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Breast cancer detection using free circulating DNA

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Annotation. Context: The prevalence of cancer and the mortality rate associated with it are on the rise, positioning it as the second most prevalent cause of death globally. Female breast cancer and male prostate cancer are the most severe forms of cancer worldwide, particularly in Africa. However, there is a deficiency in the advancement of biomarkers for the timely identification and prediction of these illnesses. The aim of this study was to assess the integrity of circulating cell-free DNA (ccfDNA) and ascertain its potential utility as a diagnostic and/or prognostic biomarker. Circulating cell-free DNA (ccfDNA) is fragmented DNA that is released into the blood plasma and then undergoes destruction. Apoptosis is the sole source of ccfDNA in individuals who are in a state of well-being, leading to the generation of consistently sized and shorter fragments of DNA. In individuals with cancer, necrosis produces cell-free DNA fragments that are both longer and irregular in size, as well as shorter fragments that result from apoptosis. The clinical efficacy of DNA integrity, as measured by the proportion of longer DNA fragments to total DNA, could be substantial in identifying the advancement of breast and prostate cancer. Methodology: The study included a sample of 64 females, divided equally into two groups: 32 breast cancer patients and 32 controls. In addition, there were a total of 61 guys, consisting of 31 individuals diagnosed with prostate cancer and 30 individuals serving as controls. Each participant

provided 5 ml of peripheral blood, from which the sera were collected. The sera underwent real-time quantitative polymerase chain reaction (qPCR) to quantify the amounts of ALU 115 and 247. Additionally, the DNA integrity was assessed by calculating the ratio of ALU247 to ALU115.

Keywords: sera, polymerase chain reaction, cancer, DNA, Female breast cancer, prostate cancer.

The prevalence of cancer and its associated fatality is increasing and has emerged as a significant public health issue. Presently, it ranks as the second most prevalent cause of mortality worldwide and accounted for around 8.82 million fatalities in the year 2015[1]. In 2012, the World Health Organization's International Agency for Research on Cancer predicted that by 2031, there will be around 21.8 million new instances of cancer and 14 million cancer-related deaths worldwide. These projections were based on factors such as population growth and longer lifespans. Incidence and mortality rates of cancer in Africa have consistently increased over time, with breast cancer being a major cause of death among females and prostate cancer among males [1,3]. Breast cancer is the most prevalent form of cancer in women worldwide, and it is also the leading cancer among women in Ghana, with a high fatality rate [3]. In 2012, the World Health Organization (WHO) documented that approximately 2000 women from Ghana were diagnosed with the condition, and out of those, 1000, or 50%, unfortunately passed away. In Ghana, breast cancer have been recognized as the second most common cause of cancer-related fatalities. Approximately 2900 instances are diagnosed each year, and at least one out of every eight women with the condition succumbs to it [3].

Considering these alarming trends, it is crucial to adopt a more pragmatic strategy to manage and address cancer. An inherent challenge in Africa regarding cancer management is the delayed detection and reporting of cases, as well as the inadequate resources available in health facilities to effectively address the disease. Effective management of cancer results in higher percentages of patient survival, especially when the illness is identified at an early stage [10]. This highlights the necessity for thorough investigation into innovative and effective techniques for identifying the condition. These innovative techniques for detection and/or result prediction should be cost-effective, easily implementable, reliable, and internationally acknowledged. Additionally, it provides an opportunity to gather DNA from tumors or tissues without the requirement of intrusive operations. Circulating cell-free DNA (ccfDNA) is fragmented DNA that is released into the bloodstream [11–13]. Iqbal et al. [14] reported that Tan et al. [15] first published the rediscovery of circulating cell-free DNA (cfDNA) in human plasma in 1966. Tan et al. identified cfDNA in individuals with autoimmune diseases. In 1977, Leon et al. [16] discovered cell-free DNA (cfDNA) in cancerous tumors. The initial identification of cfDNA was first reported by Mandel and Metais [16] in 1948. The source of cell-free circulating DNA (ccfDNA) in individuals without any health issues is only derived from programmed cell death, known as apoptosis, which leads to the production of consistently sized and shorter fragments of DNA. In cancer patients, necrosis leads to the production of extended DNA fragments, alongside the shorter pieces produced by apoptosis. The user's input is "[12]." Therefore, the identification of elevated levels of extended DNA fragments in the bloodstream has been recognized as a dependable signal for the presence of malignant tumor DNA [18–20]. Cancer patients display higher levels of circulating cell-free DNA (ccfDNA) in their blood serum compared to healthy individuals. Hence, the diagnostic and predictive significance of DNA integrity, particularly the ratio between longer and shorter fragments, has been examined in relation to cancer. There is a hypothesis that the levels of this

factor are elevated in cancer patients, particularly those with metastatic instances, in comparison to those with non-metastatic cases. Recent findings indicate that it can reliably predict the progression of tumors and the dissemination of cancer cells to adjacent lymph nodes in patients with first breast cancer. The application of DNA, whether it is found within the cell or circulating throughout the body, as a biomarker in clinical medicine has achieved significant advancements in the biomedical field. This advancement enables the timely detection, prediction of outcome, and assessment of treatment progress (12,13,16,17). The use of circulating DNA as a biomarker offers a practical and cost-effective means to overcome the infrastructure limitations faced by many developing countries. This study assessed the levels of circulating cell-free DNA (ccfDNA) and the quality of DNA in the blood sera of individuals diagnosed with breast cancer. Subsequently, these measurements were compared to those of individuals who were considered to be in a state of optimal health.

Methods

blood sample from breast cancer patients
master mix
TBE buffer
agarose
loading die
extraction DNA
syper green
alcohol 70%

Table (1) primer design for breast cancer

Primer	Sequencing
Forward sequencing	TGCCTGTGTACAGCGCTTTA
Reverse sequencing	GCACAGTGCTGGTTCACTTG

Subjects and research locations The study included females who had received a clinical diagnosis of breast cancer, as well as healthy women who willingly agreed to participate in the study. The recruitment of breast cancer patients was conducted at The Surgical Department at (KBTH) in Accra, Ghana. The department has the highest bed capacity in the hospital, accommodating up to 612 beds. The Surgical Specialist clinic receives an average of 15,100 cases per year, the Genito-Urinary clinic receives 10,600 cases per year, and the Neurology clinic receives 4,600 cases per year. The normal controls consisted of apparently healthy persons who had undergone routine medical screening at the separate health facilities. They exhibited no evident signs of disease or any clinical indications of any type of malignancy. Selection of participants We employed convenience sampling to encompass Every patient with prostate and cancer of the breast who visited the corresponding healthcare facilities from February to May 2017 and agreed to

participate in the study. The study encompassed individuals aged 18 and above who were recently diagnosed with breast or prostate cancer, as well as those undergoing treatment for these conditions. Participants classified as newly diagnosed were individuals who had recently received a diagnosis of either breast or prostate cancer, as determined by appropriate medical and laboratory assessments, and had not yet commenced any sort of medical intervention. Patients undergoing therapy included individuals whose medical condition had been diagnosed prior to the commencement of the trial, and the determination of DNA integrity. The calculation of DNA integrity involved determining The ratio between ALU247-qPCR and ALU115-qPCR. The ALU115-qPCR data provided information on the total amount of free DNA present in the serum. The ALU247-qPCR values quantified the total amount of DNA produced from cells that did not undergo apoptosis. The annealing sites of ALU115 are included by the annealing sites of ALU247. The qPCR ratio, which serves as an indicator of DNA integrity, is 1.0 when the template DNA is intact and 0.0 when all template DNA is completely fragmented into bits smaller than 247 base pairs [14].

Statistical analysis of data The data were statistically analyzed using the Statistical Package for Social Sciences version 20 software for Windows. The data was depicted utilizing the measures of central tendency, specifically the mean, and the measure of dispersion, specifically the standard deviation. A student t-test was used to compare the research parameters between groups in order to detect any disparities. A p-value that is less than or equal to 0.05 is considered to have statistical significance. The mean ± standard deviation (SD) of the average arithmetic logic unit (ALU) values from two replicates in each of two independent experiments were calculated and displayed, rounded to two decimal places. The study received ethical approval from the Ethical and Protocol Review Committee of the School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana (SBAHS: MD./10550649/AA/5A/2016– 2017) and the Institutional Review Board (IRB) of the Korle-Bu Teaching Hospital (STC/IRB/000100/2016). Participants were disqualified if they were presently receiving any form of medical intervention, such as chemotherapy or radiotherapy. The study comprised cancer patients who were referred to the healthcare facilities by certified physicians for a variety of laboratory tests. The study excluded participants with concurrent malignancies. All participants provided a written informed consent form. Demographic and pertinent clinicopathological data of all patients were also collected.

Procurement of blood samples and subsequent generation of serum Each participant in the research had 5 ml samples of venous blood drawn from their median cubital vein using labeled serum separator tubes. Following a 15-minute interval, each sample underwent centrifugation with a force equivalent to 1000 times the acceleration due to gravity for a duration of 15 minutes to obtain the serum. The serum was extracted and partitioned into smaller aliquots for preservation at a temperature of -20 °C until it is required. Control samples were obtained from healthy individuals who were carefully selected to have the same gender and age as the experimental group. Sample preparation for quantitative polymerase chain reaction (qPCR) The cryopreserved serum samples were thawed at ambient temperature while being maintained on ice. The qPCR sample preparation was performed according to the methodology described by Iqbal et al. [14]. A preparation buffer was created by mixing 2.5% Tween-20, 50 mmol/L Tris, and 1 mmol/L EDTA. To counteract the potential interference of proteins that interacted with the template DNA or DNA polymerase and could compromise the accuracy of the qPCR data, an equal volume of 20µl preparation buffer was added to each 20µl serum sample. Afterwards, a quantity of 20µg of Proteinase K (Inqaba Biotec, South Africa) was introduced into the mixture to facilitate protein digestion. The enzymatic breakdown occurred at a temperature of 56 °C for a duration of 50

minutes. Subsequently, the mixture was subjected to heat inactivation at a temperature of 95 °C for a duration of 5 minutes. The solution was subjected to centrifugation with a centrifugal force equivalent to 1000 times the acceleration due to gravity for a period of 5 minutes. Afterwards, a volume of 0.2µl of the liquid obtained above the sediment was used as the template for each quantitative polymerase chain reaction (qPCR) process. The optimal parameters for conducting quantitative polymerase chain reaction (qPCR) and quantifying ALU fragments were established. The qPCR reaction combination for each sample consisted of a template sequence, 0.2µmol/L of forward and reverse primers (Inqaba Biotec, South Africa) for both ALU115 and ALU247, one unit of iTaq DNA polymerase, 0.02 µL of fluorescein calibration dye, and a 1× concentration of SYBR Green. The overall volume of the reaction was 20 µL, containing a concentration of 5 mmol/L of Mg2+. The real-time PCR amplification procedure commenced by activating the DNA polymerase at a temperature of 95 °C for a duration of 10 minutes. This was then followed by 35 cycles of denaturation at 95 °C for a duration of 30 seconds, and subsequent annealing at 64 °C for a duration of 30 seconds.

The sample underwent an extension process at a temperature of 72 °C for a length of 30 seconds using the QuantStudio5 Real-time PCR equipment. The exact amount of DNA in each sample was determined using a standard curve, which was created using diluted genomic DNA obtained from the peripheral blood leukocytes of healthy donor volunteers. The dilutions spanned a range of 10 nanograms to 0.01 picograms. The targeted objective was achieved by employing the peripheral blood leukocytes of a healthy donor as an external benchmark. Every plate contained a negative control, including a sample lacking DNA that can be targeted. To enhance the sensitivity of DNA measurement, a qPCR technique was used to amplify the consensus ALU sequences (ALU115 and ALU247) by utilizing primer sets. Two sets of ALU primers were created: the primer set for the 115 bp amplicon (ALU115) amplified both shorter (truncated by apoptosis) and longer DNA fragments, while the primer set for the 247 bp amplicon (ALU247) only amplified longer DNA fragments. The primer sequences for ALU115 were as follows:

forward: 5 -CCTGAGGTCAGGAGTTCGAG-3 and reverse: 5

-CCCGAGTAGCTGGGATTACA-3 ; ALU247 primers were

forward: 5 -GTGGCTCACGCCTGTAATC-3 and reverse: 5 -CAGGCTGGAGTGCAGTGG-3 . β-actin was used as normalizer for all qPCR assays. The sequences of β-actin primers

used were forward: 5 -GACCTCTATGCCAACACAGT-3 and

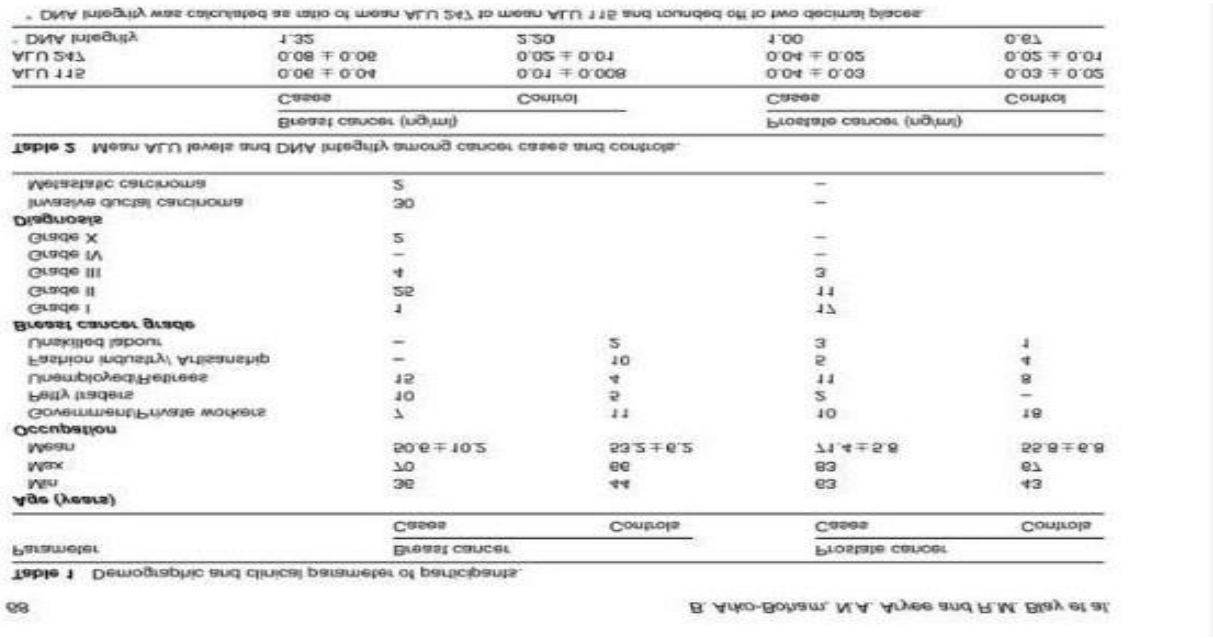
reverse: 5 -AGTACTTGCGCTCAGGAGGA-3

Result and Discussion

Results

Demographic characteristics of the study participants The study's breast cancer cohort comprised 64 females, with 32 individuals diagnosed with breast cancer and 32 serving as controls. The breast cancer patients, denoted as cases, had ages ranging from 36 to 70 years, while the healthy individuals, referred to as controls, had ages ranging from 44 to 62 years. The mean ages of the patients and controls were 50.6±10.2 years and 53.2±6.2 years, respectively. 51.0% of the total cases were within the age range of 41-50 years, making it the most common age group. Conversely, the majority of controls fell within the age range of 51-60 years, constituting 50.0% of the overall sample. The prostate cancer cohort had a total of 61 male participants, with 31

individuals diagnosed with prostate cancer and 30 individuals serving as control subjects. The mean age of individuals diagnosed with prostate cancer was 71.4±5.8 years, with a range of 63 to 83 years. By contrast, the mean age of the healthy control group was 55.8±6.8 years, with a range of 43 to 67 years.



Discussion

The objective of the study was to examine the amounts of circulating cell-free DNA (ccfDNA) in persons diagnosed with breast and prostate cancer, as well as in those without any known health conditions. Previous research [19,25–27] have revealed that serum is preferable over plasma because it contains higher levels of ccfDNA. This enhances the suitability of the serum for its intended function. The mean age of patients diagnosed with breast cancer was 50.6±10.20 years, whereas the mean age of patients diagnosed with prostate cancer was 71.4±5.8 years. The results are consistent with existing trends, which indicate that 81% of all instances of female breast cancer are detected in women who are 50 years old or older [28]. Prostate cancer primarily affects older persons, specifically guys aged 60 years or older [29,30]. The blood levels of circulating cell-free DNA (ccfDNA) and the patterns of DNA integrity that we observed in our study among both cancer patients and healthy controls were consistent with earlier research [12–14,19,26,27]. This consistency was particularly evident in respect to the prostate and endometrial cancer groups. Furthermore, we found that these levels and patterns were associated with the severity of the disease. Among the group of individuals with prostate cancer, the cases showed higher levels of DNA integrity compared to the controls. Additionally, this particular characteristic was found to have a positive correlation with the progression of the disease stage. The ALU 115, a marker for cell-free DNA concentration in the bloodstream, was found to be higher in patients with prostate cancer compared to persons in a healthy state. Elevated levels of ALU 247, a biomarker for cell-free DNA released by dying cells (both apoptotic and necrotic), were observed in prostate cancer patients in comparison to healthy individuals. The observed phenomena is likely attributed to an elevated concentration of truncated DNA fragments that are discharged by dying cells into the bloodstream of individuals with prostate cancer, as opposed to healthy males [12,14,15,19,31]. A notable discovery was the heightened concentrations of ALU species in those diagnosed with breast cancer as opposed to those diagnosed with prostate cancer (Tables 2&3).

While this study did not investigate the finding and an immediate cause is not evident, we propose the following hypothesis: (1) Females may have higher levels of ALU than males due to differences in hormones, which could be altered by conditions before and after menopause. (2) The influence of aging on the expression of ALU. The mean age of female persons diagnosed with breast cancer was lower compared to male participants diagnosed with prostate cancer. The variables in question were not analyzed in this study and hence require further examination. The DNA integrity in prostate cancer patients exhibited a statistically significant increase, with a mean value of 1.00, in comparison to healthy men who displayed a mean value of 0.67. The DNA integrity in healthy males was diminished, possibly due to a decline in necrotic activity in body tissues, resulting in a reduction in the concentration of longer DNA fragments in the bloodstream. After examining the DNA integrity at various stages of prostate cancer, it was discovered that stage III displayed the greatest amount, followed by stage II, and ultimately stage I. The occurrence of this phenomena can be ascribed to the increased amount of DNA that is discharged into the bloodstream due to tissue death, which escalates with the seriousness of the illness. Prior research [12–14,31] has seen similar patterns and has determined that there is a significant and relevant association between DNA integrity and the growth and advancement of prostate and endometrial cancer. Among the patients diagnosed with breast cancer, the control group had higher DNA integrity than the cases. This conclusion contradicts the observed pattern in the prostate cancer group of this study and previous published literature. In general, the concentrations of both ALU species 115 and 247 were shown to be higher in breast cancer patients as compared to persons who are in good condition. Nevertheless, this rise did not align with any alterations in DNA integrity. The difference in DNA integrity between the patients and the control group, however, was not statistically significant ($p>0.05$). An inherent limitation of this study is the very small sample size (32 breast cancer patients), which may result in divergent conclusions. In addition, our study did not investigate the presence of comorbidities in people, which could also contribute to the rise of ALU 247 species through necrosis. In addition, the correlation between DNA integrity in breast cancer stages II and III did not conform to the patterns seen by other researchers. The DNA integrity in stage III was much higher than in stage II. While not all the factors that contribute to the observed pattern are immediately obvious, some of the highlighted limitations could be useful in guiding future study endeavors. Additional work is warranted for two factors: (1) the effect of cancer treatment on ccfDNA levels and DNA integrity, and (2) the effects of malaria and HIV on ccfDNA levels and DNA integrity. While not specifically analyzed in this study, we propose that these factors may have an influence on ccfDNA levels and DNA integrity, potentially accounting for the observed discrepancies in our results. Ghana demonstrates a significant frequency of malaria and HIV, both of which are recognized to have adverse effects on the immune system.

Conclusion

This study demonstrates that levels of ALU species 115 and 247 in the serum are higher in patients with breast and prostate cancer compared to healthy individuals. Prostate cancer patients exhibited more DNA integrity than the control group, however breast cancer patients displayed lower DNA integrity compared to their control counterparts. DNA integrity had a positive correlation with disease severity and higher staging, namely in prostate cancers rather than breast cancers. These findings closely align with previously published studies, providing additional support for the potential use of ccfDNA and DNA integrity as diagnostic and prognostic tools in cancer. The area has significant potential and warrants further research dedication. However, we emphasize that our research are preliminary and require an expansion to a bigger sample size for validity.

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