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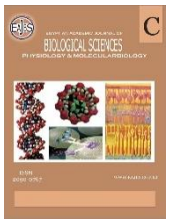

Bioinformatics in Determination of Fertility Among Nigerian Women

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ABSTRACT

Genetic studies on infertile patients have revealed various chromosomal irregularities, alterations in single genes, and genetic polymorphisms affecting different biological pathways linked to 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. This study aims to identify mutations around SNP rs12976445 on gene miRNA125a and investigate their possible association with infertility in Nigerian women. 100 adult women, aged 20 to 51 years, seeking assisted reproductive treatment in selected fertility centers in Lagos were recruited with 100 age-matched controls with proven fertility. Each participant was given a written informed consent and self-administered questionnaire. 5 milliliters blood samples were withdrawn from the ante-cubital vein with new syringes and needles for the extraction of deoxyribonucleic acid (DNA) samples for genomic studies. DNA samples were then amplified (using standard Polymerase Chain Reaction (PCR) protocols) and visualized using agarose gel electrophoresis. The single nucleotide polymorphism (SNP) rs12976445 was analyzed through genotyping using the BaeGI restriction enzyme. Mutations were observed in the studied SNP rs12976445 especially among the infertile participants. 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.

INTRODUCTION

Infertility is as a condition affecting the human reproductive system (either male or female) characterized by the inability to conceive after 12 months or more of regular, sexual intercourse without protection (Zhou *et al*. (2020). Infertility affects 11% and 20% of couples of reproductive ages in the world and in Nigeria respectively (Bosch *et al*., 2020). Its impact is particularly important in Africa especially in Nigeria, where premium is placed on having a child; female infertility accounts for up to 70% of general infertility (Omokanye *et al.,* 2016).

Research indicates that 12% to 15% of couples are unable to conceive after one year of regular unprotected sexual intercourse, and after two years, 10% of couples still have not had a live birth (Mallepaly *et al*., 2017). The prevalence of infertility is rising across the developed world. The primary cause for this is the trend of delaying pregnancy till later in life. Infertility in female can be as a result of variety of factors, including genetic issues, hormonal imbalances, and environmental factors. Additionally, conditions such as pelvic inflammatory disease, uterine fibroids, age-related changes, blocked fallopian tubes, and hostile cervical mucus can contribute to difficulties in achieving pregnancy (Barygina *et al*., 2015; Adewunmi *et al.*, 2017).

Genetic studies on infertile patients have uncovered various chromosomal irregularities, alterations in single genes, and genetic polymorphisms across different biological pathways associated with inability to give birth (Pu *et al*., 2014). MicroRNAs (miRNAs) are short, non-coding Ribonucleic acid (RNAs) that regulate gene expression by binding to complementary sequences in the 3' untranslated region (UTR) of messenger RNA (mRNA) molecules. These miRNAs are crucial for maintaining normal physiological conditions in the human body, and their abnormal expression is associated with several diseases, including female infertility (Assou *et al*., 2013).

MicroRNAs (miRNAs) are involved in regulating cell differentiation, cell cycle progression, and programmed cell death. They have been implicated in processes such as oocyte maturation and folliculogenesis (Xu *et al*., 2012). Dang *et al*. explored the differential expression of miRNAs in a study involving a group of Chinese women and found 22 miRNAs significantly upregulated and 29 miRNAs significantly downregulated in 140 patients with premature ovarian failure (POF) compared to 140 controls. Notably, miR-22-3p was significantly downregulated in POF patients, and a negative correlation was observed between serum miR-22-3p levels and follicle-stimulating hormone (FSH) levels. The study then proposed that miR-22-3p might influence pituitary FSH secretion, as its expression has been detected in the pig pituitary where its reduced levels could contribute to the pathogenesis of POF (Dominguez *et al.,* 2014). MiR-125a has been found to significantly influence cancer development.

Mutation in MiRNA-125a is associated with conception failure in human. Mutations can manifest as single nucleotide polymorphisms (SNPs), which are variations in the DNA sequence where a single nucleotide differs from the wild type in at least 1% of the population (Su *et al*., 2015). The SNP rs12976445 in miR125a has been identified as a potential factor influencing the production of miR125a. Furthermore, the role of gene miRNA125a in conception among Nigerian women is yet to be elucidated. Hence, the need for investigation on the genetic factors that affect female infertility in our environment.

MATERIALS AND METHODS

1- Ethical Approval and Participants*:*

Ethical approval for the study was granted by the Health Research Ethics Committee of Lagos University Teaching Hospital (LUTHHREC), under reference number ADM/DCST/HREC/APP/2396.

Two hundred (100 experimental and 100 control) adult women, aged 20 to 51 years, seeking assisted reproductive treatment in four selected fertility centres in Lagos, Nigeria were recruited for the study. Selection criteria for the fertility centers used for the study include accessibility, frequency of patients and locality. Participants gave their consent to the study by giving a written informed consent. A structured self-administered questionnaire was also given to participants to obtain detailed demographic and physiological information. The control group for the study was made up of women, aged 20 to 51 years, with proven fertility. Blood sample of 10.0 mL was also collected for the study.

The study included women who,

despite engaging in regular sexual intercourse for at least one year, were unable to conceive and were seeking assisted reproductive treatment at fertility clinics in Lagos, Nigeria. Women with particular ailment such as fibroid, hepatitis and HIV/AIDS were not included in the study, since these conditions may affect fertility and confound the results. The control group was made up of women, aged 20 to 51 years, with proven fertility.

2- Sample Collection and DNA Extraction:

Blood sample of 10.0 mL was obtained intravenously from each consenting respondent, seeking assisted reproductive treatment in selected fertility centers in Lagos, Nigeria. The blood samples were collected between Day 4 and 5 of menstrual cycle into EDTA sample bottles.

DNA was extracted from 100 samples of blood obtained from the subjects and purified using Quick-DNA^{TM} Miniprep Plus kit according to the manufacturer's instruction. The 100 samples of blood were randomly selected from the various age groups. Before DNA extraction, the work bench and all the materials used were thoroughly and carefully cleaned with bleach followed by 70% ethanol. The DNA extraction was carried out at room temperature 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 ^{O}C. A 200 µL sample of blood was combined with 200 µL of biofluid and cell buffer in a microcentrifuge tube. Proteinase K solution was added to the mixture, which was then thoroughly agitated and incubated at 55°C for 10 minutes. Following incubation, 420 µL of Genomic binding buffer was added to the digested sample and mixed well. The solution was then transferred to a Zymo-SpinTM IIC-XL column in a collection tube using a micropipette and centrifuged at 12,000xg for 1 minute. The collection tube, along with the flow-through, was discarded. Then, 400 µL of DNA pre-wash buffer was added to the column in a new collection tube and centrifuged for 1 minute. After discarding the flow-through and the collection tube, 700 µL of g-DNA wash buffer was added to the column and centrifuged for 1 minute. This process was repeated with 200 µL of g-DNA wash buffer, and the flow-through was discarded with the collection tube. To elute the DNA, the column was placed in a clean microcentrifuge tube, and 50 µL of DNA elution buffer was added. The mixture was incubated at room temperature for 5 minutes. This was then centrifuged for 1 minute. The column was discarded, and the eluted DNA in the microcentrifuge was then covered and stored in a freezer at -20 ^{o}C prior to subsequent DNA analysis.

3- Polymerase Chain Reaction (PCR) and DNA Analysis:

DNA samples were amplified using conventional PCR methods. The primers for amplifying miR125a were 5'-TTTTGGTC TTTCTGTCTCTGG-3' for the forward primer and 5'-TGGAGGAAGGGTATGAG GAGT-3' for the reverse primer. The PCR components were combined in the order listed in Table 1 in a thin-walled 0.2ml 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 ^{o}C for 30 seconds; 30 cycles at 94 ^{o}C for 15-30 seconds, 45 - 68 ^{o}C for 15-60 seconds and 68 ^{o}C for 60 seconds. Final extension at 68^{o}C for 5 minutes and hold at 10^{o}C for 5 hours.

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

Reagents	**Final Concentration**
2 X Master Mix with Standard Buffer	25 ul
10 µM Forward primer	1 ul
10 µM Reverse primer	1 Ul
Template DNA	10 ul
Nuclease-free water	Made up to 50µl

The amplified DNA samples were visualized using agarose gel electrophoresis. This was done to ensure that the genomic DNA was accurately extracted. 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 10X Tris Borate EDTA (TBE) buffer was introduced into a 1000ml measuring cylinder, and made up to 1000 mL with distilled water. To prepare the gel, 2 grams of agarose was mixed with 100 mL of loading buffer in a 500 mL glass bottle. The mixture was heated in a microwave until it formed a uniform 100 mL solution. Once dissolved, the gel solution was allowed to cool to 60°C and stained with ethidium bromide before casting. The 100 mL gel solution was then poured into the gel tray within the casting chamber, and 20 gel combs were inserted into the slots. After allowing the gel to set for 15 minutes, 750 mL of loading buffer was added to the gel tank. The tray was placed into the gel box, and the gel combs were carefully removed by lifting them gently. DNA ladder of 20 µL was loaded into the first lane of the gel to enhance quality of DNA fragments to be visualized. Then, 20 µL of the PCR product was loaded in subsequent gel lanes. The electrophoresis machine was then powered and allowed to run at 100 volts and 200 amperes for 45 minutes. The DNA fragments were visualized in alpha imager and recorded accurately.

The single nucleotide polymorphism (SNP) rs12976445 was analyzed through genotyping using the BaeGI restriction enzyme. 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 incubated at 37°C for 15 minutes to facilitate digestion. To halt the digestion process, heat inactivation was performed at 80°C for 20 minutes, and 10 mM EDTA was added to achieve a final concentration. The resulting digested DNA was then analyzed 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

PCR products were purified using the ExoSAP method. To prepare the Exo/SAP master mix, 50 µL of exonuclease I (Catalog No: NEB M0293L) at a concentration of 20 U/µL and 200 µL of Shrimp Alkaline Phosphatase (Catalog No: NEB M0371) at 1 U/µL were combined in a 0.6 mL microcentrifuge tube. The amplified PCR sample (10 µL) was then mixed with 2.3 µL of the ExoSAP mix and incubated at 37°C for 15 minutes. The reaction was terminated by heating the mixture to 80°C for 15 minutes.

Following purification, the PCR products were forwarded for DNA sequencing. Sequencing was carried out using the Nimagen BrilliantDye Terminator cycle sequencing kit v3.1 (BRD3-100/1000), adhering to the manufacturer's guidelines. The labeled sequencing products were then cleaned with the ZR-86 DNA sequencing clean-up kit (Catalog No: D4053). The purified samples were analyzed on an Applied Biosystems ABI 3500XL Genetic Analyzer with a 50 cm array using POP7. Sequence chromatograms were analyzed with Finch TV Analysis software.

The data analysis involves statistical analysis and bioinformatics analysis. For the statistical analysis, data were input into the spreadsheets of SPSS Version 25 for statistical analysis. The first series of analysis were summary statistics, which includes the mean, standard deviation and range. Inferential statistics was done using various statistical tools including chi-square and Fisher's exact test for measures of association involving discrete variables that were counted. Differences between means were by Student's t-test for independent samples of only two groups and no repetition of measurement were involved. Analysis of variance (ANOVA) was used to determine significance difference between means of more than two groups. However, ANOVA was used for association between continuous variable and the independent variable of interest in order to control for confounders. Multiple analysis of variance (MANOVA) was used when more than two dependent variables were analysed for their main and interaction effects. On instances that required control of confounding variables during correlation analysis, partial correlation procedure was implemented. Results of continuous normally distributed variables were as mean$\pm$ SD. Simple and partial correlations analyses respectively were used to determine association between continuous and normally distributed variables. For the bioinformatics analysis, multiple sequence alignment was carried out to give detailed nucleotide information and to show possible mutations. Single nucleotide polymorphism (SNP) identification was also carried out to properly identify the SNP of interest and detect any possible SNP. Insertions and deletions (Indel) identification was also used in mutations identifications.

RESULTS

1- Gel Electrophoresis:

The results of gel electrophoresis, before and after restriction enzyme digestion, are shown in Plate 1a and Plate 1b respectively.

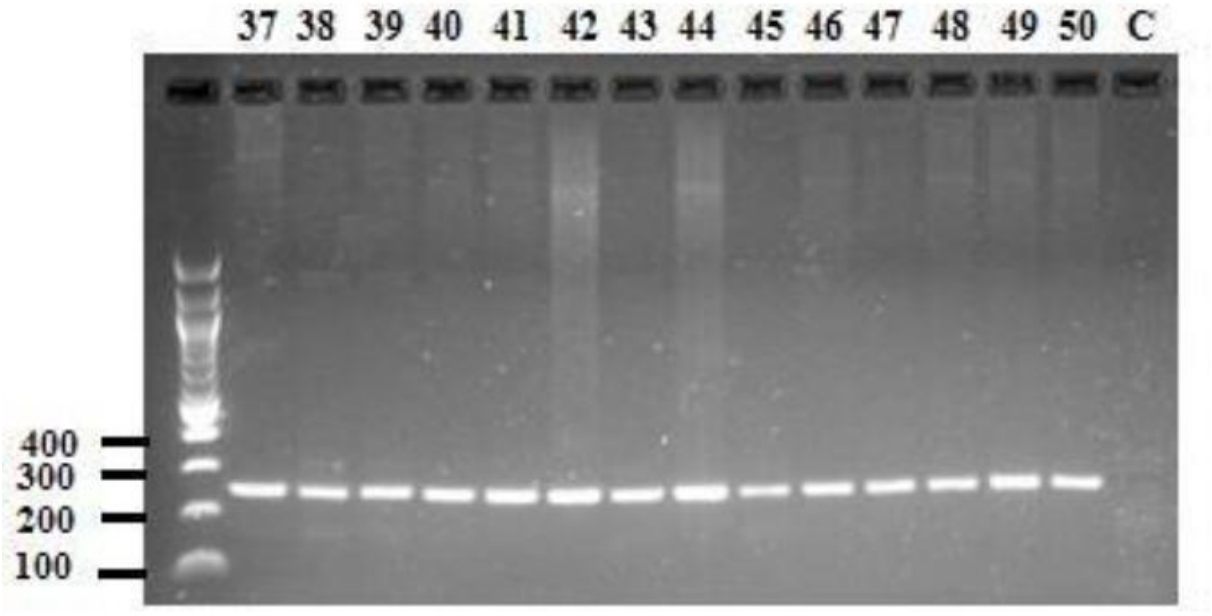

Plate 1a: Results of Gel Electrophoresis before restriction enzyme digestion.

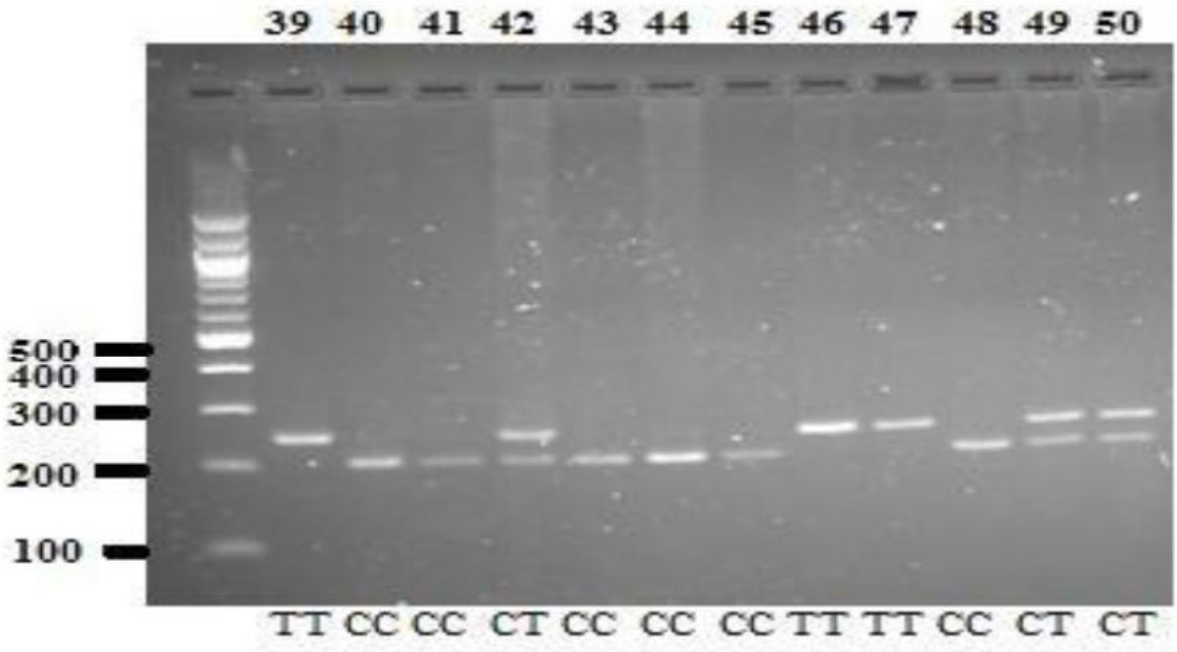

Plate 1b: Results of Gel Electrophoresis after restriction digestion.

2- Multiple Sequence Alignment*:*

The graphic summary of the sequences of significant alignment is shown below. This was derived after the application of BLAST on sequenced data. This looks a bit into the evolutionary status of the studied organism which is human (homo sapiens) considering the sequenced data as well as related organisms. It describes and compares the aligned sequences between homo sapiens and some other eukaryotic organisms in terms of scientific names, maximum score, total score, query cover and accession. Figure 2, shows and compares in details the query and the subjects in relation with the chromosome number. This was also after application of BLAST and getting the best hits differentiated from the query (Fig. 3). Results of the multiple sequence alignment of the sequenced data are shown below. There are 10 different organisms with different labels of identification and 31 columns. Mutations were reported in the studied SNP rs12976445 especially among the infertile participants. A G/A SNP and other mutations were discovered towards the left-hand panel of the image (Fig. 4).

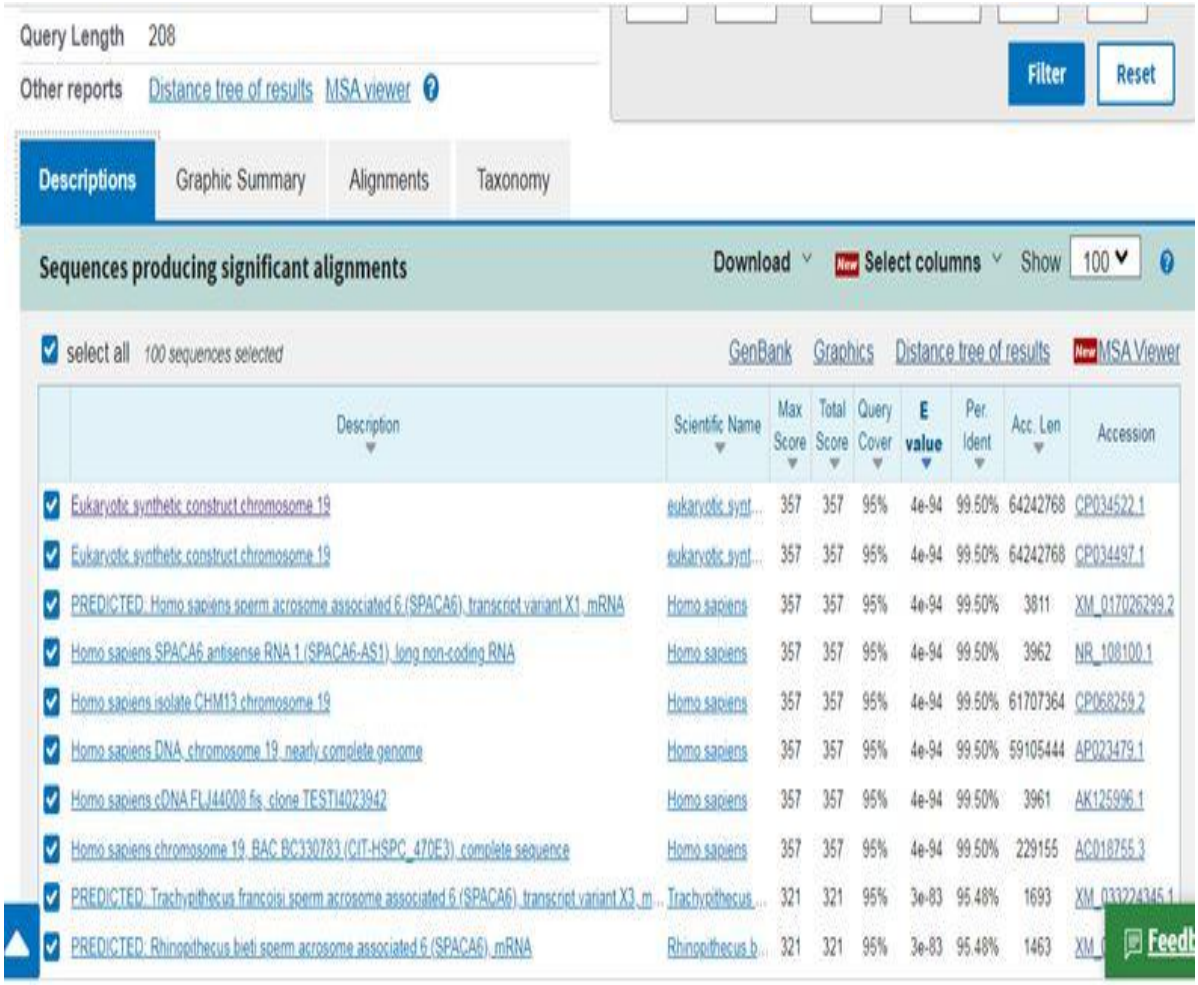

Fig. 2: Sequences with Significant Alignment**.**

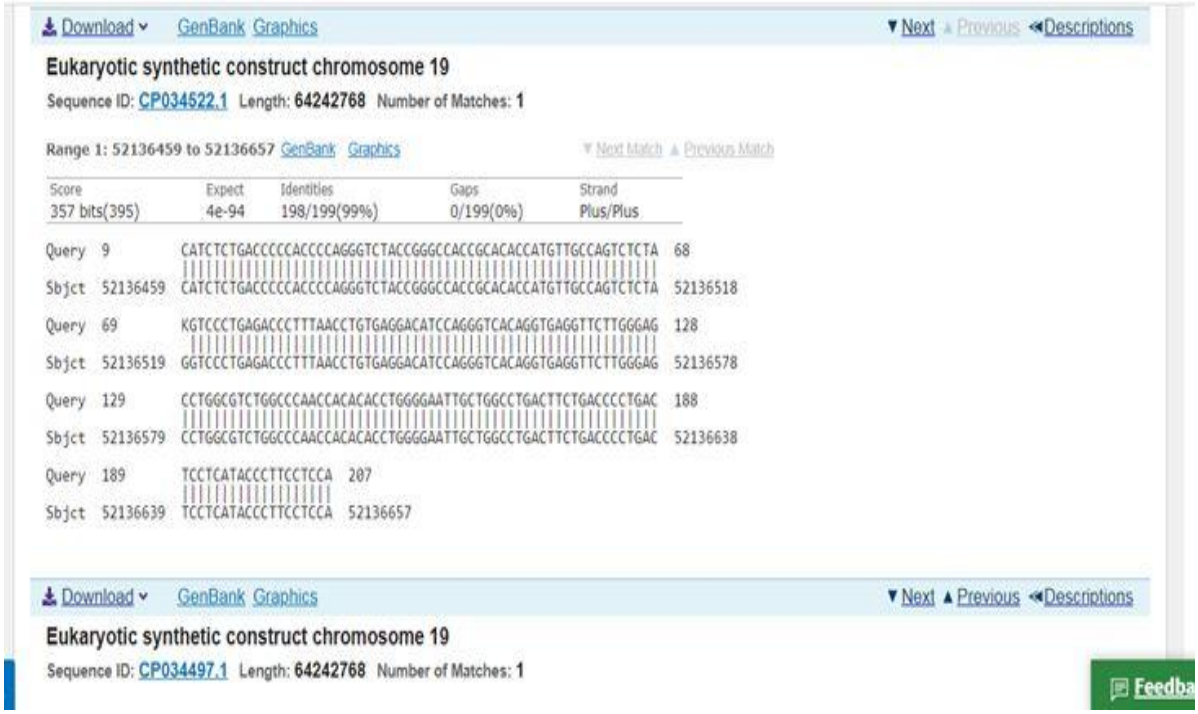

Fig. 3: Pairwise Sequence Alignment of the Query Sequence and the Best Hit**.**

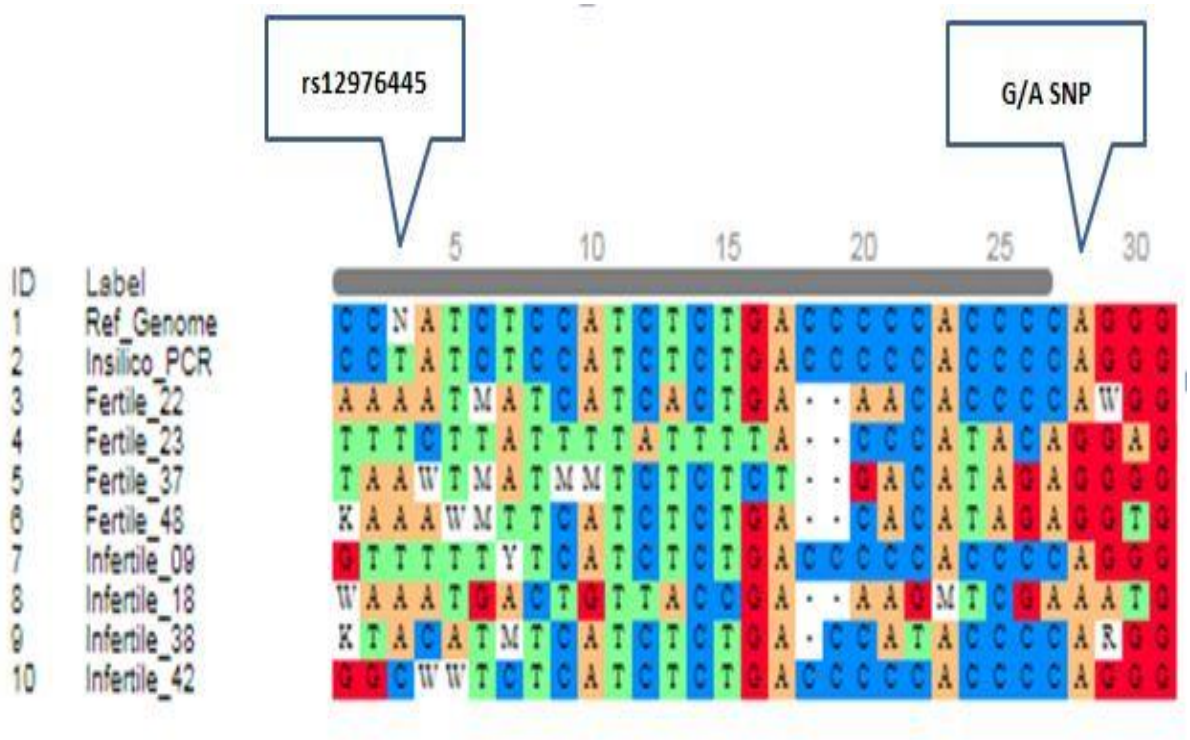

Fig. 4. Multiple Sequence Alignment Result.

DISCUSSION

MicroRNAs (miRNAs) are a group of small, non-coding regulatory RNAs believed to impact the translation of mRNAs in approximately 30% of genes across animal species (SantaMaria and Taylor, 2014). Research has demonstrated that miRNAs are significantly associated with the onset and progression of disorders associated with female infertility. (Bahmyari *et al.,* 2021) MiRNA125a has been associated with various reproductive disorders, including ovarian cancer and female infertility, across different regions worldwide, often linked to specific single nucleotide polymorphisms (Cao *et al*., 2019). Kumari *et al*. (2022) observed increased expression of miR-125a in patients with endometriosis, while a decreased expression of this miRNA was noted in patients with endometrioid endometrial cancer and ovarian cancer compared to control groups. This variation suggests that miR-125a plays a role in ErbB (erythroblastic leukemia viral oncogene homologue) signaling and influences cell migration and invasion. Furthermore, Min *et al*. (2014) identified that miR-125a plays a role in the onset of various cancers, including lung, breast, and colon cancers. Single nucleotide polymorphisms (SNPs), which are single nucleotide variations in the DNA sequence (SantaMaria and Taylor, 2014), have a substantial impact on gene expression. Specifically, the SNP rs12976445 in the miRNA125a sequence has been associated with decreased miR-125a expression in multiple diseases (Lehmann *et al.,* 2013). There are none or very few scientific reports about the roles of the rs12976445 in miR-125a in female infertility in Nigerian women. This research suggests that the rs12976445 variant in miR-125a could serve as a valuable genetic marker for predicting conception success in infertile women in Nigeria. Thus, it can serve as a diagnostic marker in molecular approach to dealing with female infertility especially in a developing country like Nigeria. 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). This is possible, although 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).

Conclusion

This study identifies a G/A SNP located downstream of SNP rs12976445 in miRNA125a, which shows a significant association with fertility. Further research is recommended to explore the potential implications of this newly identified G/A SNP on male and/or female infertility.

Declarations:

Ethical Approval: Ethical approval for the study was granted by the Health Research Ethics Committee of Lagos University Teaching Hospital (LUTHHREC), under reference number ADM/DCST/HREC/APP/2396.

Conflict of interests: The authors declare no conflicts of interest.

Authors Contributions: All authors contributed equally, and have read and agreed to the published version of the manuscript.

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Availability of Data and Materials: The data presented in this study are available on request from the corresponding author.

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