Integrative Cancer Science and Therapeutics

Research Article ISSN: 2056-4546

Assessment of DNA methylation patterns in breast tumours and apparently healthy subjects in Anambra state, Nigeria

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Abstract

Epigenetic cell alteration is an environmentally induced epimutation that could lead to tumourigenesis. Thus we considered its assessment to be of utmost importance in regional areas with serious environmental challenges such as Nigeria with respect to increasing rate of breast tumours. Fasting blood sample was collected from 24 subjects with benign breast tumour, 25 subjects with malignant breast tumour and 50 apparently healthy control subjects, for cell free DNA extraction. The DNA was subjected to epigenomic tests to determine the methylation patterns using Enzyme Linked Immuno-Assay (colorimetric) method (Epigentek USA). Significant association between tumors and Demethylation (hypomethylated DNA and Unmethylated DNA) was observed (X^2= P=0.000); also significant association between tumors and hypermethylation was observed (X^2= P=0.014). Up to 10(20%) out of the 50 apparently healthy control subjects were demethylated. Increased expression of aberrant DNA Methylation was observed in late stages of breast malignant tumour. Epigenomic Cell alteration is indicated to be a significant influential risk factor to breast tumourigenesis, and of significant increase in this locality. Therefore, being an abnormal genomic development, its detection in apparently healthy subjects, is an indicator for intrinsic genomic alteration and could be a marker for development of tumour micro-environment if not tumourigenesis, thus could be used for early detection of tumourigenesis even earlier than physical breast examination.

Introduction

Epigenetics refers to functionally relevant modifications to the genome that do not involve a change in nucleotide sequence. Such modifications include chemical marks that regulate the transcription of the genome [1,2]. There is now evidence that environmental events can directly modify the epigenetic state of the genome. Thus studies with rodent models suggest that during both early development and in adult life, environmental signals can activate intracellular pathways that directly remodel the "epigenome," leading to changes in gene expression and neural function. These studies define a biological basis for the interplay between environmental signals and the genome in the regulation of individual differences in behavior, cognition, and physiology [2]. This makes the study of epigenetics very important in areas where environmental challenges are eminent.

Breast tumours can be divided into those with identified genetic risk factors as inherited germline mutation from parents to offspring and those who are not genetically predisposed but encounter epimutational changes in their life time. While the former group accounts for only around 10% of total breast cancer cases, the later accounts for over 90% of total breast cancer cases [3]. The majority of breast cancers are sporadic caused by more complicated reasons such as the activation of mitogenic signaling pathways and loss of tumor suppressor expression epigenetically etc., [4].

DNA methylation which is one of the epigenetic mechanisms, which involves the addition of a methyl group to unmodified DNA, is described as an epigenetic change resulting from environmental influence, because it is a chemical modification to DNA not a change brought about by a DNA mutation [5]. Normal methylation of DNA would mark the DNA and enable such actions as turning genes on or off. Thus if epigenomic alteration persists in a tumour suppressor

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key words: *DNA methylatiion patterns, breast tumours, healthy subjects, epigenetic cell alteration, tumourigenesis*

Received: May 22, 2017; **Accepted:** June 19, 2017; **Published:** June 22, 2017

gene, expression of such gene could be altered and the cell may lose the normal pattern of interpretation of DNA instructions. It has been well demonstrated that the decrease in global DNA methylation (demethylation) is one of the most important characteristics of cancer [6]. Shift in normal DNA methylation could result to unmethylation, hypomethylation and hypermethylation [7]. Thus, the quantification of 5-mC content or global methylation in tumour cells and normal cells could provide very useful information for detection and analysis of breast tumour and and its onset.

Environmental, as used by cancer researchers, means any cause that is not inherited genetically. Thus the term environment refers not only to air, water, and soil but also to substances and conditions at home and at the workplace, including diet, smoking, alcohol, drugs, exposure to chemicals, sunlight, ionizing radiation, electromagnetic fields, infectious agents, etc. Lifestyle, economic and behavioral factors are all aspects of our environment" [8]. Thus, if these environmental stimuli persists in the life of an apparently healthy individual, it is fundamental that normal epigenomic process may be perturbed emanating to expression of any of the abnormal DNA methylation patterns [5], even in apparently healthy individuals and thus may pose a serious epigenetic and clinical trait to individuals in highly polluted environment. We thus propose that such abnormal expressions would serve as indicator to epimutation, consequent to anomalies that are sequel to epimutational changes. Anambra State is highly industrialized. Environmental pollution and contamination of water resources are becoming a common menace in parts of Anambra State [9]. As a result of the ever increasing industrial establishments and mans general activities, physical, chemical, and biological substances are being fed into the ground and surface water environment on a daily basis [9]. This may have resulted in losses of human, material and financial resources. Volumes of these pollutants/contaminants are produced yearly through natural and anthropogenic activities such as industrial activities, agricultural practices, waste disposal systems, soil and gully erosion [9]. No safe disposal environments have yet been found for waste products that have long half-lives. High-level, medium-level and low-level waste in solid, liquid and gaseous forms are released into the environment of Anambra state at discrete intervals or at a continuous basis. They may have short or long half-lives in the environment, the consequences are health hazards [9,10]. Because of improper handling and disposal of the harmful wastes materials, we are worried about the health implications and possible influences on epigenetic Mechanisms. Thus the need to access the epigenetic status of even supposed healthy individuals. Air and water pollutions are on the fastest increase in the area [10,11].

Breast cancer is the most commonly diagnosed cancer in women worldwide [12]. It is the leading cause of death in women amongst other cancer cases in Nigeria [13]. Tremendous progress have been made over the last decades at understanding the biology of breast cancer, however the mechanism for growth and progression of breast cancer with acquisition of invasive and metastatic phenotypes and therapeutic resistance are still not fully understood [14,15]. It is crucial to understand how and why these sporadic breast cancers arise and progress in order to develop preventive strategies against this devastating disease rather than depend on excision of the breast in the name of cure.

DNA methylation is important in the regulation of inflammatory genes. Hypermethylation of promoter regions of genes is typically associated with transcriptional silencing while hypomethylation obstructs gene expression [16]. With evidence of environmental pollutions by other researchers, we decided to check the status of different patterns of DNA methylation in subjects with breast tumours and apparently healthy subjects in this locality. The mention of the environmental pollutants in this study suggests that presence of these pollutants where un avoided, may continue to fuel persistence of toxins, microbial antigens, pesticide residues and other biological and chemical harmful substances in the system, that could perturb normal epigenomic process.

Materials and methods

The subjects were grouped into three. They included 24 female subjects with benign breast tumour (benign subjects), 25 female subjects with malignant breast tumour (Cancer subjects) and 50 female subjects without breast tumour (Control Subjects). The benign and malignant breast tumour subjects were attending clinic at the surgical unit of Nnamdi Azikiwe University Teaching Hospital Nnewi (NAUTH), Anambra State. The 50 healthy female control subjects were confirmed free from breast tumours by the physician [17]. The diagnosis of breast tumour was established by histo-pathological examination of biopsy and detection of cancer associated antigen 15-3 (CA 15-3). Staging of cancer was done according to American Joint Committee for Cancer (TNM classification). None of the subjects had received any breast tumour chemotherapy or surgery prior to the study. All the subjects were screened clinically and biochemically to exclude Human Immunodeficiency Virus and autoimmune. Approval for the study was obtained from the ethics committee of Nnamdi Azikiwe University Teaching Hospital with number NAUTH/CS/66/vol.6/026. Informed consent was obtained from the subjects before participation. All the subjects were administered questionnaire to obtain medical history and demographic information.

Inclusion criteria

Female subjects with benign or malignant breast tumour but not under chemotherapy and had not under gone breast surgery. Female subjects without tumour and are not under cytotoxic drug or any anti-immune therapy such as in auto-immune disease.

Exclusion criteria

Immuno-deficient patients or patients with HIV infection, Female subjects without cancer but are under cytotoxic drug.

Specimen collection

Up to 5ml of fasting blood sample was adequately drawn by veni-puncture from all the participants. Criteria for adequate blood sample collection were considered to suit the various parameters required to be tested in this study. The blood samples were put in plain vacutainer tubes. The blood samples were allowed to clot at room temperature, for 30 minutes. The retracted clot was removed by centrifugation (Sorvall RC5C HS-4 rotor at 1500 x g for 15 min) at room temperature and the formed serum was carefully separated and put into another tube. DNA extractions were carried out from the serum immediately and were stored at -20°C and analyzed within one week of storage.

Methods

Extraction of serum DNA [18]

Procedure: The procedure was as described by the manufacturer (Epigentek, USA). Briefly, 1 ml of digestion solution was added to digestion powder. The tube was vortexed until solution is clear. Up to 500µl of DNA isolation buffer then 20µl of the mixed (digestion

solution/digestion powder) solutions were added to 500µl of serum sample, and mixed very well. The mixture was incubated at 65°C for 10 minutes. Meanwhile, a spin column was placed into a 2 ml collection tube. Maximum of 500µl of mixture was transferred to the column and Centrifuged at 12,000 rpm for 30 seconds. The flow through was discarded and the column replaced to the collection tube and the remaining volume of mixture was transferred to the column, centrifuged again at 12,000 rpm for 30 seconds. The flow through was again discarded and the column replaced to the collection tube.

Up to 300µl of 70% ethanol was added to the spin column and centrifuge at 12,000 rotor per minute (rpm) for 20 seconds. The flow through was discarded and the column replaced to the collection tube. Up to 200µl of 90% ethanol was added to the column and centrifuge at 12,000 rpm for 20 seconds. The flow through was discarded and the column replaced to the collection tube. Another 200µl of 90% ethanol was added to the column and centrifuged at 12,000 rpm for 40 seconds. The column was now placed in a new 1.5ml vial and 100µl of DNA Elution Solution was added directly to the column filter and centrifuged at 12,000 rpm for 20 seconds to elute DNA. DNA is now ready for use. The eluted DNA was stored at –20°C until use.

Methylated DNA Quantification (Colorimetric) [18]

Assay procedure: The procedure was as directed by the manufacturer (Epigentek, USA)

DNA binding: Eighty micro-litre (80µl) of binding solution was added to each well for DNA binding. Then 1µl of negative control, 1µl of diluted positive control were added, followed by 20µl (100ng) of the isolated DNA sample. The solutions were mixed by shaking the plate on the bench several times, ensuring that the solutions coat the bottom of the well evenly. The strip plate was covered with plate seal and incubated at 37°C for 90 minutes. The binding reaction solution was removed from each well and the wells were washed three times with 150µl of diluted wash buffer.

Methylated DNA capture: Capture antibody was diluted at 1:1000 dilution) with diluted wash buffer. 50µl of the diluted capture antibody was added to each well, then covered and incubated at room temperature for 60 min. After 60 minutes, the diluted capture antibody solution was removed from each well. The wells were washed three times with 150µl of diluted wash buffer. Detection antibody was diluted at 1:2000 dilution) with the diluted wash buffer, then 50µl of the diluted detection antibody was added to each well, then covered and incubated at room temperature for 30 min. the diluted detection antibody solution was remove from wells, and the wells were washed four times with 150µl of diluted wash buffer). Enhancer solution was diluted at 1:5000 dilution with the diluted wash buffer. Afterwards, 50µl of the diluted enhancer solution was added to each well. The wells were covered and incubate at room temperature for 30 minutes. The diluted enhancer solution was removed from each well and the wells were washed five times with 150µl of diluted Wash Buffer).

Signal detection: Total of 100µl of developer solution was added to each well and incubated at room temperature for 10 minutes away from light. Colour change was monitored in the sample wells and control wells. Then 100µl of stop solution was added to each well to stop enzyme reaction when colour in the positive control wells turned medium blue. The solution was mixed by gently shaking the frame on the bench and waited for 2 minutes to allow the colour reaction to be completely stopped. The colour change to yellow after adding stop solution and the absorbance was read on a micro-plate reader at 450 nm within 2 to 15 min.

5-mC (5-carbon of cytosine ring where DNA methylation occurs) calculation and relative

quantification. To determine the relative methylation status of two different DNA samples, calculation of percentage of 5-mC in total DNA was carried out using the following formula (Jin et al., 2010).

$$5\text{-}mC\% = \frac{(SampleOD - ME3OD) \div 100}{ME4OD - ME3OD \times 2 \div 5} \times 100$$

2*: is a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50% of 5-mC.

S= the amount of input sample DNA in ng = 100ng

P= the amount of input positive control in ng = 5ng

Positive control is a methylathed polynucleotide containing 50% of 5-methylcytosine

Negative control is an unmethylathed polynucleotide containing 50% of cytosine

Results

Pattern of DNA methylation in breast tumour subjects and control subjects

DNA methylation pattern was determined in 24 benign tumour subjects, 25 malignant tumour subjects, and 50 control subjects. The rate of occurrence of DNA hypomethylation in subjects with benign breast tumour 7(29.2%); malignant breast tumour 12(48%) and control subjects 5(10%) was statistically significant P=0.000. The rate of occurrence of DNA hypermethylation in subjects with benign breast tumour 2(8.3%) and malignant breast tumour 4(16%), was statistically significant P=0.014. The occurrence of unmethylated DNA in subjects with benign breast tumour 9(37.5%); malignant breast tumour 8(32%) and control subjects 5(10%) was statistically significant P=0.000. Also the occurrence of normal methylated DNA detected in subjects with benign breast tumour 6(25%); malignant breast tumour 1(4%) and control subjects 40(80%) was statistically significant P=0.000 (Table 1).

In subjects with malignant tumour, the rate of occurrence of hypomethylation 12(48%); hypermethylation 4(16%); unmethylation 8(38%) and normal methylation 1(4%), was significantly different P=0.000. In subjects with benign tumour, the rate of occurrence of hypomethylation 7(29.2%); hypermethylation 2(8%); unmethylation 8(37.5%) and normal methylation 6(25%), was significantly different P=0.000. Furthermore, in control subjects, the rate of occurrence of hypomethylation 5(10%); unmethylation 5(10%) and normal methylation 40(80%), was significantly different P=0.000. Hypermethylation pattern of DNA methylation shift was not detected in control subjects (Table 1).

Staging of the subjects with malignant breast tumour and their pattern of DNA methylation

The staging of the malignancy was done according to American Joint Committee on Cancer (2010). Out of the 25 subjects with malignant tumour, 5 (20%) were at stage 2b, 9(36%) were at stage 3b, 7(28%) were at stage 3c while 4(16%) were at stage 4. Out of the 5subjects in stage 2b, DNA hypomethylation was detected in 1(20%) subject, DNA unmethylation was detected in 3(60%) subjects, normal DNA methylation was observed in 1(20%) and DNA hypermethylation was detectesd in 0(0%). Out of the 9 subjects in stage 3b, DNA hypomethylation was detected in 6(66.7%) subjects, DNA unmethylation

was detected in 1(11.1%) subjects, normal DNA methylation was observed in 0(0%) and DNA hypermethylation was detectesd in 2(22.2%). Out of the 7 subjects in stage 3c, DNA hypomethylation was detected in 4(57.1%) subjects, DNA unmethylation was detected in 2(28.6%) subjects, normal DNA methylation was observed in 0(0%) and DNA hypermethylation was detected in 1(14.3%). Out of the 4 subjects in stage 4, DNA hypomethylation was detected in 1(25%) subjects, DNA unmethylation was detected in 2(50%) subjects, normal DNA methylation was observed in 0(0%) and DNA hypermethylation was detected in 1(25%) (Table 2).

Discussion

This study explored Global DNA Methylation-Based signatures in determining the pattern of methylation in benign breast tumour, malignant breast tumour and healthy female subjects. This work revealed that 75–96 percent of breast tumour/cancer development could develop during individual's life time and attributable to epigenetic cell alteration. About 75% of the subjects with benign tumour had epigenomic aberration or irregularities (DNA methylation), while 96% of the subjects with malignant tumour had epigenomic aberration. This is in line with the report that only 5-10% of cancers are due to gene abnormalities inherited from parents and that about 90-95% of cases are attributed to epigenetic factors, 'wear and tear' of life in general [3]. DNA methylation is vital during development, and aberrant DNA methylation, both hypermethylation and demethylation, have been associated with aging, cancer and other diseases [19]. Therefore, methods to study DNA methylation are important tools in biological research. DNA methylation shift seem to be an important epigenetic event in breast tumourigenesis and by using global methylation assay, it is possible to identify these markers for diagnostic and therapeutic purposes in this disease.

Tumor node metastasis (TNM) which is a measure of stages of cancer has been used to measure cancer spread and prognosis [20], and is commonly used in clinical setting. The stages range from 0 to IV, with stage 0 being in situ, stage I being early stage invasive cancer, and stage IV being the most advanced disease. Larger size, nodal spread, and metastasis have a larger stage number and a worse prognosis [21]. Stage 0 is a pre-cancerous or marker condition, either ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS). Stages 1–3 are within the breast or regional lymph nodes. Stage 4 is 'metastatic' cancer that has a less favorable prognosis [20]. Based on these, this study revealed 4 different stages of breast cancer viz-a-viz 2b, 3b, 3c and 4. This indicated that majority of the breast cancer cases did not present themselves for medical attention at the early stage. The prevailing nature of demethylation in subjects with malignant breast tumour could be a source of evidence to nodal spread and progression, as higher numbers of hypomethylation/unmethylation were found in stages 3b and 3c and 2b. Thus it seems that the most critical stages of breast cancer in this locality in terms of physiological changes manifesting in epigenetic cell alteration are stages 3b and 3c.

Table 1. Pattern of DNA methylation in subjects with breast tumour and control subjects

	Benign N=24	Malignant N=25	Control N=50	P-value
Hypomethylated	7(29.2%)	12(48%)	5(10%)	0.000
Unmethylated	9(37.5%)	8(38%)	5(10%)	0.000
Hyper methylated	2(8%)	4(16%)	0(0%)	0.014
Normal methylation	6(25%)	1(4%)	40(80%)	0.000

Significant association between tumors and Demethylation (hypomethylated DNA and Unmethylated DNA) (X^2= P=0.000); significant association between tumors and hypermethylation (X^2= P=0.014); significant association between healthy control and normal methylation (X^2= P=0.000). Statistical significant P-value is P=<0.05

Table 2. Staging of the subjects with malignant breast tumour and their pattern of DNA methylation

	Stage 2b N=5	Stage 3b N=9	Stage3c N=7	Stage4 N=4
Hypomethylated	1(20%)	6(66%)	4(57%)	1(25%)
Unmethylated	3(60%)	1(11%)	2(28%)	2(50%)
Normal methylated	1(20%)	0	0	0
Hypermethylated	0	2(22%)	1(14%)	1(26%)

Increased expression of DNA Methylation Aberation in stages of breast malignant tumour

DNA methylation changes in cancer cells include the loss of methylation at normally methylated sequences (hypomethylation) and the gain of methylated sequences at sites usually unmethylated (hypermethylation) [22]. In this study, presentation of normal DNA methylation is low in subjects with breast tumours, while DNA methylation shift such as hypomethylation, unmethylation and hypermethylation were significantly high in subjects with malignant and benign breast tumours. Szyf, 2012 reported that in vertebrates almost every CpG site is methylated [23], with the exception of those in CpG islands where transcription takes place and that specifically, nearly 80% of CpG sites are methylated in the human genome. However, in cancer cells, dramatic and opposing changes to the epigenetic landscape often occur and these changes consist of global hypomethylation of the DNA and localized hypermethylation of CpG island-associated promoters. It is important to note that presentation of hypermethylation may not be peculiar with CpG islands associated promoter regions as this work revealed high level of hypomethylation and low level of hypermethylation in subjects with breast tumours using global methylation assay. Earlier, the work of Tan et al. [24], though pancreatic cancer, detected hypermethylation and hypomethylation in using global methylation profiling assay.

Specifically shift in DNA methylation was more profound in tumour subjects with only 4% of malignant breast tumour and 25% of benign breast tumour subjects having normal DNA methylation unlike in control subjects with 80% having normal DNA methylation. The prevailing state of demethylation (unmethylation and hypomethylation) in subjects with benign and malignant tumour (66.6%) and (80%) respectively and the indifferent occurrence of unmethylation in malignant subjects and benign subjects give room for concern.

DNA hypomethylation can cause the undesirable activation of dormant repeat elements and lead to altered expression of associated genes. DNA hypomethylation can cause genomic instability and may contribute to mutations and chromosomal recombinations [22]. DNA demethylation has an important role in cancer by turning on the expression of pro-metastatic genes, such as the heparanase gene, *MMP2* (which encodes matrix metalloproteinase-2) and *uPA* (which encodes urokinase plasminogen activator) [23]. A causal role for demethylation in cancer metastasis is supported by the fact that treatment of non-metastatic breast cancer cells with demethylating agents increases their invasiveness [25], and that treatment of invasive breast cancer and liver cancer cell lines with agents that reverse unmethylation results in inhibition of invasiveness and metastasis [26].

DNA hypomethylation was found in this study to be a gradual development stemming from few healthy subjects to greater number of tumour/cancer subjects. It was earlier reported that the epigenetic signature of any cell provides valuable information about its cellular

state, its developmental potential, and its overall health [27]. Detection of methylation shift in the present study amongst the healthy subjects is considered to be an indication that DNA methylation would be an early indicator for breast tumour development in women. Considering the fact that while physical breast examination detects lumps in the breast, serum detection of methylation status, most importantly demethylation (unmethylation or hypomethylation) would give earlier alert even before any lump is developed. However it is Important to note that this may be attributed to other forms of tumour development. Aberrant methylation can begin very early in tumor development and mediate most of the important pathway abnormalities in cancer development including loss of cell cycle control, altered function of transcription factors, altered receptor function, disruption of normal cell–cell and cell–substratum interaction, inactivation of signal transduction pathways, loss of apoptotic signals and genetic instability [28]. Thus the use of circulating DNA (cfDNA) or liquid biopsy in this study, could have added more values to the importance of early tumour detection and thus auguments early DNA methylation aberration detection, as the use of solid biopsy can only be possible when tumour must have developed [29]. Using methylation haplotypes, Guo et al. [30] demonstrated quantitative estimation of tumor load and tissue-of-origin mapping in the circulating cell-free DNA of 59 patients with lung or colorectal cancer. Thus we report that a blood test could be used for early tumor and early enough to detect pre-tumour genomic aberration even before the tumour develops. Circulating tumour DNA (CtDNA) can be distinguished from circulating DNA from healthy cells by the presence of genomic aberrations that correspond to those found in the tumor, such as tumor-specific mutations or methylation [31].

The association of global DNA hypomethylation with the early stages of carcinogenesis or with tumour progression would provide cancer markers that would be very useful in the clinic [32,33]. In addition, the knowledge that metastatic genes are activated by hypomethylation will be necessary for development of personalized treatment programs that are molecularly based. Translation of research findings to the clinical setting will be facilitated by the recent developments in high-throughput and high-resolution methods for DNA methylation analysis [34]. Besides since DNA methylation is subjects to environmental influences, its study, would be wholesome to include the prevalence of microbial agents and other pollutants in its analysis, however depending on locality.

So far, little is known about the functional implications of the precise spatial distribution patterns of DNA methylation among tissues/individuals. Functional analysis of individual CpG dinucleotides is desirable for better understanding of normal cellular physiology, inter-individual variation of DNA methylation, disease pathogenesis and for the optimization of cancer biomarker searches for robustness and precision [35].

Conclusion

About 75% methylation shift in benign tumour and 96% methylation shift in malignancy show that epimutation is far on the increase in this locality with documented evidence of high environmental pollution.

Demethylation (hypomethylation and unmethylation) could be used for early detection of tumourigenesis even earlier than physical breast examination.

DNA methylation shift such as unmethylation and hypomethylation can occur in apparently healthy subjects.

Recommendation

Adoption of DNA methylation test using blood sample for regular laboratory (routine) check, could serve as a recent innovative approach for detection of early tumour development even before the tumour forms. Thus could serve a very good purpose for prevention of cancer development

Acknowledgement

We wish to appreciate the staff of Nnamdi Azikiwe University Teaching Hospital Chemical Pathology and HIV units, for their understanding and corporation during this study

Authors' contributions: Michael Chukwudi Ezeani, conceived the study. Michael Chukwudi Ezeani, Charles Chinedum Onyenekwe and Samuel Chukwuemeka Meludu designed the study. Michael Chukwudi Ezeani Ujuamala Uloma Ezeani and Daniel Chukwuemeka Anyiam performed the experiments and acquired the data. Michael Chukwudi Ezeani, Grace Amilo, Chiemelu Dickson Emegakpor and Gabriel Chianakwanam analysed the data. Michael Chukwudi Ezeani and Ujuamala Uloma Ezeani wrote the manuscript. All authors edited and approved the manuscript.

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