

RESEARCH ARTICLE

Circulating DNA in Egyptian Women with Breast Cancer

Iman Hassan Ibrahim^{1*}, Mahmoud M Kamel², Mohamed Ghareeb³

Abstract

The commonest cancer in Egyptian females occurs in the breast. cfDNA is a non-invasive marker for tumor detection and prognostic assessment in many types of cancer including breast cancer. This study aimed to assess the role of cfDNA and its fragmentation pattern in breast cancer prognosis and treatment response. Forty female patients with malignant breast tumors and a comparable group of healthy blood donors were enrolled prospectively. cfDNA levels and fragmentation patterns were investigated after cfDNA extraction, gel electrophoresis and gel analysis. The percentage of breast cancer patients positive for cfDNA (92.5%) was significantly higher than that of controls (55%). Also, mean concentration of cfDNA was significantly higher than in the control group ($P < 0.05$). Most Her-2 positive patients had long cfDNA fragments, this being significant as compared to Her-2 negative patients ($P < 0.05$). Metastasis was also positively linked to significantly higher cfDNA ($P < 0.05$) and the mean cfDNA integrity index was significantly higher in non-responders compared to treatment responders ($P < 0.05$). In conclusion, both qualitative and quantitative aspects of cfDNA and its different fragments in breast cancer patients could be related to prognosis, metastasis and treatment response. Long cfDNA fragments could be particularly useful for prediction purposes.

Keywords: Breast cancer - circulating DNA - DNA fragments - treatment response

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Introduction

Breast cancer is the most frequent malignancy with high morbidity and mortality among women worldwide accounting for about 25% of female cancers worldwide (Ferlay et al., 2015). Mortality in breast cancer patients (14.7% of female cancer deaths worldwide) is mostly caused by metastasis which is related to poor prognosis of breast cancer patients (Fang et al., 2013). In Egypt, breast cancer is the most common cancer in females accounting for 38.8% of all female cancers (Ibrahim et al., 2014). Carcinogenesis, progression and treatment response of breast cancer are multifactorial processes affected by several genetic, hormonal, environmental factors, and lifestyle (Porter, 2009). Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (hER-2) are among the most important predictive markers in breast cancer. Both ER and PR tests are important in hormonal therapy decision making. HER2 overexpression is associated with high histological grade, occurrence of necrosis and p53 mutation, and it is inversely associated with the expression of hormone receptors (Steinman et al., 2007). Triple-negative breast cancer (TN) is cases that do not express ER, PR or hER-2 accounting for 15-26% of all breast cancer female patients. TN breast cancer cases usually show poor prognosis and poor treatment response (Pal et al., 2011).

Tumor cells release DNA fragments into the circulation,

which can be found in the cell-free fraction of blood together with DNA fragments from normal cells [cell-free DNA (cfDNA)]. Elevated levels of cfDNA are observed in advanced cancer patients, and have been suggested for the diagnosis of breast (Huang et al., 2006) and other cancers (Mead et al., 2011). Levels of cell-free DNA in serum or plasma of cancer patients were reported to be higher than those in healthy controls (Stotzer et al., 2014).

Early diagnosis of breast cancer and accurate identification of hematologic metastasis can improve the success of treatment. The cfDNA concentrations in breast cancer were reported to be higher when compared with healthy individuals and patients with occult tumor or monitoring after surgery (Tangvarasittichai et al., 2015). Both serum levels of cfDNA and its integrity were found to be potential prognostic biomarkers in patients of primary breast cancer (Iqbal et al., 2015), thus could be used to monitor cancer in real time.

DNA fragmentation pattern differs in cancer patients than normal. In healthy individuals, the main source of cfDNA in circulating blood is through apoptosis (mostly short fragments), whereas in cancer patients it results from both apoptosis as well as by necrosis (mostly long fragments). Therefore, elevated levels of longer fragments of DNA in blood could be a good marker for the presence of malignant tumor DNA (Diehl et al., 2008). Thus, DNA integrity (the ratio of longer to shorter cfDNA fragments), has been assessed for its diagnostic and prognostic

¹Biochemistry Department, Faculty of Pharmacy for Girls -Al Azhar University, ²Clinical Pathology Department, ³Department of Medical Oncology, National Cancer Institute, Cairo University, Cairo, Egypt *For correspondence: emanhssn@yahoo.com.

potential in cancer patients (El-Shazly et al., 2010). DNA integrity has been reported to be significantly higher in patients with metastatic breast cancer as compared to patients with locally confined breast cancer and benign controls (Stotzer et al., 2014).

This study aims to assess the potential role of serum cfDNA levels and fragmentation in breast cancer prognosis and treatment response.

Materials and Methods

Ethical approval: The project and data forms were approved by the Regional Research and Ethics Committee at the National Cancer Institute (NCI), Cairo University, Egypt. Written informed consent was obtained from all participants involved in our study.

Subjects: A total number of 40 female patients with de novo malignant breast tumor admitted at the Department of Surgery in National Cancer Institute, Cairo University were enrolled in this study. Patients' age ranged from 28 to 78 (49.77±12.84). Control group were age matched healthy female blood donors.

Clinical examination and treatment: All patients have been subjected to full history, clinical examination and metastatic workup including chest radiograph, abdominal sonar and bone scan. Baseline Echocardiography and CBC, liver and Renal chemistry were required before starting treatment. Biopsy to document invasive breast cancer and to do hormonal and molecular subtypes was done for every patient. Patients with early breast cancer were offered surgery (radical or conservative) followed by adjuvant treatment. Adjuvant chemotherapy - when indicated - included anthracycline based regimen (FAC/FEC) and Taxanes. Adjuvant hormonal therapy included tamoxifen and/or aromatase inhibitors (for postmenopausal). Loco-regional radiotherapy was given for any of T3 lesion, N2 and/or conservative surgery. Patients with advanced breast cancer at presentation were offered neoadjuvant chemotherapy and patients diagnosed as metastatic disease at presentation were offered palliative chemotherapy, radiotherapy and/or hormonal therapy according to their hormonal status, tumor burden, site of metastasis and performance status. Patients were followed up to assess treatment response.

Methods: Five ml peripheral blood sample were collected from each patient and divided into 2 tubes; one for DNA extraction and the other tube for serum separation for biochemical parameters assay following standard laboratory methods. Tests for ER, PR and hER-2 were performed on 10% formalin-fixed paraffin embedded blocks for each patient.

After serum separation, cfDNA was extracted manually from serum using phenol/chloroform/ethanol method in the presence of glycogen (Stirling, 2003). The yield of cfDNA of this method was higher than that obtained by a commercially available kit and the traditional phenol/chloroform/ethanol method without glycogen.

cfDNA fragmentation pattern is visualized by 1.5% agarose gel electrophoresis. Bands of cfDNA fragments were quantified and analysed by GelQuant.NET software (Biochemlabsolutions, University of California, San Francisco, USA) and PyElph (Pavel and Vasile, 2012). cfDNA integrity index is calculated as the ratio of longer to shorter DNA fragments.

Statistical analysis: Data were assessed with GraphPad prism (version 5) software using Student t- test and Z- test. Chi-square test was used to calculate the significance of cfDNA concentration and fragmentation in different studied groups. Results were expressed as means±standard error mean, and p value less than 0.05 was considered statistically significant.

Results

Results of clinical examination and metastatic workup (including chest radiograph, abdominal sonar and bone scan) are summarized in table (1).

Regarding serum cfDNA, 92.5% of breast cancer patients were positive for cfDNA compared to about 55% in healthy controls showed detectable cfDNA, this difference is significant at p<0.05. Analysis of the gel by GelQuant.NET and PyElph software for cfDNA quantification revealed that the mean concentration of cfDNA is significantly higher than that of control group (Table 2, Figure 1).

In breast cancer group, fragmentation pattern of cfDNA showed short fragments (about 200 bp) in 92.5 % of breast cancer patients, this is significantly higher

Table 1. Clinical Data for Malignant Breast Cancer Patients

Menopause	Pre-menopausal	21 (52.5%)		
	Post- menopausal	19 (47.5%)		
Pathology and Grade	IDC	IDC	IDC II+ DCIS III	
	31	5	4	
Site	Left	Right	Bilateral	
	28	11	1	
ER	Positive:	+	++	+++
		11	9	
	Negative		11	
PR	Positive:	+	++	
		16	7	
	Negative		12	
Her2	Positive	7		
	Negative	33		
Lymph node involvement:	Positive	12		
	Negative	28		

Table 2. Percent Positive cfDNA Cases and Mean cfDNA Concentrations in Studied Groups and in Subgroups of Breast Cancer Patients

Group		cfDNA % positive cases	cfDNA (Mean \pm SEM)	cfDNA integrity index (Mean \pm SD)
Breast cancer	Breast cancer	92.5	335 \pm 40.9	-
	Control	55	19.6 \pm 5.4	-
	P-value	p < 0.05	p < 0.05	-
Breast Cancer Patients	Patients with long+short fragments	92.5	448.6 \pm 33.5	0.9 \pm 0.13
	Patients with short fragments only	55	124.1 \pm 54.7	0
		p < 0.05	p < 0.05	p < 0.05

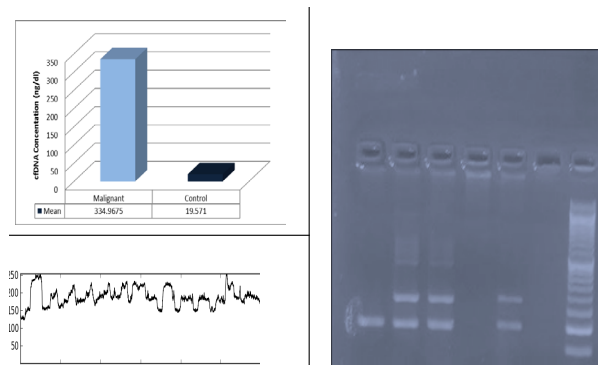


Figure 1. a) Mean Concentrations of cfDNA (in ng/ml) in Malignant and Control Groups (p<0.05). b) cfDNA Band Analysis showing Intensity of cfDNA for some Breast Cancer Cases. c) cfDNA Fragmentation. Lane 1 (left) shows short fragment only (200 bp), lanes 2,3,5 showing both long (400 bp) and short fragments, lanes 4,6 showed no cfDNA bands, and the last lane for DNA marker. Lanes 2,3 showed smears suggesting the presence of longer fragments

than the frequency of long fragments (400 bp) which had been detected in only 55 % of breast cancer patients (p<0.05). Also, mean cfDNA concentration is significantly higher in patients with long cfDNA fragments compared to those with short fragments only (p<0.05) (Table 2). Control group showed only short DNA fragments. Upon calculating the cfDNA integrity index, breast cancer patients showed significantly higher cfDNA integrity index compared to healthy controls (Table 3).

Regarding the disease onset, in pre- and post-menopausal breast cancer patients, DNA integrity showed no significant difference between post-menopausal patients compared to pre-menopausal patients. All cfDNA negative cases were post-menopausal, yet this is statistically non-significant due to small group size.

Patients with metastasis showed significantly higher cfDNA concentrations (mean \pm SEM = 455.5 \pm 41.5) compared to patients with no metastasis (mean \pm SEM = 279.1 \pm 53.1) (p<0.05) (Figure 2). Neither lymph node involvement nor tumor size showed any significant relation to cfDNA concentration or integrity in breast cancer patients.

Her-2 expression analysis showed that about 85% of Her-2 positive cases had long cfDNA compared to about 36% of Her-2 negative patients (P<0.05) (Figure 3).

In triple negative (TN) breast cancer cases, 85.7% of them were positive for cfDNA, 42.8 % of them showed long DNA fragments. There was marked but statistically

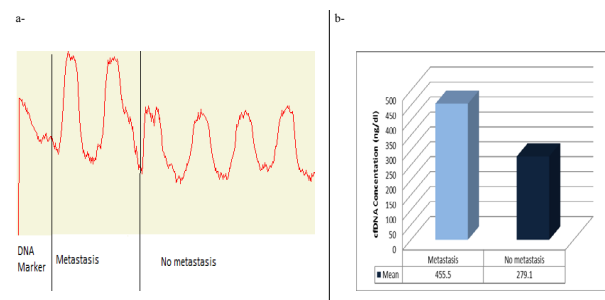


Figure 2. a) cfDNA % Intensity Higher in Patients with Metastatic Breast Cancer. b) Mean cfDNA Concentrations in Breast Cancer Patients with and without Metastasis

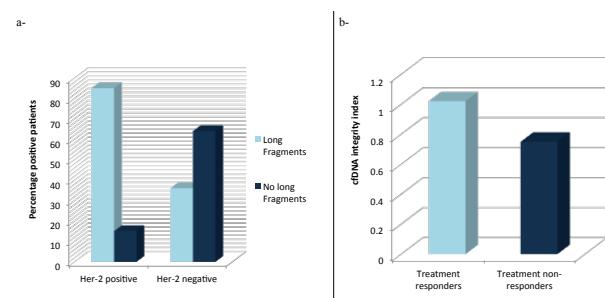


Figure 3. a) Percentage Positive Patients for cfDNA Long Fragments in Her-2 Positive and Negative Patients. b) Mean cfDNA Integrity Index in Breast Cancer Treatment Responders and Non-Responders

non-significant difference between cfDNA concentrations in these patients compared to other breast cancer patients.

All treatment non-responders were positive to cfDNA compared to 91.2 % in treatment responders. All treatment non-responders showed long DNA fragments compared to 40 % in treatment responders (P<0.05). Mean cfDNA integrity index is significantly higher in non-responders (mean \pm SD = 1.02 \pm 0.2) compared to treatment responders (mean \pm SD = 0.75 \pm 0.18) (P<0.05) (Figure 3).

Discussion

Breast cancer is one of the major health problems for females worldwide. Several factors affect tumor behaviour, progression and treatment response (von Minckwitz et al., 2011). In Egypt, breast cancer comes on top of all malignancies with poor outcome compared to international figures (Ibrahim et al., 2014). Age at diagnosis of breast cancer is about 10 years younger in Arab countries than that in Western countries (El Saghir

Breast cancer is associated with different genetic and epigenetic events, such as DNA strand integrity, gene amplifications, gene mutations, DNA methylation, and microsatellite abnormalities. These alterations detected in the primary tumor may also be found in plasma/serum cfDNA of patients with breast cancer (Hashad et al., 2012). cfDNA levels in serum/plasma seem to be an interesting universal marker of malignancy, and numerous studies have been performed to evaluate its value in several tumour entities. cfDNA is of apoptotic or necrotic origin. Apoptotic cfDNA is fragmented into 180-200 bp, whereas cfDNA from necrotic origin is of higher molecular weight (Ellinger et al., 2008). It was previously reported that malignancy of the tumor leads to a higher degree of necrosis, corresponding to an increase in circulating tumor DNA. It was suggested that DNA fragments found in the circulation are derived from necrotic neoplastic cells that had been engulfed by macrophages (Diehl et al., 2008). Later, Lo et al. (2010) provided another explanation of cfDNA fragmentation as they reported that plasma DNA molecules showed a predictable fragmentation pattern due to nuclease activity which had been related to the progression of several cancers.

The current study utilized direct visualization method of cfDNA. Generally cfDNA is present in relatively low serum concentrations. Different cfDNA quantification methods, including spectrophotometric methods, fluorescent dyes, or quantitative PCR-based methods produce different results because these measurements target either total or only amplifiable DNA (Devonshire et al., 2014). Also, extraction of cfDNA fragments from gel might lead to loss of some fragments (Pavel AB and Vasile, 2012). Thus, cfDNA fragment analysis software including GelQuant. Net and PyElph directly from the gel could provide convenient method for determination of DNA band intensity and fragmentation pattern among other uses (Hares et al., 2015).

In the current study, the mean cfDNA concentration of healthy controls is 19.6 ± 5.4 which is consistent with results of previous studies (Fleischhacker and Schmidt, 2007), the mean cfDNA concentration of breast cancer patients was significantly higher. These results are similar to those of Hashad et al. (2012) who reported that cfDNA levels in breast cancer patients are significantly higher than in women with benign lesions and in control groups. Also, a more recent study suggested that circulating cfDNA provides a better overall representation of the malignant disease and could be a reliable source of diagnostic DNA, which could replace the use of tumor tissue in a diagnostic setting (Kuo et al., 2014). High levels of cfDNA were reported to be associated with worse survival in solid tumors (Ocana et al., 2016).

Regarding cfDNA fragmentation in breast cancer patients, 55% of breast cancer patients had both short and long fragments. This might be caused by high rates of necrosis in these patients. It was noticed that these patients have also significantly higher mean serum cfDNA compared to patients with short fragments only. Mean serum cfDNA integrity index was significantly higher in breast cancer patients compared to controls. These

results are similar to those reported previously that mean cfDNA integrity was significantly higher in patients with breast cancer patients than in healthy women and was associated with lympho-vascular invasion, lymph node metastasis, and tumor size (Stotzer et al., 2014). Also, Leszinski et al. (2014) reported similar results in colorectal cancer. On the contrary, another study reported decreased cfDNA integrity index in breast cancer patients. They explained this contradiction by different primer sets used in detecting long and short fragments in PCR-based methods (Madhavan et al., 2014). This might give a great advantage by direct visualization techniques of cfDNA analysis to avoid this conflict. Combination of direct visualisation and PCR-based method could be useful (Mouliere et al., 2015).

Patients with metastasis showed significantly higher mean serum cfDNA compared to patients without metastasis. Previously, a significant correlation was reported between cfDNA and metastatic breast cancer utilizing different techniques (Agostini et al., 2012; Dawson et al., 2013). In the current study, cfDNA didn't correlate with tumor size. Previously, Mouliere et al. (2011) reported that size of the tumors did not significantly correlate with the concentration of detectable cfDNA.

Upon analysing cfDNA status in ER, PR, Her-2 positive and negative cases, long cfDNA fragments are detected in significantly higher percentage patients with positive Her-2 compared to Her-2 negative cases. This could be explained by the reported correlation of HER2 signaling with several clinical and therapeutic implications including tumor growth, tumor cell migration, and necrosis (Curigliano et al., 2015). Long cfDNA are usually of necrotic origin.

TN group shows marked (although statistically non-significant due to small number) increase in cfDNA serum levels and integrity index.

Non-responders to treatment showed significantly higher mean serum cfDNA integrity index compared to responders. These results are in consistency with previous studies reporting poor prognosis of cancer patients related to cfDNA levels and integrity (Ocana et al., 2016).

In conclusion, both qualitative and quantitative assay of cfDNA and its different fragments in breast cancer patients could be related to patients' prognosis, metastasis and treatment response. Direct visualization of cfDNA fragments via gel electrophoresis and gel image quantification using suitable software could serve as a tool to monitor and predict tumor prognosis and treatment response in breast cancer patients.

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