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The role of circulating cell-free DNA and its integrity as a biomarker for diagnosis of breast cancer using ALU (247/115) bP sequences

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Abstract

Diagnosis of breast cancer by using sensitive and specific biomarkers is necessary. Cell- free DNA (cf-DNA) is a candidate biomarker in various cancers. Contrasting, shorted uniformed DNA released from apoptotic non-diseased cells, DNA released from malignant cells varies in size. DNA integrity is a ratio between 247 and 115 bp. This study aimed to evaluate the diagnostic values of cf-DNA using ALU -247 and ALU- 115 and DNA integrity in peripheral blood of breast cancer patients as a noninvasive marker. Also, to determine correlations between ALU-247 and ALU-115, DNA integrity, cancer antigen (CA)15-3 and carcinoembryonic antigen (CEA) with each other in breast cancer patients and in different stages of breast cancer. This study included 100 females, divided into 3 groups. The first group consisted of 20 apparently healthy females as the control group. The second group included 20 patients with benign breast lesions. The third group included 60 patients with breast cancer. Serum levels of both ALU-247 and ALU-115 as well as cf-DNA integrity were statistically significant higher in breast cancer patients as compared to the control group ($p=0.018$, $p<0.001$ and $p=0.009$ respectively). Compared to the control group, ALU-247 had the best diagnostic sensitivity for diagnosis of breast cancer (86.78%) with 75% specificity with area under the curve of 0.848. We concluded that measuring ALU-247, ALU-115 and DNA integrity in peripheral blood would be a promising novel approach for diagnosis and early detection of breast cancer.

Keywords: Breast cancer, Cell frees DNA, DNA integrity.

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Introduction

According to Dolatkhah et al., 2020,[1] breast cancer is the second most common cancer worldwide and the most common cancer in women. According to statistics that were made by the International Agency for Research on Cancer (IARC) in December 2020, breast cancer has now surpassed lung cancer as the cancer that is diagnosed the most frequently

worldwide.[2] It was the fifth leading cause of cancer-related death worldwide in 2018, causing an estimated 2.1 million cancers. Breast cancer may affect one in nine women in developed nations and one in twenty women in less developed nations.[3] Breast cancer accounts for 18.9% of all cancer cases in Egypt (32.04 percent of women and 2.2 percent of men), with an age-adjusted rate of 49.6 cases per 100,000 people. Breast cancer has a 97% chance of surviving five years in its early, treatable stage. However, once it spreads to other parts of the body, women's chances of surviving for five years drop by 20%.[4]

Previous studies showed that early detection of breast cancer and given the right treatment, death rates from the disease could be significantly reduced over time.[5] The current standard for breast screening is mammography; however, it is less effective for subjects younger than 40 and with dense breasts, less sensitive to small tumors and does not provide any indication of the eventual outcome of the disease.[6]

In addition to mammography, ultrasound has been utilized as a medical imaging tool. Although magnetic resonance imaging (MRI) can detect small lesions that cannot be detected by mammography, it is also costly and has low specificity, which can result in over diagnosis. The most accurate method for observing the progression of tumors or their response to treatment is positron emission tomography (PET).[7]

Tumor activity has been identified by measuring the concentration of tumor markers in the blood. A cost-effective, minimally invasive source of data for monitoring the course of the disease, determining prognosis, and assisting in treatment planning are tumor markers. The recommendations for the use of tumor markers in the prevention, screening, treatment, and surveillance of breast cancer have been updated by the American Society of Clinical Oncology (ASCO).[8] Cancer antigen 15-3 (CA 15-3), CA 27.29, carcinoembryonic antigen (CEA), estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), urokinase plasminogen activator (uPA), plasminogen activator inhibitor 1 (PAI-1) and multiparameter assays for gene expression are among the tumor markers that were found to have clinical utility and were recommended for use in practice.[8] P53, cathepsin D, cyclin E, and nesting are among the other categories that are utilized in breast cancer screening, but there is insufficient evidence to support their routine use in clinical practice.[9]

As a liquid biopsy in the peripheral blood, circulating molecular biomarkers are increasingly being utilized due to their accessibility, early detection, and reproducibility. As a method of detection and prognosis for various types of cancer, circulating tumor cells, circulating DNA, and microRNAs have been investigated.[10]

The majority of cell free plasma DNA in healthy individuals is composed of DNA repeat sequences that include long and short interspersed nucleotide elements.[11] Cell free DNA (cf-DNA), are short fragments of nucleic acids that are present in the circulation. The release of cf-DNA into the blood stream occurs from various sources, including normal cell types like hematopoietic and stromal cells. It is likely that a significant portion of the cf-DNA is bound to protein molecules, possibly as nucleosomes. In addition, it is released by metastatic deposits and circulating tumor cells.[12]

Various etiological conditions, including trauma, stroke, burns, sepsis, and autoimmune diseases, have been linked to elevated levels of circulating cf-DNA. Also elevated rates of cf-DNA in the blood of cancer patients may be caused by elevated rates of cellular proliferation in tandem with elevated rates of various forms of cell death, which are characteristic biological features of tumor growth.[13] Multiple studies also have indicated elevated levels of cf-DNA in breast cancer,[14-16] and several other malignancies as colorectal cancer, lung cancer, testicular cancer, prostate cancer, ovarian cancer and other solid tumors.[10]

Many gene sequences such as the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, ß-globin-gene, human telomerase reverse transcriptase (hTERT), LINE1 (long interspersed nucleotide elements) were studied as biomarkers in breast cancer.[17] The

Arthrobacter luteus (ALU) sequences were chosen because they are the most common and active repeated elements in the human genome. They typically have a length of 300 nucleotides and make up more than 10% of the genome.[10, 18, 19] *Umetani et al.,*2006, described primers as well as a quantitative PCR method for measuring ALU- 115 and ALU -247, in which the smaller ALU -115 fragments were incorporated into the larger ALU- 247 fragments.[20,21]

Apoptosis is the primary source of cell-free DNA in the blood of healthy individuals, whereas necrosis and apoptosis coexist in cancer patients.[22] As a result, it has been suggested that elevated blood levels of longer DNA fragments (ALU-247) are a useful indicator of the presence of a malignant tumor.[20, 22, 23] As the annealing sites of ALU-115 are located within the annealing sites of ALU-247, the ratio of ALU-247 to ALU-115 is referred to as DNA integrity. Thus, it has been assessed for its diagnostic and prognostic potential role in cancer patients.[24]

Consequently, this study aimed to investigate the role of plasma cf-DNA (ALU-247 and ALU-115) and DNA integrity in peripheral blood of breast cancer patients as a noninvasive marker, and to determine correlations between ALU-247 and ALU-115 integrity, CA15-3 and CEA with each other in breast cancer patients and in different stages of breast cancer.

Subjects and Methods

This study included 100 female who were admitted to the General Surgery Department, Surgical Oncology Department, Assiut University Hospitals and South Egypt Cancer Institute, Assiut University. They were divided into 3 groups. The first group consisted of 20 apparently healthy female who were included as the control group. Their ages ranged from 25 to 70 years. The second group included 20 patients with benign breast lesions, their ages ranged from 25 to 70 years. The third group included 60 patients with breast cancer, their ages ranged from 30 to 70 years. They were divided into two subgroups (3A) with early stages of breast cancer (stage I and II) and subgroup (3B) with advanced breast cancer (stage III and IV). Blood samples were taken from these patients before the initiation of any chemotherapeutic or surgical treatment.

At the hospital, tumors are confirmed histologically and staged according to TNM staging, 7th edition (T, tumor size; N, regional lymph nodes; and M, distant metastasis), American Joint Committee of Cancer staging system.[(25)] Therefore, histological data as well as tumor estrogen, progesterone, and Her-2 receptors were obtained from hospital records.

The laboratory work and interpretation were carried out at the Clinical Pathology Department, Assiut University Hospital. The study protocol was reviewed and ethically approved by the Institutional Review Board of the Faculty of Medicine, Assiut University (approval dated April 2018). An informed consent was taken from each participant, before included in the study. Patients with breast cancer that had received chemotherapy, radiotherapy, or surgical treatment, and patients with history of malignant tumors in other organs were excluded from the study.

From each participant, venous blood sample (10 ml) was withdrawn and divided into several aliquots. The first blood aliquot (2 ml) was placed into tri-sodium citrate coated tube, for assessment of prothrombin time, concentration, and international normalization ratio (INR). They were assessed by using a fully automated coagulation system (Sysmex CS 5100, Siemens Healthineers, USA), according to the manufacturer's instructions. The second blood aliquot (2 ml) was placed into Ethylene-Diamine Tetra Acetic Acid (EDTA) coated tube, for complete blood count which was done by using a fully automated hematology analyzers (Cell Dyn Ruby, Abbott Diagnostics, USA, and ADVIA 2120, Siemens Healthineers, USA), according to the manufacturer's instructions. The third blood aliquot (6 ml) was used for separation of serum. A serum sample (2 ml) was used for routine laboratory investigations. Another serum sample (2 ml) was used for carcinoembryonic antigen (CEA) and cancer antigen 5-3 (CA15-3) measurements. Finally, a serum sample (2 ml) was used for cf-DNA extraction, centrifuged at 123xg for 10 min, and

then serum was carefully transferred into a DNase–free tube for extraction of DNA. Serum was stored at -80 °C until assayed.

Laboratory investigations

Determination of CEA and CA15-3

They were determined by sandwich chemiluminscence immunoassays using an automated analyzer (ADVIA Centaur Auto-Analyzer, Siemens Healthineers, USA), according to the manufacturer's instructions. The reagents included purified polyclonal rabbit anti-CEA or monoclonal mouse anti-CA15-3 antibodies labeled with acridinium ester, monoclonal mouse anti-CEA or anti-CA15-3 antibody covalently coupled to paramagnetic particles, as appropriate. The measuring range for CEA was 0.5–100 ng/ml, and 5-200 U/ml for CA15-3.

Measurement of cf-DNA Concentration and Integrity by Quantitative PCR of ALU Repeats

Absolute quantification of serum cf-DNA was done in the Molecular Biology Unit, Clinical Pathology Department, Assiut University Hospital.

Quantification of ALU-274, ALU -115 sequences

Quantification of ALU-247 and ALU-115 repeats was performed by Real-time PCR. Cf-DNA concentration and integrity in serum samples were examined by measuring ALU repeats (ALU-115 bp, ALU-247 bp). The primer for 115 bp amplicon (ALU-115) amplifies both shorter and longer DNA fragments, while the primer for the 247 bp amplicon (ALU-247) amplifies only longer DNA fragments.

The ALU-115 primers (Catalog Number: 10629186, Invitrogen, Life technologies, Thermo Fisher, Scientific Inc. USA) were: Forward: 5/-CCTGAGGTCAGGAGTTCGAG-3/ and Reverse: 5/-CCCGAGTAGCTGGGATTACA-3/. And the ALU-247 primers (Catalog Number:10629186, Invitrogen, Life technologies, Thermo Fisher, Scientific Inc. USA) were: Forward: 5/-GTGGCTCACGCCTGTAATC-3/ and Reverse: 5/-CAGGCTGGAGTGCAGTGG-3/.

The real-time PCR was achieved using commercial kits, (Catalog Number: 204143, FastStart Universal Quantitect SYBR Green, QIAGEN, USA). The final reaction volume of 25 µl contained 12.5 µl of 1× SYBR Green Master Mix, 0.5 µl of 0.2 µM PCR forward primer, 0.5 µl of 0.2 µM PCR reverse primer, 5 µl DNA template, and 6.5 µl of RNase-free water. The reaction mixture was mixed thoroughly but gently and dispensed into the proper place/ well in the 7500 Fast Real-Time PCR System (Applied Biosystems, USA) after sealing with appropriate provided caps. The real-time PCR amplification was performed with pre-activation of DNA polymerase at 95 °C for 10 min, followed by 40 cycles each of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and then followed by melt curve stage.

The ALU-247 and ALU-115 standard curves of gently prepared genomic DNA obtained from peripheral blood of the control volunteers with serial dilutions from 10 ng to 0.01 pg were used to determine the absolute corresponding concentration of the ALU-247 and ALU-115 in each sample. Finally, the cf-DNA integrity was considered as ALU-247/ALU-115 ratio. The DNA integrity was 1 if template DNA was not truncated and 0 if DNA was completely truncated to fragments smaller than 247 bp.

Statistical analysis

Data were collected and analyzed by using SPSS (Statistical Package for the Social Science, version 20, IBM, and Armonk, New York). The Shapiro test was used to determine compliance of the data to normal distribution. Quantitative data with normal distribution are expressed as mean ± standard deviation (SD) and compared with Student t test and ANOVA. Quantitative data with abnormal distribution are expressed as median (interquartile range) and compared by Mann-Whitney U test and Kruskal Wallis. Nominal data are given as number (n) and percentage (%). Chi^2 test was implemented on such data. Diagnostic accuracy of serum CEA, CA15-3, ALU-115, ALU-247, and DNA integrity in diagnosis of breast cancer and advanced breast cancer was determined by the receiver operator characteristics (ROC) analysis. Level of confidence was kept at 95% and hence, significance was considered at $p< 0.05$.

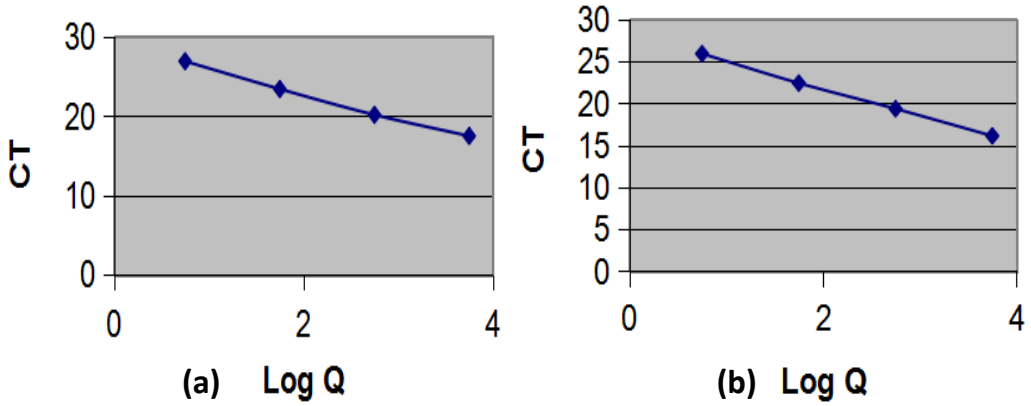

Figure 1. Standard curve for (a) ALU-247 and (b) ALU-115.

Results

Sociodemographic data of the studied groups

Regarding age, marital status, history of pregnancy, history of breastfeeding, menstruation, positive family history and history of oral contraceptive, there were no differences between the different studied groups.

Diagnosis among the 20 patients with benign lesions

Half of these patients had breast abscess 10 (50%). Fibro adenosis and mastitis were present in 4 (20%) and 3 (15%) patients, respectively. In addition, two patients (10%) had duct ectasia and one patient had macromastia (5%).

Characteristics of patients with breast cancer

Of the 60 patients with breast cancer, 8 (13.3%), 27 (45%), 21 (35%) and 4 (6.7%) patients had breast cancer stage I, II, III and IV, respectively. The majority of these patients had T2 stage (51.7%), but 15 (25%) patients T1 stage, and 14 (23.3%) patients T3 stage. While 12 (20%), 23 (38.3%), 15 (25%) and 10 (16.7%) patients had N1, N2 and N3 stage, respectively. It was found that 51 (85%) patients with distant metastasis cannot be assessed, 4 (6.7) patients had no distant metastasis, and 5 (8.3%) patients had distant metastasis. Based on histopathological examination, 53 (88.3%) patients had intraductal carcinoma while 7 (11.7%) patients had intralobular carcinoma. Estrogen, progesterone, and Her-2 receptor were positive in 14 (23.3%), 11 (18.3%) and 4 (6.7%) patients, respectively. Of the 60 breast cancer patients, 4 (6.7%), 47 (78.3%), and 9 (15%) patients had breast cancer grade I, II and III, respectively. Table 1

Table1. Characteristics of the 60 patients with breast cancer.

Studied parameter	No (%)
Stage	
I	8 (13.3%)
II	27 (45%)
III	21 (35%)
IV	4 (6.7%)
T stage	
T1	15 (25%)
T2	31 (51.7%)
T3	14 (23.3%)

Table1. Continued.

Studied parameter	No (%)
N stage	
N0	12 (20%)
N1	23 (38.3%)
N2	15 (25%)
N3	10 (16.7%)
Distant metastasis	
Mx (Distant metastasis cannot be assessed)	51 (85%)
M0 (No distant metastasis.)	4 (6.7%)
M1 Distant metastasis	5 (8.3%)
Grade	
Grade-I	4 (6.7%)
Grade-II	47 (78.3%)
Grade-III	9 (15%)
Histopathological types	
Intraductal carcinoma	53 (88.3%)
Intralobular carcinoma	7 (11.7%)
Positive estrogen receptors	14 (23.3%)
Positive progesterone receptor	11 (18.3%)
Positive Her-2 receptor	4 (6.7%)

Data expressed as frequency (percentage); T describes the size of the tumor and whether it has nearby tissue, N describes regional lymph nodes that are involved, and M describes distant metastasis.

Biomarker level differences between studied groups

Data for CEA, CA15-3, ALU-115, ALU-247, and DNA integrity among studied groups are shown in Table2. There was no difference in CEA was found between the different studied groups (p>0.05). Patients with breast cancer had a statistically significantly higher level of CA15-3 in comparison to the control group (p= 0.032). No difference in CA15-3 was noted among other study groups.

Patients with breast cancer had statistically significantly higher ALU-115 in comparison to the control group (p=0.018). Also, breast cancer group and these with benign breast lesions had statistically significant higher ALU-247 and DNA integrity levels in comparison to the control group (p<0.001 and p=0.009, respectively). Otherwise, no differences were found among the other groups. Table 2

Table 2. Comparison between levels of studied biomarkers among the studied groups.

Biomarkers	Normal controls (n= 20)	Benign breast lesions (n =20)	Breast cancer patients (n= 60)	*p-Value*
CEA (ng/mL)				
Median	1.80	1.60	3.00	NS
Range	(0.8-4.0)	(0.8-8.0)	(0.5-100.0)	
CA15.3 (U/mL)				
Median	17.50	18.00	25.00 *	0.032
Range	(5.9-30.0)	(7.0-90.0)	(5.0-200.0)	
ALU-115 (ng/mL)				
Median	157.00	313.00	351.00 *	0.018
Range	(59-604)	(61-1108)	(13-1789)	

Table 2. Continued.

Biomarkers	Normal controls (n= 20)	Benign breast lesions (n =20)	Breast cancer patients (n= 60)	*p-Value*
ALU-247 (ng/mL)				
Median	13.00	60.00 *	62.50 *	<0.001
Range	(4-83)	(8-330)	(4-1345)	
DNA integrity				
Median	0.11	0.19 *	0.21 *	0.009
Range	(0.01-0.40)	(0.09-0.56)	(0.01-3.84)	

Data are expressed as median (range); Significant difference as compared to the normal control group.
P > 0.05 is not significant (NS).

Levels of studied biomarkers (CEA, CA15-3, ALU-115, ALU-247, and DNA integrity) based on breast cancer stage

Patients with early stage of breast cancer (subgroup 3A) had statistically significant higher CEA and CA15-3 level in comparison to those with benign lesions and control group (*p*=0.041 and *p*=0.030, respectively) (Table 3). Otherwise, no differences in CEA and CA15-3 levels were found among other groups. Also, levels of ALU-115, ALU-247 and DNA integrity were statistically significantly higher in benign breast lesions (group2), early-stage breast cancer patients (subgroup 3A) and advanced breast cancer (subgroup 3B) had level of in comparison to controls (group1) (*p*= 0.018, *p*<0.001 and *p*= 0.019, respectively). Table 3

Table 3. Comparison between levels of studied biomarkers among studied groups based on breast cancer stage.

Variables	Group 1 (n= 20)	Group 2 (n =20)	Group 3A (n= 35)	Group 3B (n =25)	*p-Value*
CEA (ng/mL)					
Median	1.80	1.60	3.00* Δ	2.00	0.041
Range	(0.8-4.0)	(0.8-8.0)	(0.6-100.0)	(0.5-50.0)	
CA15-3 (U/mL)					
Median	17.50	18.00	26.00* Δ	25.00	0.030
Range	(5.9-30.0)	(7.0-90.0)	(6.5-200.0)	(5.0-200.0)	
ALU-115 (ng/mL)					
Median	157.00	313.00*	398.00*	355.00*	0.018
Range	(59-604)	(61-1108)	(61-1755)	(13-1789)	
ALU-247 (ng/mL)					
Median	13.00	60.00 *	65.00*	45.00*	<0.001
Range	(4-83)	(8-330)	(4-575)	(9-1345)	
DNA integrity					
Median	0.11	0.19 *	0.29*	0.21*	0.019
Range	(0.01-0.40)	(0.09-0.56)	(0.01-3.84)	(0.02-2.62)	

Data expressed as median (range) Group 1: Control, Group 2: Benign breast lesions, Group 3: breast cancer; Subgroup 3A: early-stage breast cancer patients; Subgroup 3B: advanced breast cancer. *Significant difference as compared to (group 1) *p*<0.05; Δ Significant difference as compared to (group 2) *p*<0.05.

Diagnostic performance of breast cancer biomarkers for malignant cases

To determine the optimum diagnostic cut-off value and evaluate the sensitivity of circulating plasma serum CEA, serum CA15-3, ALU-115, ALU-247 and DNA integrity for diagnosis of breast cancer (malignant cases vs. study controls) the ROC analysis was performed. The ROC curve was constructed to compare their diagnostic performance, in such a way that the higher area under the curve (AUC) corresponds to a better diagnostic test. Figure 1

The AUC was 0.638, 0.677 for CEA and CA15-3, respectively (p=0.021 and p=0.004, respectively). The AUC was 0.730, 0.848 and 0.710 for ALU-115, ALU-247 and DNA integrity respectively (p<0.001 for all). Table 4

Table 4. Diagnostic criteria of studied tumor biomarkers for prediction of breast cancer.

Indices	Studied biomarkers				
	CEA	CA 15.3	ALU- 115	ALU- 247	DNA integrity
AUC	0.638	0.677	0.730	0.848	0.710
Sensitivity, %	66.8%	70.0%	75.0%	86.7%	70.0%
Specificity, %	55.0%	50.0%	65.0%	75.0%	60.0%
PPV %	81.6%	80.8%	86.5%	91.2%	84.0%
NPV %	35.5%	35.7%	46.4%	65.2%	40.0%
p Value	0.021	0.004	<0.001	<0.001	<0.001

PPV: positive predictive value; NPV: negative predictive value. *$P \leq 0.05$ is significant.

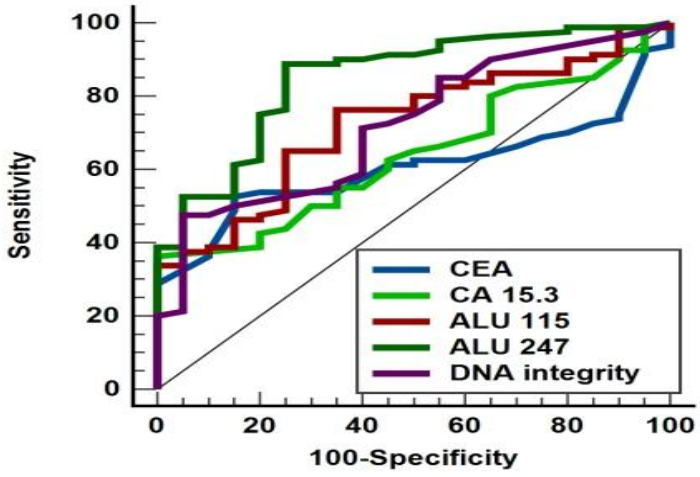

Figure 2. ROC curve for studied tumor biomarkers (CEA, CA15-3, ALU- 115, ALU- 247, and DNA integrity) for prediction of breast cancer versus controls.

Discussion

The present work aimed to evaluate the diagnostic values of cf-DNA and DNA integrity in patients with breast cancer, also to study correlations between cf-DNA, DNA integrity, CA15-3 and CEA with each other in breast cancer patients.

In this study patients with breast cancer had statistically significant higher level of CA15-3 in comparison to the control group (p=0.032). The

results of our study regarding CA15-3 were consistent with these of Rashad et al., 2014, who reported that the mean serum CA15-3 was significantly increased in patients with breast cancer as compared to the control group (p<0.034).[26] Our results were also consistent with those of Al-Hilaly et al., 2017, who reported that the mean serum CA15-3 was significantly higher in patients with breast cancer as compared to control groups (p<0.0001).[27] Also our results are parallel to those reported in the study of Zaleski et al., 2018, who observed that the tumor markers CA15-3 was significantly higher in serum of breast cancer patients as compared with apparently healthy women.[28]

The ALU-247 and ALU-115 are used to distinguish between necrotic cell death as well as apoptotic cells. During apoptosis, DNA fragmentation leads to DNA pieces just about 185–200 bp; the longer ALU-247 is considered a necrotic product, whereas the shorter ALU-115 corresponded to the total amount of DNA. Since necrotic cell death is mainly related to tumor progressive process, so the longer DNA is planned to be a promising marker for cancer.[12, 29]

Also, it was found in our study that patients with early stage of breast cancer had significant higher CEA and CA15-3 in comparison to those with benign lesions and control group (p= 0.041 and p=0.030, respectively). This consistent with Lian et al., 2019, who reported that serum CA15-3 in patients with early stages of breast cancer were higher than those of healthy volunteer group and patients with benign breast diseases (p< 0.05 for each).[30] Similar to these results is the study of *Dolscheid-Pommerich et al., 2017,* who reported that both CA15-3 and CEA were significantly higher in patients with breast cancer compared to patients with benign diseases (p=0.022 and p=0.019, respectively).[31] In contrast to our results as regard CEA and CA15-3, Stötzeret et al., 2013, reported that locally confined tumors could not be distinguished from the control groups of healthy women and from those with benign breast diseases using these markers. However, women with metastatic breast cancer had significantly higher median CEA levels than healthy women, women with benign breast diseases, and patients with locally breast cancer.[32] Moreover, Li et al., 2020, reported that the elevated levels of CA15-3 and CEA were related to the tumor burden and higher levels may indicate vascularization of the tumor with an increased likelihood of occult systemic metastases, elevated CA15-3 and CEA concentration at initial presentation could be predictive of poor breast cancer outcome. Therefore, CA15-3 and CEA may be combined with known prognostic variables for clinical practice in assessing patients' outcomes, and directing treatment modalities in pursuit of better prognoses as well as determining personalized treatments for patients with different molecular subtypes.[33]

As our study revealed that the serum level of both ALU-247 and ALU-115 as well as cf-DNA integrity were statistically significantly higher in breast cancer patients as compared to control group (p=0.018, p<0.001 and p=0.009, respectively). These results agreed with those of a study done by Elhelaly et al., 2020, as DNA integrity showed significant higher median concentration in breast cancer cases in comparison to controls (p<0.001) with no significant difference between benign cases and controls.[16]

Also, these results are in accordance with the study of Adusei et al., 2021, they found that serum levels of both ALU-115 and ALU-247 were elevated in breast cancer patients compared to healthy controls (p=0.028 and p<0.0001, respectively). DNA integrity was higher in breast cancer patients compared to controls (p= 0.522). DNA integrity was lower in healthy individuals, probably due to low necrotic activity in body tissues, thereby lowering the concentration of longer DNA fragments in the blood stream.[15]

In this study, we found that patients with benign lesions, early and advanced stages of breast cancer had statistically significant higher ALU-115, and ALU-247 levels and DNA integrity in comparison to the control group, (p=0.018, p<0.001 and p=0.019, respectively). These results are consistent with the data obtained from a study by Hussein et al., 2019, indicated that ALU-247, ALU-115 expression, and cf-DNA integrity concentration showed a trend to

increase with breast cancer stage, where the mean values of these parameters were significantly higher in breast cancer patients with stages II, III, and IV than healthy subjects (p=0.001, p=0.002 and p=0.009, respectively). These results may be due to the released DNA from tumor cells into the circulation which is elevated by lymph vascular invasion because blood or direct lymphatic flow through the tumor cells allows spreading of viable tumor cells and increases diffusion of DNA released from dead tumor cells into the circulation. Consequently, the circulating cf-DNA may be directly associated with the turnover rate of tumor cells and tumor development, representing biologic tumor aggressiveness. Thus, the circulating cf-DNA integrity may be suitable for monitoring of breast cancer progression.[19] On the other hand, other studies found that cf-DNA levels can be low in cancer patients due to low cell death rates and a low half life time of cf-DNA in the plasma as a result of high DNA clearance.[18] In a study done by Edge et al., 2010, it was found that the mean DNA integrity was lower in breast cancer patients than in the controls though the difference did not reach statistical significance (p>0.05). Moreover, breast cancer patients had significantly higher ALU-115 level than the controls (p = 0.005). In the breast cancer group, the mean ALU-247 value was statistically significantly higher than the control group (p = 0.01). Among the stages, ALU-247 level was significantly lower in stage II than in stage III (p = 0.035).[25]

The receiver operating characteristic curve has been widely used in assessment of diagnostic and prognostic power of diverse markers. The values of plasma ALU-247, ALU-115, cf-DNA integrity, and CA15-3 in diagnosis of breast cancer were evaluated by the ROC curve analysis. The use of the area under the ROC curve was useful in elucidation of the validity of a specific marker in the early detection of breast cancer.

In our study, it was found that ALU-247 had the best diagnostic sensitivity for diagnosis of breast cancer versus control group (86.78%) with 75% specificity at AUC of 0.848 followed by ALU-115 with 75% sensitivity and 65% specificity at AUC of 0.730 followed by the DNA integrity with 70% sensitivity, 60% specificity at AUC of 0.710, followed by CA15-3 with 70% sensitivity and 50% specificity at AUC of 0.677, followed by CEA with 66.8 % sensitivity, 55% specificity at AUC of 0.638. Our results were consistent with *Hussein et al., 2019,* who reported that for plasma ALU-247, AUC =0. 795, p = 0.004; for ALU-115, AUC = 0.782, p = 0.007; for DNA integrity, AUC = 0.825, p = 0.002, while for plasma CA15-3, AUC = 0.980, p < 0.001. These results indicate the validity of using plasma ALU-247, ALU-115, DNA integrity, and plasma CA15-3 as diagnostic markers for breast cancer.[19]

In a study done by *Elhelaly et al.,* 2020, to verify validity of cf-DNA concentration and DNA integrity in discrimination between benign cases and breast cancer cases, the ROC curve was conducted. cf-DNA concentration and DNA integrity showed good (AUC= 0.860) and fair (AUC=0.727) discrimination, respectively between benign cases and breast cancer cases. cf-DNA concentration ≥ 74 ng/ml and DNA integrity ≥ 0.44 were diagnostic of breast cancer with specificity of 90% for both and PPV of 85.3 and 81.5%, respectively; but with lower sensitivity 67.4 and 51.2 %, respectively.[16]

It is concluded that measuring serum level of ALU-247, ALU-115 and DNA integrity was significantly elevated in breast cancer patients compared to patients with benign breast lesions, and healthy controls. Measuring ALU-247, ALU-115 and DNA integrity in peripheral blood could be a promising novel approach for diagnosis and early detection of breast cancer that has the advantages of being convenient, non-invasive, and it may provide new diagnostic information.

Author Contributions

HAA and ZAA, writing, review and editing, methodology, analysis and interpretation of data. MZA, project administration, conception and design of the study and supervision. KMR, methodology, analysis and interpretation of data. TMK, review and editing, clinical evaluation of patients and resources. FMM, data collection writing the original draft, review, editing and practical work.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The protocol of the study was ethically reviewed and approved by the Institutional Review Board of the Faculty of Medicine, Assiut University (approval dated April 2018). (Clinical trial: NCT034740160).

Informed consent

A signed consent form was obtained from each study participant before included in the study.

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