Egypt. J. Chem. **Vol. 66**, No. 5 pp. 341 - 348 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Plasma cell-free DNA integrity as a molecular diagnostic tool for breast cancer: non-invasive genomic studies among Egyptian women

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Abstract

The sulfur recovery unit (SRU) is an important part of gas processing and crude oil refinery plants. The primary purpose of Liquid biopsy could be an alternative to the traditional tumor biopsies being less invasive and easy to obtain. This research aimed to verify if cfDNA integrity and concentration in plasma could be clinically useful as putative biomarkers in early breast cancer diagnosis using SYBR green based qPCR being simple and of low cost compared to Taqman technique.

This study was conducted on 80 breast cancer patients (40 early and 40 late breast cancer cases) to investigate the utility of cfDNA in cancer staging, 40 cases with benign breast lesions and 40 healthy donors. Plasma CfDNA integrity was determined by measuring ALU115 and ALU247 using real-time qPCR and ROC curve was performed to determine the best cut-off value for breast cancer detection.

Our finding showed that, cfDNA integrity in breast cancer was significantly higher than in both benign and healthy groups. Integrity index was correlated to the Tumor, Node, and Metastasis (TNM) stage.

This study suggested that the plasma DNA integrity could be a promising molecular diagnostic biomarker of breast cancer. The findings of this study are a step towards validating a non-invasive assay for the early diagnosis of breast cancer.

Key words: cfDNA, integrity index, ALU, breast cancer. .

Introduction

Breast cancer is the most often diagnosed cancer in women worldwide accounting for 25% of all cancers [1].Among Egyptian women, Breast cancer is the most frequent cancer type according to the Egyptian National Cancer Institute's official statistics with 28,000 confirmed cases per year [2]. While radiological screening programs including mammography have proven to be effective in breast cancer detection in it's early stages, no valuable blood biomarkers have yet been established.

Liquid biopsies, especially those including plasma cfDNA, are rapidly emerging as a valuable and minimally invasive approach to the traditional tumor biopsy and, it could be in some cases, even a potential alternative approach [3]. CfDNA analysis has the potential to detect or monitor tumors in patients without clinically evident disease [4].

Tumor necrosis is common in solid malignant tumors, and it results in DNA fragments with varying strand lengths due to random and partial digestion of genomic DNA by various deoxyribonucleases. Cell death in normal tissues, on the other hand, occurs via apoptosis resulting in smaller and uniform DNA fragments of 185–200 bp [5].

The *ALU* repeat family accounts for the majority of the plasma cfDNA in healthy individuals. [5]. *ALU* family is short stretch of repetitive DNA elements representing the most abundant repeated sequence in the human genome with more than one million copies per human diploid genome. They were named after the *ALU I* (Arthrobacter luteus) restriction enzyme site within the *ALU* sequence [6].

CfDNA can be used to identify genetic changes that are cancer specific [7]. These biological characteristics distinguish tumor derived cfDNA from normal cfDNA and ensure that cell-free DNA is a specific biomarker that provides individualized information for detecting residual tumor or monitoring disease progression during treatment.

Due to it's high sensitivity, the integrity index of cfDNA, that is measured as the ratio of long to short

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Receive Date: 29 September 2022, Revise Date: 09 November 2022, Accept Date: 20 November 2022.

DOI10.21608/EJCHEM.2022.165165.7029

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fragments, has recently been proposed as a promising specific molecular biomarker for tumors [8]. Sample collection for measuring integrity index is more convenient and eliminates the need for tumor biopsy. As a result, minimally invasive cfDNA analyses could one day take the role of the currently used cancer tissue and blood biomarkers [9].

In the present study, the aim was to assess the utility of cell free DNA as an alternative tool to diagnose breast cancer. We studied the serum levels of longer fragments of DNA (*ALU247*) and shorter fragments (*ALU115*) as well as its integrity in patients with breast cancer and benign masses compared to healthy control. cfDNA integrity was calculated as the ratio of longer DNA (*ALU 247*) to shorter DNA fragments truncated by apoptosis (*ALU 115*).

Although some clinical studies have suggested that cell-free DNA may be a valuable tool for cancer detection, the scientific community has not yet come to a consensus on how to apply these findings due to different target choice. This work underscores the value of cell-free DNA Integrity for breast cancer detection using SYBR Green qPCR being a sensitive and cost effective method to detect ALU sequence which represent the most abundant sequence in the human genome.

Experimental

Subjects

This case control study was conducted on 120 patients (80 cases with histologically confirmed breast cancer and 40 with benign breast lesions) compared to 40 apparently healthy controls recruited from Cairo University Hospitals between June and December 2020.

This study protocol was approved by ethical committee at Faculty of Medicine, Cairo University (approval number: MD-12-2020). All study participants signed informed consent forms.This study met the following inclusion criteria: patients with breast cancer diagnosed by histopathology. Clinical examination and fine needle aspiration cytology were used to diagnose benign breast lesions. Control subjects were apparently healthy and age matched donors.

The exclusion criteria were as follows: patients with autoimmune diseases and/or tissue injuries, patients receiving radio or chemotherapy.

All cases involved in this study were subjected to full history taking & clinical examination, as well as imaging techniques including: mammography for both benign & malignant breast lesions, CXR for cases with malignancy, bone and liver scan to rule out metastasis, receptor study (ER & PR), grading and histopathology for breast lesions together with molecular genetic study which includes: extraction of cfDNA, measurement of cfDNA concentration and genetic analysis for determination of cfDNA integrity using SYBR green based quantitative real time PCR (qPCR).

Methods

Venous blood (4 ml) was withdrawn, from each participant, in a sterile EDTA vacutainer. Plasma was then separated within 2 hours of collection via centrifugation at 8000 rpm for ten minutes followed by harvesting the plasma supernatant, then centrifugation at 16000 rpm for 10 minutes to ensure complete exclusion of any cellular elements. The plasma samples were aliquoted and stored at -80°C until used for cfDNA extraction.

Cell free DNA extraction and analysis were tested using QIAamp* DNA Mini Kit (Catalog number: 51304).DNA concentration (ng/µl) was determined by using Nanodrop spectrophotometer at 260nm. The absorbance ratio A260/A280 nm was calculated for purity of DNA, and any sample with a ratio less than 1.7 was rejected.

PCR amplification of cfDNA:

Quantitative Real-time PCR (qPCR) was used for detecting, amplifying, and quantifying a targeted DNA sequence. To quantify the levels of *ALU 115* and *247* bp DNA amplicons, two primer sets amplifying *ALU* sequences were used. The ratio (*ALU247 /ALU115*) of the qPCR findings was used to determine DNA integrity.

Sequence of *ALU 115* primer was as follows: forward: 5′-CCTGAGGTCAGGAGTTCGAG-3′, reverse: 5′-CCCGAGTAGCTGGGATTACA-3′. As for *ALU 247* primer sequence was: forward: 5′-GTGGCTCACGCCTGTAATC-3′ reverse: 5′-CAGGCTGGAGTGCAGTGG-3′.

PCR was performed in 25 µl of total reaction volume including 12.5 µl of Maxima SYBR Green PCR master mix (Thermo Scientific, USA), 1 µl from each primer of both amplicons, 5.5 µl nuclease free water and 5 µl from each isolated DNA sample. qPCR was carried out using the Applied biosystems StepOne™ (Applied Biosystems, Foster City, CA, USA) under the following conditions: precycling heat activation of DNA polymerase at 95 °C for 15 min, followed 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 60 seconds and extension at 72 °C for 30 seconds. The caliberation curve was then constructed by performing serial dilutions of Human Genomic DNA (Thermo Fisher scientific, Catalog

number: 4312660) to create a four-point standard curve for each of *ALU 115* and *ALU 247*.

The cycle threshold of qPCR for both *ALU 115* and *ALU 247* was obtained at the end of the run for each sample. The results were interpreted using Absolute Quantification and the Standard Curve Method. The amounts of *ALU* in DNA of an unknown sample were quantified using this method by comparing the CT of the unknown sample to a standard curve with known concentrations. The ratio of Q247/Q115 was used to calculate cfDNA integrity.

Statistical analysis

Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Median, minimum and maximum were used to summarize the quantitative data. In categorical data, Chi square (2) test, the frequency (count) and relative frequency (percentage) were used. Non-parametric tests (Mann-Whitney and Kruskal-Wallis tests) were used to compare quantitative variables. The Spearman correlation coefficient was used to calculate correlations between quantitative variables. To determine the best cutoff value of significant characteristics for cancer detection, a ROC curve was constructed with analysis of the area under the curve. Statistical significance was defined as P-value less than 0.05.

Results

Patient characteristics:

In this case control study, 160 subjects were enrolled; 80 patients diagnosed with breast cancer with mean age 52.51 ± 10.84 years; 40 patients with benign breast masses with mean age 41.68 ± 7.38 years; and 40 apparently healthy females with mean age 40.78 ± 7.62 years.

Characteristics of benign and malignant cases:

Benign breast lesions included 35 premenopausal patients (87.5%) and 5 postmenopausal (12.5%) with average tumor size 3.24 ± 1.22. With respect to pathological type, patients with fibroadenoma were 36 (90.9 %), 2 patients with hamartoma (5%) and only 1 patient with phylloides tumor and one lipoma case (each represents 2.5%)**.** Average tumor size in benign group was 3.24 ± 1.22 cm while in breast cancer group was 4.56 ± 2.38 cm.

Clinical and histopathological data of patients suffering from breast cancer including family history, menstruation, tumor size, tumor subtype, grading, LN involvement, receptor-status, Her2/neu status, metastasis and TNM stage are showed in table (1). Average tumor size in Breast cancer group was 4.56 ± 2.38 cm while in benign group was 3.24 ± 1.22 cm**.**

Table (1): Clinicopathological characteristics of breast cancer patients

	Breast Cancer patients	***No***	***%***
Family History	Positive	8	10%
	Negative	72	90%
Menstruation	Pre-menopausal	39	48.8%
	Post-menopausal	41	51.2%
Tumor size	T1 (<2 cm)	16	20%
	T2 (2-5 cm)	35	43.75%
	T3 (> 5 cm)	29	36.25%
Tumor type	Invasive Duct Carcinoma	70	87.5%
	Invasive lobular Carcinoma	7	8.8%
	Mixed Duct and Lobular Carcinoma	3	3.7%
Tumor Grade	G II	65	81.2%
	G III	15	18.8%
LN	N0	14	17.5%
	N1	48	60%
	N2	16	20%
	N3	2	2.5%
ER	Positive	61	76.2%
	Negative	19	23.8%
PR	Positive	54	67.5%
	Negative	26	32.5%
Her 2-neu	Positive	26	32.5%
	Negative	54	67.5%
Metastasis	M0	73	91.2%
	M1	7	8.8%
Tumor Stage	Stage I	6	7.5%
	Stage II	34	42.5%
	Stage III	33	41.2%
	Stage IV	7	8.8%

Molecular Results:

Data listed in table (2) showed that plasma levels of *ALU 115*, *ALU 247* and integrity index were significantly higher in breast cancer cases than in non-malignant (both benign and control groups) (P < 0.001)**.** While the integrity index between the healthy controls and benign group showed that there is no significant difference between the two groups as illustrated in figure (1).

Table (2): *ALU115*, *ALU 247* and integrity index among control, benign and BC groups

	BC group	Benign group	Control group	P value
ALU115(ng/ml)	93.15(1.95-1022)[a]	28.23(0.8-301.43)[b]	4.2(0.51-56.78)[c]	< 0.001
ALU247(ng/ml)	80.32(1.04-999.36)[a]	11.94(0.61-218.9)[b]	1.07(0.07-30.77)[c]	< 0.001
Integrity index	0.93(0.30-1.17)[a]	0.48(0.23-1.12)[b]	0.35(0.08-1.46)[b]	< 0.001

*Data is presented as Median (minimum-maximum).

**P value <0.05 is considered significant. Groups bearing the same initials are not statistically significant.

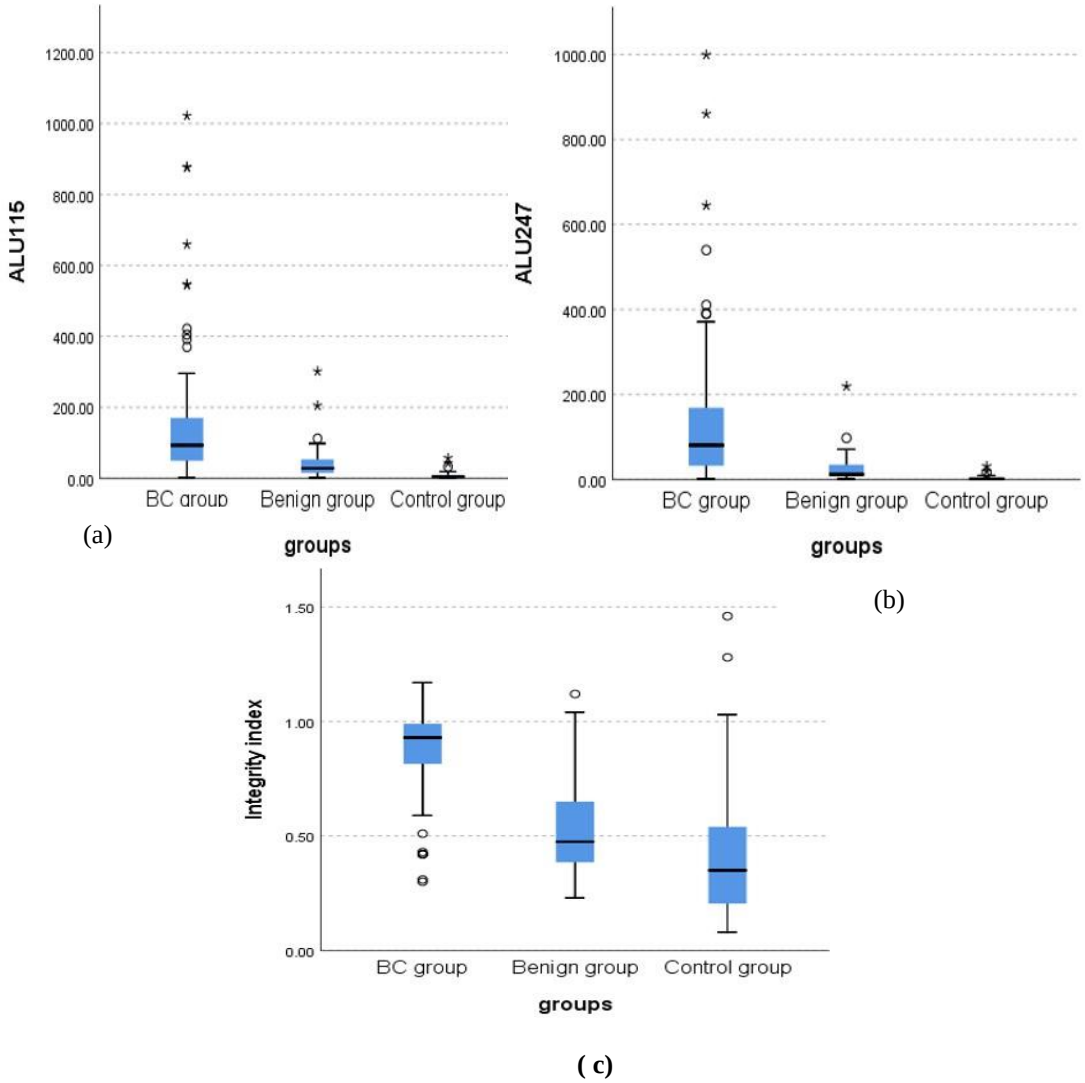

Figure (1): Median level of *ALU115* (a), *ALU247* (b) and *ALU* index (c) among the three groups

Regarding tumor size: cases were divided into three categories according to the TNM staging system: T1 tumors (less than 2 cm), T2 tumors (between 2 and 5cm) and T3 tumors (more than 5 cm). Regarding the plasma DNA integrity index there was a statistical difference (P=0.041), however no statistical difference in plasma levels of *ALU115*, *ALU247* (p=0.165, p=0.149 respectively). There is a positive Correlation between integrity index and tumor size (r2=0.229).

Regarding pathological type, even though invasive lobular and mixed carcinomas are less common, it showed a greater concentration of plasma *ALU115* and *ALU247* but there was no statistically significant difference (p=0.093, p=0.163 respectively)**.**

Regarding tumor grade, plasma level of *ALU115* and *ALU 247* were higher in grade 3 than grade 2 with statistically significance (p=0.005, p=0.013 respectively), while no significance differences in integrity index among tumor grades (p=0.921).

As regards lymph node status (LNs), 14 (17.5%) cases were node negative, while 66 (82.5%) were node positive cases. According to the TNM staging system, node positive cases were divided into N1 (from 1 to 3), N2 (from 4 to 9) and N3 (≥10 nodes) based on the number of affected nodes. The majority of the cases that tested positive for lymph nodes belonged to the N1 group, accounting for 60% of the studied cases, followed by the N2 category (20%) and the N3 category (2.5%). Levels of ALU115 and ALU247 showed increase in N2 and N3, but no statistical significance was found (p= 0.27, p= 0.42 respectively). However, there was a statistical significance in integrity index (p= 0.008).

Regarding distant metastasis, all patients with breast cancer underwent bone scans to check for metastases at the time of diagnosis. In our study, we found metastases in 7 cases out of the 80 breast cancer patients representing 8.8%. When compared to the non-metastatic group, *ALU115* and *ALU247* levels were significantly higher in metastatic group. However, no statistical difference was found on comparing the integrity index between the two groups.

Immuno-histochemical study of breast specimens showed that 61/80 (76.2%) of the samples tested positive for estrogen receptor, with 17 cases testing negative for the progesterone receptor, while 54/80 (67.5%) were positive for progesterone receptor. No statistical significance was observed on comparing *ALU115*, *ALU247* or integrity index between receptor positive and negative cases (p=0.839, p=0.959, p=0.157 respectively for ER), (p=0.504, p=0.383, p=0.339 respectively for PR).

As regards Her2-neu immunostaining 30/80 (37.5%) were positive, 9 (11.25%) were triple negative and 15 (18.75%) were triple positive. No significant difference was observed on comparing *ALU115*, *ALU247* or integrity index between Her2-neu positive and negative groups (p=0.789, p=0.766, p=0.511 respectively).

Regarding tumor stage, according to the AJCC, the majority of the patients in the study had stage II tumors, accounting for 42.5% of all cases, followed by 41.2% stage III tumors, 7% stage I tumors and 8% stage IV tumors. ALU 115, ALU247 and ALU index showed increased median values with advanced stages and a statistical significance (p=0.044, p=0.036, p=0.046 respectively).

Receiver operating characteristic curve (ROC)

The ROC curve was used to compare the diagnostic values of *ALU115* representing the total amount of cfDNA and cfDNA integrity between *malignant* and *non-malignant groups* (benign and healthy control) based on the area under the curve (AUC). The diagnostic value of a test is better with a greater AUC (Table 3) (Figure 2).

For *ALU115*, it showed AUC=0.855 (95%CI: 0.797-0.914), at cut-off ≥45.155ng/ml, sensitivity was 77.5%, specificity was 83.8%, PPV=82.67%, NPV=78.82% and diagnostic accuracy=80.63%. Regarding DNA Integrity index, it showed AUC=0.883(95%CI: 0.824-0.942), at cut-off ≥0.735, sensitivity was 82.5 %, specificity was 91.3%, PPV=90.4%, NPV=83.9% and diagnostic accuracy=86.88%

Table (3): Sensitivity & specificity of the biomarkers in diagnosing malignant vs. non-malignant

	Cut off*	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
ALU115(ng/ml)	45.155	77.5	83.8	82.67	78.82	80.63
ALU247(ng/ml)	44.375	71.3	95	93.44	76.77	83.13
Integrity index	0.735	82.5	91.3	90.41	83.91	86.88

*Positive response if greater than or equal to cut-off value.

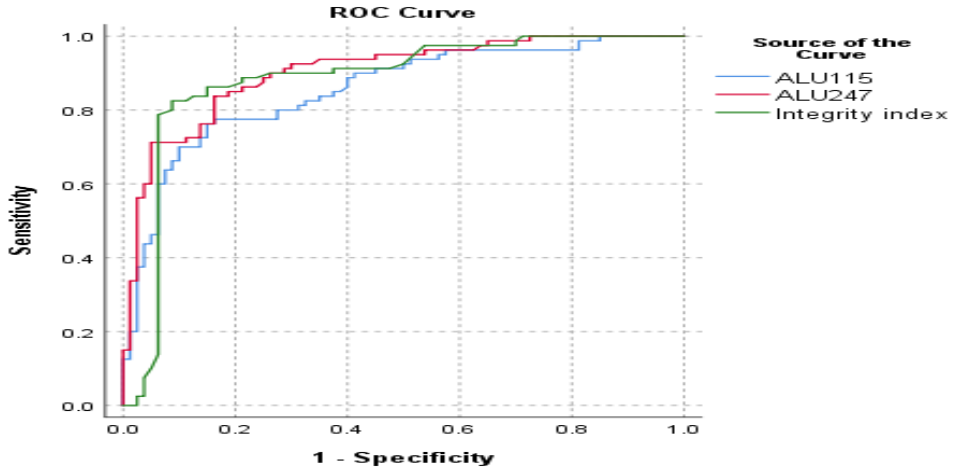

Figure (2): ROC curves of plasma *ALU 115*, *ALU 247*and integrity index for the discrimination between breast cancer and both benign and control groups.

Discussion

Liquid biopsies, as opposed to current surgical biopsies, are an appealing and more practical routine cancer alternative surveillance method. As cfDNA being readily available in the plasma of cancer patients, various attempts have been made to assess their clinical utility.

CfDNA is an extracellular DNA that is mostly derived through apoptosis and necrosis. Apoptosis can cause normal tissues to emit a small quantity of homogeneous short-segment DNA (less than 200 bp). The composition and concentration of cfDNA may differ with the pathological state**.** The cfDNA level in peripheral blood is abundant during the tumor-cell necrosis process that it cannot be completely digested by deoxyribonuclease. As a result, the lengths of the genomic DNA fragments vary, with long DNA fragments predominating (typically >200 bp), as described by [10].

The presence of a higher percentage of fragmented DNA in the cancer group is based on the fact that tumor cells are uncontrollable compared to normal cell deaths. As the disease progresses, an enhanced DNA integrity would be expected representing the increased tumor load and removal of damaged DNA, providing a method of diagnosing cancer patients and differentiating them based on disease staging and progression as well [11]. Accordingly, this study was designed to investigate the use of *ALU*-derived cfDNA concentrations and *ALU* index as a means of distinguishing cancer patients from those without the disease.

In this study the median value of cfDNA concentration (*ALU 115*) was significantly higher in breast cancer group than in both benign and control groups. The cfDNA found in samples of healthy persons may originate from hematopoietic cells, reflecting the processes of regulated cell turnover in these cells as illustrated by Moss [12].

Our results are supported by the finding of Stötzer [13] who reported that median values of cfDNA in healthy females were significantly lower than in patients with benign diseases, however there was no significant difference obtained on comparing benign and malignant diseases. In accordance with our findings, astudy by Tang [14] found a significantly higher concentration of cfDNA between malignant and non- malignant groups. Close results were also previously reported by Iqbal [15] who found that the levels of *ALU115* were significantly higher in patients than in healthy controls.

Concerning cfDNA integrity index *(ALU247/115)*, its median value in our study was significantly higher in breast cancer group when compared to control groups. However, there was no statistical significance found between benign and control groups this could be due to benign breast diseases are frequently associated with inflammation, and inflammation is also known to raise cfDNA levels in the blood.

These results were slightly different from that reported by Stötzer [13] who stated that the ratio of plasma *ALU247/115* were higher in patients with locally confined BC and MBC than healthy controls but not vs benign breast lesions . However, a study by Arko-Boham [16] on *ALU115* and DNA integrity index in Prostate and breast cancer patients involving only a small sample reported that DNA integrity was lower in breast cancer patients compared to their controls.

In our study, in order to evaluate the discriminating power of the *ALU* index, ROC curve analysis was conducted ,our results are in line with Park [17] using

different primer sets for shorter *ALU* fragments (58 bp) representing the total amount of *ALU* DNA and longer *ALU* fragments (263 bp) represents DNA derived from malignant lesions.

Our findings suggest that the *ALU247/115* index was stronger than *ALU115* in breast cancer detection being stronger in sensitivity, specificity, PPV, NPV, and total efficiency of BC diagnosis. Because the *ALU* index correlates with tumor cell death, it is preferable to absolute cfDNA concentrations, which do not reflect DNA release.

Regarding the clinico-pathological characteristics, the present study found a statistical correlation only between cfDNA (ALU 115) and metastasis and tumor grade, however, no statistical correlation was found with age, menstruation, and other characteristics including: tumor size, lymph node pathological type and receptor study. The result is in accordance with the previous study of Cheng [18] who found a significant increase in cfDNA with distant metastases.

cfDNA appears to be reasonably linked to tumor size and metastasis, because tumor markers are thought to reflect the number of malignant cells as well as their ability to enter the bloodstream.

No association was detected between the levels of circulating cfDNA and the scoring of ER or PR or Her2/neu similar to another study [11], however previous studies reported a significant difference between cfDNA and Her2/neu [19]. This could be attributed to the fact that all the molecular subtypes were included in this study; a more homogeneous set of molecular subtypes could have produced greater predictive values.

The present study observed that *ALU* index was correlated to the size of breast cancer and lymph node metastasis similar to the findings by Cheng [11], who demonstrated that serum DNA integrity predicted lymph node metastasis, similarly Umetani [20] reported that the mean serum DNA integrity index was significantly higher in the presence of LN metastasis.

The disparities in predictive and prognostic utility of cfDNA reported in the literature are due to the lack of standardized methodologies as well as using different amplicons for its quantification. It is necessary to harmonize the approaches used in cfDNA measurement and to include a well-powered sample size in the study in order to validate its clinical relevance.

The present study pointed out that plasma cell free DNA concentration and DNA integrity index in breast cancer among other cells could be a valuable biomarker in diagnosis of breast cancer. It could be integrated into a screening program to detect early BC having the ability to distinguish between breast cancer, benign breast lesions and healthy females.

Conclusion

This study concluded that the Plasma levels of *ALU 115*, *ALU 247* and integrity index were statistically higher in breast cancer cases than in non-malignant (both benign and control groups) with integrity index being superior to cfDNA concentration in discriminating breast cancer cases. The findings of this study paves the way to introduce plasma cfDNA integrity as a potential diagnostic marker for breast cancer detection.

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