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GENETIC DETERMINANTS OF FEMALE INFERTILITY IN NIGERIA

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ABSTRACT

Background: Genetic analyses of infertile patients have been identified with several chromosomal abnormalities, single gene mutations and genetic polymorphisms from different biological pathways, associated with infertility. Abnormal micro ribonucleic acid (miRNA) expressions have been linked to female infertility and many human diseases. Mutation, especially in form of single nucleotide polymorphisms (SNPs), has been linked with failure of conception in humans.

Objective: To identify mutations around SNP rs12976445 on gene miRNA125a and investigate their possible association with infertility in Nigerian women.

Methods: Fifty (50) adult women, aged 20 to 51 y, seeking

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assisted reproductive treatment in selected fertility centers in Lagos were studied with 50 age-matched controls with proven fertility. Each of participants gave a written informed consent, and self-administered questionnaire was given to them. 5 mL blood samples were withdrawn from the ante-cubital vein with new syringes and needles for the extraction of deoxyribonucleic acid (DNA) samples genomic studies. DNA samples were then amplified (using standard PCR protocols) and visualized using agarose gel electrophoresis. Single nucleotide polymorphism (SNP) rs12976445 was genotyped using the restriction enzyme BaeGI.

Result: *Mutations were observed in the studied SNP rs12976445 especially among the infertile participants.*

Conclusion: *This study shows that SNP rs12976445 on miRNA125a might be a useful genetic determinant of conception among infertile women, and a diagnostic molecular marker in treatment of female infertility in Nigeria.*

Keywords: *miRNA125a, infertility, mutation, reproduction, SN*

INTRODUCTION

Infertility is a reproductive system disease in women and men, and it is defined as the failure to achieve a pregnancy after 12 months of regular unprotected sexual intercourse (Zhou *et al.*, 2020). Infertility affects 20% and 11% of couples of reproductive ages in Nigeria and the world respectively. Its impact is particularly important in Africa especially in Nigeria, where premium is placed on having a child; female infertility now accounts for about 70% of general infertility (Omokanye *et al.*, 2016).

Causes of female infertility include ovulation problems, hormonal imbalance, cervical problems, fallopian tube damage and uterine complications. Age contributes significantly to infertility as infertility increases with advancing age in women; thus, the incidence of older women seeking infertility treatment is on the increase (Bosch *et al.*, 2020). Assisted Reproductive Technology (ART) is the application of laboratory or clinical technology to gametes and/or embryos for the purposes of reproduction. ARTs also involve all procedures that include *in vitro* handling of human oocytes, sperm and embryo for the purpose of establishing a pregnancy (Omokanye *et al.*, 2017).

Several chromosomal abnormalities, single gene mutations and genetic polymorphisms associated with infertility have been identified by genetic analyses (Pu *et al.*, 2014). The miRNAs play important roles in human body physiological homeostasis; meanwhile, abnormal miRNA expressions are related to female infertility and many human diseases (Assou *et al.*, 2013). Single nucleotide polymorphism (SNP) rs12976445 in miRNA125a has been identified for possible alteration in production of miRNA125a. Furthermore, the role of gene miRNA125a in conception among Nigerian women is yet to be clarified. The study is aimed at identifying mutations around SNP rs12976445 on gene *miRNA125a* and investigating their association with infertility in Nigerian women.

METHODOLOGY

Ethical Approval for the Study

Ethical approval was obtained from the Lagos University Teaching Hospital

Health Research Ethics Committee (LUTHHREC): Number ADM/DCST/HREC/APP/2396.

Participants for the Study

Fifty (50) adult women aged 20- to 51-year-old, seeking assisted reproductive treatment in selected fertility centres in Nigeria were studied with 50 age-matched women with proven fertility as controls. The participants gave a written informed consent. The participants were served self-administered questionnaire to obtain their personal and clinical history.

Inclusion Criteria

Women with inability to conceive in spite of regular sexual intercourse for a minimum of one year, seeking assisted reproductive treatment in fertility clinics in Lagos, Nigeria were recruited.

Exclusion Criteria

Women living with fibroid, hepatitis, HIV/AIDS, cervical complications, ovarian, ovulation, fallopian tube and/or endometrial diseases, sexually transmitted diseases, and hormonal imbalance were not recruited, since these conditions are usually not compatible with fertility and may affect the findings in this study.

Sample Collection

Five milliliter (5.0 mL) of blood was withdrawn, new needles and syringes, from the ante-cubital vein of the participants between Day 4 and 5 of their menstrual cycles into EDTA sample bottles.

DNA Extraction and Purification

DNA was extracted from the 100 samples of blood withdrawn from the participants and purified using Quick-DNATM Miniprep Plus kit (D3025) according to the manufacturer's instruction (ref). Before DNA extraction, the work bench and all the materials used were thoroughly and carefully cleaned with 4% sodium hypochlorite w/v followed by 70% v/v ethanol. The DNA extraction was carried out at room temperature (25 °C) in a fume cupboard as follows: 20 mg of proteinase K solution was diluted with 1,060 µL of storage buffer and stored at -20 °C. Sample of 200 µL was added to a micro centrifuge and 200 µL biofluid and cell buffer was added. Proteinase K

solution was then added to the mixture. The solution was mixed thoroughly and then incubated at 55 °C for 10 min. Genomic binding buffer of 420 µL was added to the 420 µL digested sample and mixed thoroughly. The mixture was transferred to a Zymo-SpinTM IIC-XL column in a collection tube using a micropipette after which the mixture was centrifuged at 12,000 x G for 1 min. The collection tube was discarded with the flow through. 400 µL of DNA pre-wash buffer was added to the column in a new collection tube and centrifuged for 1 min. The collection tube was emptied and discarded after the centrifugation process. Genomic DNA (g-DNA) wash buffer of 700 µL was added to the solution in the column and centrifuged for 1 min. The collection tube was again emptied and discarded; g-DNA wash buffer of 200 µL was added to the column in a new collection tube and centrifuged for 1 min. The collection tube was discarded with the flow through. To elute the DNA, the column was transferred into a clean micro centrifuge tube. DNA elution buffer of 50 µL was added and the mixtures in the tube were incubated at room temperature for 5 min. This was then centrifuged for 1 min. The column was discarded, and the eluted DNA in the microcentrifuge was then covered and stored in a freezer at -20 °C prior to subsequent DNA analysis.

Polymerase Chain Reaction (PCR)

DNA specimens were amplified using standard PCR protocols. The PCR forward and reverse primers corresponding to miRNA125a amplification were 5'-TTTTGGTCTTTCTGTCTCTGG-3' and 5'-TGGAGGAAGGGTATGAGGAGT-3' respectively. The PCR components were combined in the order listed in table 1 in a thin walled 0.2 mL reaction tube on ice. Table 1 also analysed concentrations of reagents used for the PCR. The reaction tubes were placed in heat block and thermal cycling proceeded with initial denaturing step at 94 °C for 30 sec; 30 cycles at 94 °C for 15-30 sec, 45-68 °C for 15-60 sec and 68 °C for 60 sec. Final extension at 68 °C for 5 min and hold at 10 °C for 5 h.

Table 1: Concentrations of Reagents used for the Polymerase Chain Reaction

Reagents	Final Concentration
2 X Master Mix with Standard Buffer	25 ul
10 μ M Forward primer	1 ul
10 μ M Reverse primer	1 U1
Template DNA	10 ul
Nuclease-free water	Made up to 50 μ l

Agarose Gel Electrophoresis

The amplified DNA samples were visualized using agarose gel electrophoresis. This was done to ensure that the genomic DNA was accurately extracted. Quick-load purple 100 bp DNA ladder was used. The casting chamber was leveled for 1 gel and the trays were placed in casting chamber. A high-quality electrophoresis grade agarose was used. To prepare the loading buffer, 100 mL of 10 X Tris Borate EDTA (TBE) buffer was introduced into a 1000 mL measuring cylinder and made up to 1000 mL with distilled water. To 2 g of agarose, 100 mL of the loading buffer was added in a 500 mL glass bottle. The agarose was dissolved by boiling in a microwave oven until a homogenous solution of 100 mL was formed. The dissolved gel solution was allowed to cool to 60 °C. The gel solution was stained with ethidium bromide prior to casting. Gel solution of 100 mL was poured into the gel tray in the casting chamber and 20 gel combs were placed in the slots of the gel tray. The gel was allowed to set for 15 min and 750 mL of the loading buffer was poured into the gel tank. The tray was immersed into the gel box, the gel combs were carefully removed by lifting them up gently. DNA ladder of 20 μ L was loaded into the first lane of the gel. Then, 20 μ L of the PCR product was loaded in subsequent gel lanes. The electrophoresis machine was then connected to the power pack and allowed to run at 100 V and 200 A for 45 min. The DNA fragments were visualized in alpha imager and recorded accurately.

Restriction Enzyme Digestion

Single nucleotide polymorphism (SNP) rs12976445 was genotyped using the restriction enzyme *BaeGI*. Other materials used include DNA sample of interest, restriction digest buffer, gel loading dye, electrophoresis buffer, pipettes and pipette tips. The components for the restriction enzyme digestion were combined in the order listed in Table 2 below in a thin walled 0.2 mL reaction tube on ice. Table 2 also showed concentrations of reagents used for the PCR. The reaction was then incubated at digestion temperature of 37 °C for 15 min. The digestion was stopped by heat inactivation (80 °C for 20 min) and addition of 10 mM final concentration EDTA. The digested DNA was then visualized using gel electrophoresis.

Table 2: Concentrations of Reagents for Polymerase Chain Reaction (Restriction Enzyme)

Reagents	Final Volume
Template DNA	1µg (20µl)
10 X Reaction buffer	5µl
Restriction enzyme (BaeGI)	10 units/1µl PCR
Nuclease-free water	Made up to 50µl

RESULTS

Data Analysis and Bioinformatics

The data analysis involves statistical and bioinformatics analyses. For the statistical analysis, data were input into the spreadsheets of SPSS Version 25. For the bioinformatics analysis, single nucleotide polymorphism (SNP) identification was carried out to properly identify the SNP of interest and detect any possible SNP.

Frequency of SNP rs12976445 Genotypes and Alleles

Plate 1a shows DNA bands of some samples after gel electrophoresis and control before restriction enzyme digestion. Plate 1b shows the DNA bands of each sample after restriction enzyme digestion using a BaeGI restriction enzyme specific to the rs12976445 variant. The restriction analysis revealed that 5 out of 50 participants were TT homozygotes (10%), 21 (42%) were TC heterozygotes variants and 24 (48%) were CC homozygotes variants. Plate 1b shows that SNP CC homozygote variant was more abundant (48%) than SNP TT homozygote variant (10%) in the test group of participants compared to heterozygotes TC variant of miRNA125a and rs12976445.

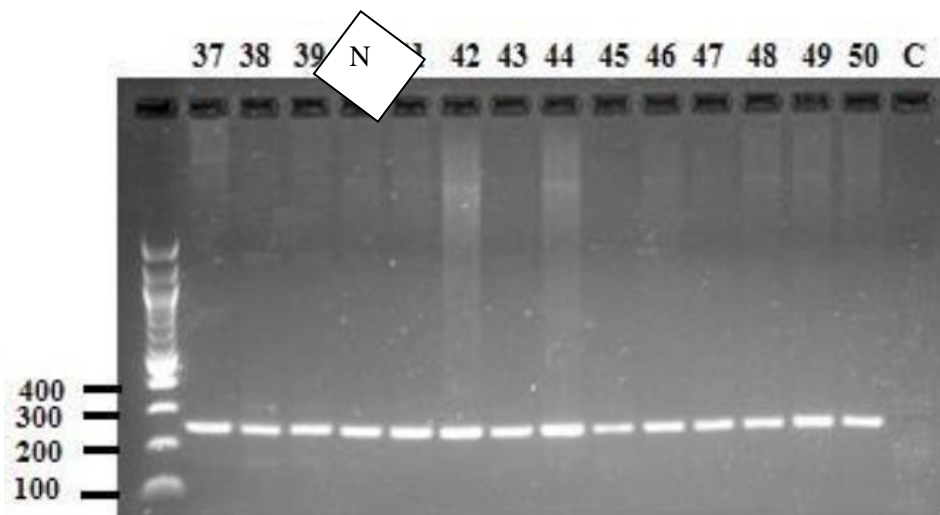


Plate 1a: Results of Gel Electrophoresis before restriction digestion. Lanes 37-50 represent amplicon of size 247 bp of the segment of miRNA125A. This was compared to the ladder 100 bp on lane N. Lane C is the control.

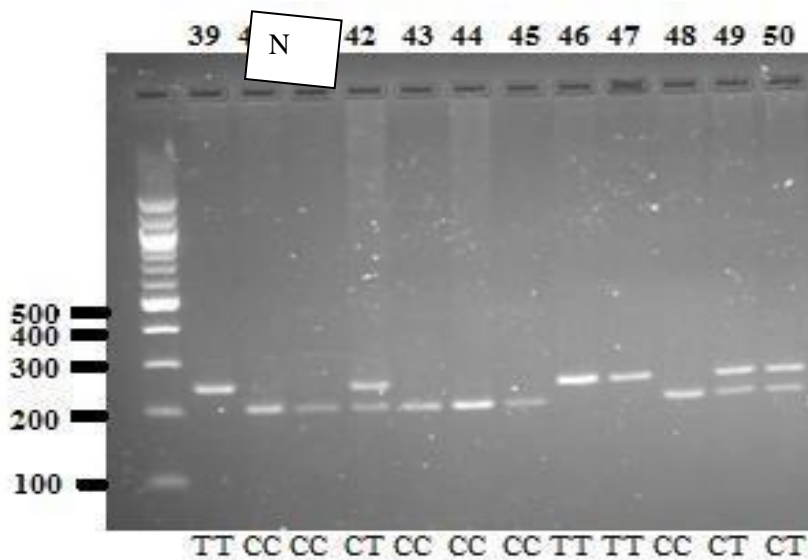


Plate 1b: Results of Gel Electrophoresis after restriction digestion.
Lanes 39-50 represent amplicon of size 247 bp of the segment of miRNA125A. This was compared to the ladder 100 bp on lane N.

DISCUSSION

The end of a woman's reproductive lifespan is marked by the occurrence of menopause, defined as being the last menstruation that occurs for a woman, but is caused by the exhaustion of the ovarian reserve (Perry *et al.*, 2013). Menopause before the age of 40 years is defined as premature ovarian failure (POF), also known as primary ovarian insufficiency. POF is a common disease, occurring in 1% of all women and 0.1% of women below the age of 30 years (Shelling, 2010). Genetic analyses of POF patients have identified several chromosomal abnormalities, single gene mutations, and genetic polymorphisms, from multiple different biological pathways, associated with POF development (Pu, 2014).

The analysis of microRNAs (miRNAs) is another promising strategy recently applied to POF gene research. miRNAs are small noncoding single-stranded RNA molecules, approximately 22–24 nucleotides in size, that are involved in posttranslational gene regulation through repression or cleavage of specific RNA targets (Bernstein *et al.*, 2001). miRNAs regulate cell differentiation, cell cycle progression, and apoptosis and, through the use of mouse models, have been shown to play a role in oocyte maturation and folliculogenesis

(Murchison *et al.*, 2007). One of the upregulated miRNAs, mir-23a, was demonstrated to promote granulosa cell apoptosis via the repression of X-linked inhibitor of apoptosis (XIAP) expression, suggesting the differential expression of mir-23a may be a potential contributor to POF development (Yang *et al.*, 2012).

More recently, Dang *et al* investigated the differential expression of miRNAs in a large cohort of Chinese women, and identified 22 significantly upregulated and 29 significantly downregulated miRNAs in 140 POF patients, compared to 140 controls. Among them, mir-22-3p was significantly downregulated in POF patients, and a negative association between serum mir-22-3p and FSH levels was identified. The researchers suggest mir-22-3p may regulate pituitary FSH secretion, as its expression has been identified in the pig pituitary, whereby the decreased expression may contribute toward POF pathogenesis (Dang *et al.*, 2015). These findings highlight how defects in the miRNA regulatory networks can impact on folliculogenesis. Further studies with larger sample sizes and different ethnicities are warranted to investigate the role of miRNAs in POF pathogenesis.

MiRNA125a has been linked to many reproductive disorders such as ovarian cancer as well as female infertility in many parts of the world with identification of single nucleotide polymorphisms (Cao *et al.*, 2019), but there are none or very few scientific reports about the roles of the rs12976445 in mir125a in female infertility in Nigerian women. The 48% SNP CC homozygote, and 10% SNP TT homozygote variants are likely to play roles in female infertility (Cao *et al.*, 2019). There is possibility of these two SNPs obtained in the test participants to express series of reproductive hormones to suppress ovarian reserve or ovulation (Masood *et al.*, 2021). We do not know how this might have been possible, because it is beyond the scope of our study. However, low productions of follicle stimulating hormone. estrogen and progesterone have been found associated with high ovarian response (Coccia and Rizzello, 2008), higher secretions of luteinizing hormone and/or testosterone have been reported as major contributors to ovarian reserve failure (de Koning *et al.*, 2000; Steckler *et al.*, 2005). Furthermore, prolactin over secretion have been associated with menstrual anomalies and development of ovarian follicle to Graafian follicle by inhibiting follicle stimulating and luteinizing hormone (Yoshimura *et al.*, 1992; Kostrzak *et al.*,

2009). The miRNA125a SNPs are possible to be in association with several diseases of the reproductive tissues owing to the upregulation of miRNA125a SNPs with pathophysiological consequences on reproductive tissues of infertile woman. The list of such reproductive diseases includes ovarian cancer; breast cancer; endometriosis; polycystic ovarian syndrome; primary ovarian insufficiency; and hormonal imbalance (Masood *et al.*, 2021).

This study reveals that expressions rs12976445 in Mir125a might be a useful genetic determinant of conception among infertile women in Nigeria.

CONCLUSION

MiRNA125a can serve as a diagnostic marker in molecular approach to dealing with female infertility especially in a developing country like Nigeria.

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Appendix

Query Length 208

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Descriptions Graphic Summary Alignments Taxonomy

Sequences producing significant alignments **Download** **Select columns** Show 100 [?](#)

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	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
<input checked="" type="checkbox"/>	Eukaryotic synthetic construct chromosome 19	eukaryotic synt...	357	357	95%	4e-94	99.50%	64242768	CP034522.1
<input checked="" type="checkbox"/>	Eukaryotic synthetic construct chromosome 19	eukaryotic synt...	357	357	95%	4e-94	99.50%	64242768	CP034497.1
<input checked="" type="checkbox"/>	PREDICTED: Homo sapiens sperm acrosome associated 6 (SPACA6) transcript variant X1, mRNA	Homo sapiens	357	357	95%	4e-94	99.50%	3811	XM_017026299.2
<input checked="" type="checkbox"/>	Homo sapiens SPACA6 antisense RNA 1 (SPACA6-AS1), long non-coding RNA	Homo sapiens	357	357	95%	4e-94	99.50%	3962	NR_108100.1
<input checked="" type="checkbox"/>	Homo sapiens isolate CHM13 chromosome 19	Homo sapiens	357	357	95%	4e-94	99.50%	61707364	CP068259.2
<input checked="" type="checkbox"/>	Homo sapiens DNA chromosome 19, nearly complete genome	Homo sapiens	357	357	95%	4e-94	99.50%	59105444	AP023479.1
<input checked="" type="checkbox"/>	Homo sapiens cDNA FLJ44008 fis clone TEST14023942	Homo sapiens	357	357	95%	4e-94	99.50%	3961	AK125996.1
<input checked="" type="checkbox"/>	Homo sapiens chromosome 19, BAC BC130783 (CIT-HSPC_470E3), complete sequence	Homo sapiens	357	357	95%	4e-94	99.50%	229155	AC018755.3
<input checked="" type="checkbox"/>	PREDICTED: Trachyothecus francoisi sperm acrosome associated 6 (SPACA6) transcript variant X3, m...	Trachyothecus ...	321	321	95%	3e-83	95.48%	1693	XM_0333224345.1
<input checked="" type="checkbox"/>	PREDICTED: Rhinopithecus bieti sperm acrosome associated 6 (SPACA6) mRNA	Rhinopithecus b...	321	321	95%	3e-83	95.48%	1463	XM_0...

Feedb

Figure 1: Sequences with Significant Alignment