; extension, 72 ºC for 30 seconds for 35 cycles and final extension, 72 ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130 V for 30 minutes and visualized on blue light imaging system for a 900 base pair product size.

2.14. Protamine 2 Gene Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. Sequencing was performed with a final volume of 10 µL. The kit comprised 0.25 µL BigDye® terminator v1.1/v3.1, 2.25 µL 5× BigDye sequencing buffer, 10 µM Primer PCR primer, and 2-10ng PCR template per 100 bp. The sequential conditions were as follows: 32 cycles of 96 °C for 10 seconds and 55 °C for 4 minutes

2.15. Statistical Analysis

The statistical analysis was carried out using GraphPad Prism version 8.02 (San Diego, California, USA). The difference in SNP results was statistically significant at $p<0.05$

3. Results

3.1. Result of agarose gel electrophoresis showing the amplified *PRM2*

The result of the Agarose gel electrophoresis representing the PCR amplification of 10 constituent fragments of the 100-bp ladder, showed the protamine 2 DNA band as 900 base pairs (Figure 1.0). Lanes 1-10 represent the *PRM2* Bands at 900bp, while lane represents the molecular Ladder

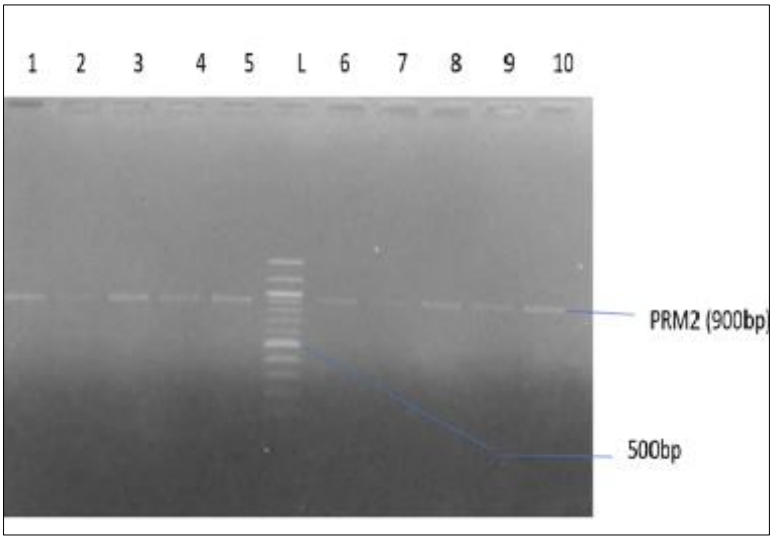

Figure 1 Agarose Gel Electrophoresis showing the Amplified PRM 2 Genes. Lanes 1-10 Represent the PRM 2 Gene Bands at 900bp, while Lane Represents the Molecular Ladder

3.2. Result of SNPs Distribution in *PRM2* of Teratospermic, Oligospermic infertile and Normospermic fertile men

Table 1 SNPs Distribution in *PRM2* of Teratospermic, Oligospermic infertile and Normospermic fertile men

S/N	Variants	SNP	TSZ (n=22)	Locus in TSZ	OZS (n=35)	Locus in OZS	NZS (n=35)	Locus in NZS
1	rs1479789045	G>C or A>G	Present	4	Present	4	Present	4
2	rs2069880888	C>T	Present	7	Absent	-	Present	16
3	rs2069881018	G>C	Present	14	Present	16	Absent	-
4	rs2069880951	A>T or T>A	Present	16	Present	18	Present	18
5	rs1434703461	C>T or G>T	Present	28	Absent	-	Present	28
6	rs1052575569	G>A or C>A	Present	32	Absent	-	Absent	-
7	rs1281533806	C>A	Present	46	Absent	-	Absent	-
8	rs549835830	G>A	Absent	-	Present	16	Absent	-
9	rs539592598	G>T or A>T	Absent	-	Present	20	Absent	-
10	rs935520555	C>G	Absent	-	Present	36	Present	36

11	rs590570800	G>C or C>T	Absent	-	Present	40	Absent	-
12	rs1382451565	C>T	Absent	-	Present	28	Present	40
13	rs2069880799	C>T	Absent	-	Absent	-	Present	16

Keynotes: SNP: single nucleotide polymorphism, TZS: Teratospermic, OZS: Oligospermic, NZS: Normospermic, >: replace

3.3. Result of Global MAF of PRM2 gene variants and functional consequences of SNPs observed in Spermatozoa of teratospermic, oligospermic infertile and normospermic fertile men

Table 2 Global MAF of PRM2 gene variants and functional consequences of SNPs observed in Spermatozoa of teratospermic, oligospermic infertile and normospermic fertile men.

S/N	Variants	Global MAF	Nucleotide Change	Function consequence
1	rs1479789045	C=0. /0 (0/14050) C=0.000026/17 (7/264690) C=0.00002914 (4/140120)	G/C> or A>G	No consequence
2	rs2069880888	A =0. /0 (0/10680) A=0.00007/1(1/264690)	C > T	No consequence
3	rs2069881018	G=0. /0 (0/10680) G=0.000004/1(1/264690)	G>C	No consequence
4	rs2069880951	A=0. /0 (0/10680) A=0.000004/1(1/264690)	A>T or T>A	No clinical consequence
5	rs1434703461	A=0. /0 (0/10680) A=0.000004/1(1/264690)	C>T or G>T	No consequence
6	rs1052575569	T=0. /0 (0/14050) T=0.000008/2(2/264690) T=0.000021/3(3/140246)	G>A or C>A	No consequence
7	rs1281533806	A=0. /0 (0/14050)	C>A	No consequence
8	rs549835830	G = 0./0 (0/14050) G =0.000026/7 (2/264690) G = 00000294/4 (2/140234)	G>C	No consequence
9	rs539592598	A =0./0 (0/14050) A =0.000007/1 (1/140262) A= 0.0000019/5 (5/264690)	G>T or A>T	No consequence
10	rs935520555	A= 0.0 (0/14050) A=0.000023/6 (6/264690) A=0.0000443/6 (6/140262)	C>G	No consequence
11	rs590570800	A = 0. /0 (0/14412) A = 0.00187/9 (12/6404) A = 0.00935/2 (2/214)	C/G>T	No consequence
12	rs1382451565	A = 0./10 (0/14050) A =0.00004/1 (1/264690) A= 0.000007/1 (1/140278)	C>T	No consequence
13	rs2069880799	A =0. /0 (0/10680) A=0.00007/1(1/264690)	C>T	No consequence

Keys: M = Minor MAF = A=0. /0 (0/14050); A = Allele TOPMED = A= 0.000004/1 (1/264690); F = Frequency GnoMAD = A = 0.000007/1 (1.40278) (NCBI database)

4. Discussion

Spermatozoa count and morphology are important and complex characteristics of the fertilization capacity of male germ cells (Agarwal *et al.,* 2014). Abnormal in shape, or the sperm count is low, may not be able to fertilize the female egg if it is immobilized (Ghadirkhomi *et al*., 2021). Problems with sperm production or quality may occur due to hormonal imbalance, damage to the urinary or genital organs, fever or heat stroke, some drugs and toxins, or some genetic disorders.

Genetic variability in the spermatogenesis process may be relevant in sperm production abnormalities and male infertility (Ghadirkhomi *et al.,* 2022). One of the most promising areas of male infertility research is the study of single nucleotide polymorphisms in critical genes involved in the spermatogenesis process (Ghadirkhomi *et al.,* 2022).

The aim of this study is find out single nucleotide polymorphisms in *PRM2* of spermatozoa of teratospermic and oligospermic infertile men. However, we included normospermic fertile men as control. The objective of the study was to compare the single nucleotide polymorphisms, if there are, between the teratospermic and oligospermic infertile men. Studies done by (Ghadirkhomi *et al.* (2022) and others have illustrated the association of SNPs and infertility in men in other regions of the world.

In our study, the sequence alignment of the 5'-UTR of PRM 2 gene of the teratospermic, oligospermic infertile men and normospermic ferile men (control) with the reference sequence showed the distribution of Single Nucleotide Polymorphisms (SNPs) in these categories. A total of 40 variants were observed in the sequence alignment of the 5'-UTR of PRM 2 gene of the spermatozoa of teratospermic infertile men, 40 variants were observed in the sequence alignment of the 5'-UTR of PRM 2 gene of the spermatozoa of oligospermic infertile men and over 30 variants were observed in the sequence alignment of the 5'-UTR of PRM 2 gene of the spermatozoa of the normospermic fertile men.

However, the blast results of PRM II gene (PRM II_F06_09) indicated the presence of 7 single nucleotide polymorphism of SNV type (single nucleotide variant: is a kind of SNP in a population genome limited to the germline DNA) in the chromosome loci 4, 16, 18, 28, 36, and 40. 8 single nucleotide polymorphism of SNV type (single nucleotide variant in the chromosome loci 4, 7, 14, 16, 28, 32, 40, in the oligospermic infertile men and 7 single nucleotide polymorphism of SNV type in the chromosome loci 4, 16, 18, 28, 36, and 40 in the control. The SNPs substitutions and resultant variants are shown in Table 1.1.

Some variants and SNPs in the *PRM2* were found common (similar) between the teratospermic, oligospermic infertile men and the normospermic fertile men when compared. The similar variants and SNPs included: rs2069880951 with nucleotide change of A>T or T>A; occurred at locus 16 in the teratospermic infertile men, locus 18 in the oligospermic infertile men and locus 18 in the control's *PRM2*. Also, variant rs1479789045 with nucleotide change G>C or A>G was found present at loci 4 in the teratospermic, and oligospermic infertile men as well as the control (Table 1.1). Also, we compared the difference between the SNPs observed in the teratospermic and oligospermic infertile men, the difference was statistically significant.

Several single nucleotide polymorphisms (SNPs) associated with various classes of male infertility have been reported (Kennet *et al.*, 2010). Kennet et al. (2010) reported several SNPs in oligospermic infertile in men, our findings revealed presence of several SNPs in the infertile men in this study. However, the SNPs discovered in their study, differ from our study. Ethnicity and environmental factors are factors that influence variations. Nabi et al. 2018 reported 6 SNPs in asthenospermic infertile men; they also reported significant difference with the control group when compared. In our findings, there was no significant difference in each group compared with the control.

In Addition, we keyed in the results of the observed SNPs in our study in the database of NCBI, the interpretation given by the NCBI database identified the SNPs as non-pathological (no clinical consequences) and the Global MAF is very minimal (Table 1.2). Although some studies have proposed some *PRM2* variants and SNPs to be pathologically associated with infertility in oligospermic and teratospermic infertile men. We did not find these novel SNPs to be pathologic.

5. Conclusion

We found 7 SNPs in the teratospermic infertile men, 8 SNPs in the oligospermic infertile men and 7 SNPs in the normospermic fertile men. The SNPs between the teratospermic infertile men and the oligospermic infertile men are significantly different. The SNPs were not related to pathogenesis of infertility according to NCBI Database. We propose

that considerably larger genome-wide investigations are required to confidently validate these SNPs and find new SNPs linked with male infertility.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The Authors have no conflicts of interest to declare.

Statement of ethical approval

The Health Research Ethics Committee, College of Medicine, University of Lagos, granted approval for the study.

Statement of informed consent

In addition, Inform consent was obtained from all individual participants prior to being recruited for the study.

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