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Single nucleotide polymorphisms in protamine 2 genes in fertile and infertile human males in Southwest, Nigeria

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Abstract

It is reported that variations in the nucleotide sequence of protamine genes play contributory role in men's fertility. The aim of this study was to evaluate single nucleotide polymorphisms (SNPs) in protamine 2 genes (*PRM2*) of infertile and fertile men in Southwest, Nigeria. In this cross-sectional study, 92 volunteers (57 infertile men and 35 fertile men) age 30-59 years who visited fertility clinics at the time of this study were recruited after thorough evaluation of their clinical fertility histories and confirmation of their fertility statuses, aided by Computer Assisted Semen Analyzer and WHO guidelines for semen analysis. Polymerase chain reaction analysis of SNPs in *PRM2* in the semen samples was determined. BLAST results of the 5'-UTR of *PRM2*, identified 16 novel SNPs in total, in the infertile and fertile men. Out of the 16 SNPs discovered, 15 SNPs were found in the infertile men, randomly distributed and 7 SNPs were discovered in the fertile men. However, there were six SNPs which were common between the infertile and fertile men. The common SNPs occurred at one or more loci in the *PRM2* in the infertile men than in the fertile men. We found one variant rs2069880799 (16C>T) present in the fertile men but missing in the infertile men. In general, the SNPs in *PRM2* were statistically different between the infertile and fertile men when compared. All the SNPs discovered, according to NCBI database were clinically non pathological.

Keywords: Single Nucleotide Polymorphism; Sequence: Fertility; Protamine 2; Mutation; DNA

1. Introduction

The European Bioinformatics Institute (EMBL-EBI, 2020) defines genetic variation as the difference in DNA sequences between individuals within a population. Variation occurs in germ cells i.e., sperm and egg, and in somatic cells. Only variation that arises in germ cells can be inherited from one individual to another and so affect population dynamics, and ultimately evolution. Findings show that each human has on average 60 new mutations compared to their parents (Oni, *et al.*, 2023; Conrad, *et al*., 2011; Science Daily, 2011).

Ultimately, genetic variation is caused by variation in the order of bases in the nucleotides in genes (polymorphism). Single nucleotide polymorphisms (SNPs) are polymorphisms that are caused by point mutations that give rise to different alleles containing alternative bases at a given position of nucleotide within a locus (Jin *et al.,* 2016). Mutations are the number one source of genetic variation. A mutation is a permanent alteration to a DNA sequence (EMBL-EBI, 2020). *De novo* (new) mutations occur when there is an error during DNA replication that is not corrected by DNA repair enzymes. Mutations and polymorphism may be beneficial, harmful, or of no effect on the organism (EMBL-EBI, 2020)

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Our study is centered on deciphering protamine 2 genetic variations in infetile and fertile men in Southwest, Nigeria. It is reported that protamines play vital role in spermatogenis (Wang *et al*., 2019) hence, influencing fertility in men. Infertility is a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse (WHO, 2024). Infertility may be caused by several different factors, such as malformations of the reproductive tract (cryptorchidism or varicocele, orchitis, karyotype anomalies (Jiang *et al.,* 2015). However, it is sometimes not possible to explain the causes of infertility.

Sperm DNA damage is the single largest cause of defective sperm function and may be the underlying cause of idiopathic male infertility (Bisht *et al.,* 2016). Protamine is the major DNA-binding protein in sperm nucleus that promotes DNA condensation and packaging in spermatozoa by histone replacement during spermatogenesis (Oni, *et al*.,2023).

During mammalian spermiogenesis, the chromatin structure undergoes substantial condensation (Cho *et al.,* 2001). The key role in this process is played by protamine 1 (*PRM1)* and protamine 2 genes (*PRM2*) (Cho *et al.,* 2003). Protamine 2 is a small, arginine rich nuclear proteins that are involved in parking deoxylribonucleic acid (DNA) in spermatozoa (Ammer *et al*., 1986). The *PRM2* are localized on chromosome 16p13.3. *PRM2* is translated as a precursor protein consisting of 103 aminino acids, which undergoes proteolytic cleavage to form 50 amino acid matured protein (Balhorn *et al.,* 1999). *PRM2* is transcribed in the post-meitotic haploid spermids, whereas the proteins are completely absent at this stage (Steger *et al*., 2000; Stewart *et al.*, 1999). The translation of *PRM2* transcripts is initiated and completed by the elongation stage of spermatid development (Kleene, 1996)

The combination of P1 and P2 with sperm nucleic acid results to more chromatin condensation, solidification, and very compact DNA. This chromatin compaction is required for normal sperm function, hydrodynamic shape, motility, and protection of genetic information. Any changes in the protamine genes may result to changes in their expression, P1:P2 ratio and sperm condensation (Aoki *et al.*,, 2006a). Abnormal spermatozoal PRM1/PRM2 protein ration can be contributed to abnormal chromatin condensation and increase DNA strand breaks, which result in male infertility (Aoki *et al.,* 2006b; Mengual *et al.,* 2003).

Several molecular epidemiological studies have been conducted to examine the association between PRM1 and PRM2 polymorphisms and male infertility in diverse populations. Mutations or polymorphisms within protamine genes induce conformational changes of the encoded proteins and alter their incorporation into sperm chromatin, leading to sperm defects (Jiang *et al.,* 2017).

The human sperm protamine deficiency correlates significantly with diminished semen quality parameters, sperm functional ability, and sperm DNA integrity (Aoki *et al.*, 2005). P2 is more important for maintaining male ferrtility. Mouse knockout models clearly demonstrated that sperm protamine haploinsufficiency directly impairs spermatogenesis and subsequent embryo development (Cho *et al.*, 2003; Cho *et al.,* 2001).

The study of human genetic variation has evolutionary significance and medical applications. For medicine, study of human genetic variation may be important because some disease-causing alleles occur more often in people from specific geographic regions.

There is continous studies to discover whether sperm cells from an individual human males possess variations in *PRM2*. The aim of the study is to evaluate single nucleotide polymorphism in protamine 2 genes sperm cells of fertile and sub-fertile men in Southwest, Nigeria.

2. Materials and Methods

2.1. Study Area

The study was carried out in Fertility Clinics in Lagos Metropolis, Lagos State, Nigeria

2.2. Ethical Approval

Before the start of the experimental study, ethical approval was obtained from Health Research Ethics Committee, College of Medicine, University of Lagos, with approval number CMUL/HREC/02/21/543. Also, written permission was obtained from various Clinics authorities and the participants were requested to fill informed consent before being recruited for the study.

2.3. Scope of Experimental Design

A total of ninety-two (92) Nigerian men (aged 30-59years) who attended Fertility Clinics in Lagos State, at the time of assessment were recruited as fertile and infertile subjects. The clinical procedures for screening semen subjects were done at the fertility Clinic and included full personal and familiar medical history to rule out heritable conditions, physical examination, and a minimum of two semen analyses performed in accordance with the World Health Organization guidelines (WHO, 2010) except for motility assessments that were done at room temperature. Spermiograms included were volume, pH, sperm concentration, four-category motility assessment, vitality, and morphology. Motility and sperm count were done in duplicate aliquots of ≥200 cells, and measures were adopted to control for acceptable differences between duplicates. Sperm concentration was performed on diluted, immobilized samples using haemocytometer chambers. Computer-assisted sperm analysis (CASA) was performed on fresh ejaculates with a Hamilton-Thorn 2030 system (software version 6.4) to obtain objective measurements of sperm kinematics (WHO, 2010). Serological tests for HIV I and II, hepatitis B and C, cytomegalovirus and syphilis were done at baseline, at the end of the donations. Semen parameters of each individual donation were measured to monitor semen quality relative to the baseline assessment.

Collection processes were the same in all subjects. According to the World Health Organization Laboratory Manual for the Examination and Processing of Human Semen, the selection was based on the total motility of less than 32% (WHO, 2010).

2.3.1. Volunteer group

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

2.3.2. Control group

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

2.3.3. Experimental group

This group consisted of fifty-seven (57) infertile males within the age range of 30-59 years

2.4. 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.

2.5. Exclusion Criteria

Male subjects with any of the following criteria were excluded from the study, varicocele, cryptorchidism, iatrogenic infertility, testis trauma, previous genital infections, and exposure to chemotherapeutics or radiation, klinifelter's syndrome, cystic fibrosis, addiction to smoking, alcohol drinking and environmental exposure like driving job, miners, bakers, and workers of chemical plants.

2.6. Semen collection and processing

After three days of abstaining from sex, semen was collected into sterile plastic containers by ejaculation. The specimens were placed on the bench at room temperature (25 °C) and allowed for 40 minutes for liquefaction. The liquefied semen specimens were centrifuged at 4500 rpm for 15 minutes. Each supernatant seminal sample was then carefully aliquot into Eppendorf tubes. The seminal plasma was then frozen at -20°C until molecular analysis.

2.7. Confirmation of Subject Status

The CASA machine was able to fully detect the motile sperms. The procedure involves the tracking of at least 109 motile spermatozoa. The CASA machine was linked to computer software that permitted data organization and statistical analysis.

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

The CASA machine was able to fully detect the motile sperms. The procedure involves the tracking of at least 109 motile spermatozoa. The CASA machine was linked to computer software that permitted data organization and statistical analysis.

2.8.1. DNA Extraction from Semen

Sperm DNA was extracted with the use of Proteinase K Storage buffer

2.8.2. Sperm DNA Quantification

The extracted genomic DNA was quantified using Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µLof sterile nuclease free water and blanked using DNA elution buffer. Two microlitres of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

2.8.3. Amplification of Protamine 2 Gene

Protamine II genes from the semen samples were amplified using the PRM II F: 5-AGGGCCCTGCTAGTTGTGA-3' PRM II R: 3'- CAGATCTTGTGGGCTTCTCG -5' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 25 microlitres for 35 cycles. 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.

2.8.4. Protamine 2 Gene Sequencing

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

2.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8.02 (San Diego, California, USA). Difference of the SNPs result was considered statistical significant at $p< 0.05$).

3. Result

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

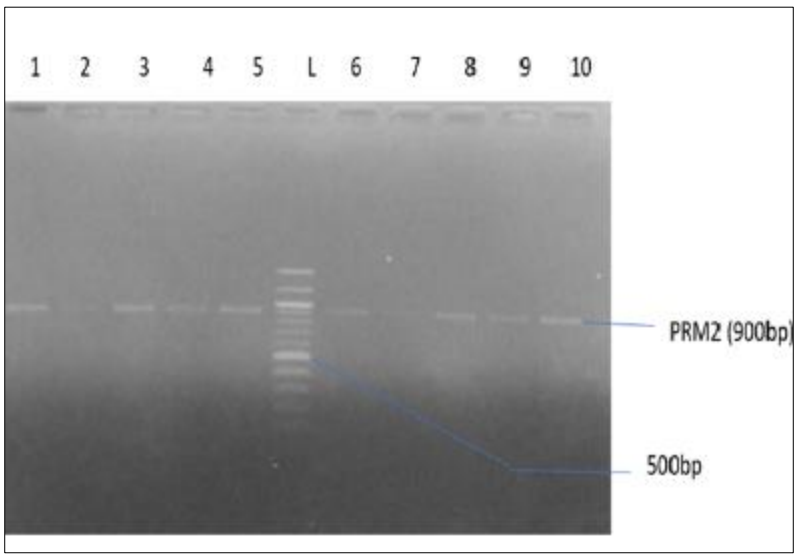

Figure 1 Agarose Gel Electrophoresis showing the Amplified PRM 2 Genes. Lanes 1-10 Represent the PRM 2 Gene Bands at 900bp, while Lane Represents the Molecular Ladder.

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

3.2. Results of SNPs found present and absent in infertile and fertile male participants

Table 1 *PRM2* genes variants in infertile and fertile male groups

Gene	Variants	SNP	Infertile group	Loci found in Infertile group	Fertile group	Loci found in fertile group
PRM2	rs2069880951	A>T /T>A	Present	16,18, 20	Present	20
	rs570570800	C>T/ G>T	Present	40, 28	Absent	-
	rs935520555	G>C	Present	36, 38	Present	36
	rs1382451565	C>T	Present	28, 38	Present	40
	rs1281533806	C>A	Present	46	Absent	-
	rs1479789045	G/C>A/ A>G	Present	4	Present	4
	rs549835830	G>C	Present	6	Absent	-
	rs997110743	G>C/C>G	Present	21	Absent	-
	rs1434703461	C>T/ G>T	Present	28, 31	Present	28
	rs2069881018	G>C	Present	14, 16	Absent	-
	rs53952598	G>T/ A>G	Present	20	Absent	-
	rs119399669	C>T	Present	60, 45	Absent	-
	rs2069880888	C>T	Present	7	Present	16
	rs1052575569	G>A/C>A	Present	32	Absent	-
	rs1236501971	C>A	Present	46	Absent	-
	rs2069880799	C>T	Absent	-	Present	16

3.3. Result of global minor allele frequency and functional consequences of SNPs in infertile and fertile human males

Table 2 Global MAF and Functional consequences of SNPs in infertile and fertile males

S/N	Variants	Global MAF	Functional consequences	Nucleotide changes
1	rs2069880951	A = 0. / 0 (0/10680) A = 0.000004/1 (1/264690)	No Clinical Functional Consequences	A>T/ or T>A
2	rs570570800	A = 0. /0 (0/14412) A = 0.00187/9 (12/6404) A = 0.00935/2 (2/214)	No Clinical Functional Consequences	C/G>T
3	rs935520555	A = 0. /0 (0/14050) 0.000023/6 (6/264690) 0.000043/6 (6/140262)	No Clinical Functional Consequences	G>C

4	rs1382451565	A = 0. /0 (0/14050) A = 0.00004/1 (1/264690) A = 0.000007/1 (1/40278)	No Clinical Functional Consequences	C>T
5.	rs1281533806	A = 0. /10 (0/14050)	No Clinical Functional Consequences	C>A
6	rs1479789045	A= 0. /0 (0/14,050) C= 0.000026/7 (7/264690) C= 0.000029/4 (4/140120)	No Clinical or Pathology Consequences	G/C> or A>G
7	rs549835830	G = 0. /0 (0/14050) G=0.000026/7 (2/264,690) G= 000029/4 (2/140234)	No Clinical or Pathology Consequences	G>C
8	rs997110743	G= 0. /0 (0/14050) G= 0.000019/5 (5/264690) G= 0.000014/2 (2/140256)	No Clinical or Pathology Consequences	C>G G>C or C>G
9	rs1434703461	A = 0. /0 (0/14050) A=0.000023/6 (6/264690) A=0.000043/6 (6/140262)	No Clinical or Pathology Consequences	C/T or G/T
10	rs2069881018	G=0. /0 (0/10680) G=0.000004/1(1/264690)	No Clinical or Pathology Consequences	G>C
11	rs53952598	0./0 = (0/14050) 0.00000 7/1 (1/140262) 0.00000 19/5 (5/264690)	Pathology Consequences	G/T> or A>T
12	rs119399669	0./00.000111/1(1/8988) 0.000004/1(1/242,424)	No Clinical or Pathology	C>G
13 14 rs1052575569	rs2069880888	A =0. /0 (0/10680) A=0.00007/1(1/264690) T=0. /0 (0/14050) T=0.000008/2(2/264690) T=0.000021/3(3/140246)	No Clinical or Pathology Consequences	C>T G>A or C>A
15. 16	rs1236501971 rs2069880799	A= 0.0(0/14050) A = 0.00007/1 (1/204256)	No pathology consequences No pathological or Consequences	C>A C>T

Keys: M = Minor MAF = A=0. /0 (0/10680; A = Allele TOPMED = A= 0.000004/1 (1/264690);F = Frequency GnoMAD = A = 0.000007/1 (1.40278); Source: (NCBI database)

Table 3 Frequency of *PRM2* gene variants in sperm cells of infertile and fertile male groups

Variant	SNP	Number	Frequency	% Frequency
rs2069880951	A>T /T>A	6	0.066	6.6
rs570570800	C>T/ G>T	3	0.033	3.3
rs935520555	G>C	6	0.066	6.6
rs1382451565	C>T	5	0.054	5.4
rs1281533806	C>A	3	0.033	3.3

rs1479789045	G>C>A/A>G	4	0.043	4.3
rs549835830	G>C	2	0.022	2.2
rs997110743	G>C/C>G	1	0.011	1.1
rs1434703461	C>T/ G>T	3	0.033	3.3
rs2069881018	G>C	3	0.033	3.3
rs53952598	G>T or A>T	1	0.011	1.1
rs119399669	C>T	1	0.011	1.1
rs2069880888	C>T	2	0.022	2.2
rs1052575569 rs2069880799 rs1236501971	G>A or C>A C>T C>A	1 1 1	0.011 0.11 0.11	1.1 1.1 1.1

4. Discussion

The study evaluated the single nucleotide polymorphisms in *PRM2* in infertile and fertile men in Southwest, Nigeria. Collectively, sixteen (16) variants with SNPs in *PRM2* were identified in the infertile and fertile male participants. Out of the 16 SNPs discovered, 15 SNPs were found in the infertile men, randomly distributed and 7 SNPs were discovered in the fertile men. However, there were six variants with SNPs which are common between the infertile and fertile men. They include: rs2069880951 (A>T), rs935520555 (G>C), rs1479789045 (G>C>A/A>G), rs1434703461 (C>T/G>T), rs2069880888 (C>T), and rs2069880799 (C>T). The common SNPs occurred at one or more loci in the *PRM2* in the infertile men than in the fertile men. Furthermore, we found one variant rs2069880799 (16C>T) present in the fertile men but missing in the infertile men (Table 1.1). In general the SNPs in *PRM2* were statistically different between the infertile and fertile men when compared.

The SNPs and their frequencies discovered in our study were different from other *PRM2* polymorphism studies done in other countries. Al Zeyadi et al. (2023) reported 3 consistent SNPs in infertile males in their study in Iraq, Nabi et al. (2018), reported 6 SNPs in asthernozoospermic in fertile men in Iran. Grassetti et al. (2012) reported 4 SNPs in infertile males in Italy. On the ground of unequal distributions of SNPs in infertile men, our finding was similar to Grassetti et al. (2012).

We found rs2069880951 (A>T/T>A), and rs935520555 (G>C) with the highest occurring frequency (Table 1.3). In a previous study, Tanaka et al. (2003) reported that prevalence of the SNPs in infertile and fertile men were similar, however, in our study, the prevalence of SNPs were not similar in the infertile and fertile males when compared. The frequency was high in the infertile men than in the fertile men.

The SNPs discovered in our study are novel and incomparable as they were no related study done in Nigeria at the time of this report. We compared our findings with similar studies done in other the countries; we found that the polymorphisms in *PRM2* vary in regions. The explanation is that gene expression, ethnicity, and environmental factors are influencers of gene variation (Jiang *et al*., 2015).

The functional consequences of the SNPs discovered in our study, were non pathological as identified by the NCBI data base and the global MAF for the variants are shown in Table 1. 2.

5. Conclusion

Sixteen (16) SNPs in *PRM2* were identified in the infertile and fertile male participants in total. Out of the 16 SNPs discovered, 15 SNPs were found in the infertile men, randomly distributed and 7 SNPs were discovered in the fertile men. However, there were six SNPs which were common between the infertile and fertile men. The common SNPs occurred at one or more loci in the *PRM2* in the infertile men than in the fertile men. We found one variant rs2069880799 (16C>T) present in the fertile men but missing in the infertile men. In general, the SNPs in *PRM2* were statistically different between the infertile and fertile men when compared. All the SNP discovered, according to NCBI data base were clinically non pathological.

Compliance with ethical standards

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Disclosure of conflict of interest

The Authors have no conflicts of interest to declare.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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