

Detection of Cell-Free DNA In the Blood of Breast Cancer Patients in an Egyptian Hospital by Real Time PCR

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Abstract: Introduction: In females the most common cancer is Breast cancer with high mortality rate so there is a strong need for early noninvasive method for diagnosis, apoptotic tumor cells may release circulating cell free DNA (CCFDNA) into the peripheral blood and detection of this released CCFDNA in blood of cancer patients may be useful in early cancer diagnosis. **Subjects and Methods:** The present study included 80 female patients and 20 healthy controls from Clinical Oncology Department, Faculty of Medicine, and Tanta University and for all subjects CCFDNA was measured by Real Time PCR. **Results:** Our study showed that CCFDNA increased with breast cancer patients when compared to patients with benign breast lesions and control groups and these differences were statistically significant, and detection of CCFDNA in breast cancer patient's blood has the highest sensitivity and specificity when compared to CEA and CA 15.3. **Conclusion:** The blood levels of CFDNA were significantly increased in patients with breast cancer compared with those of patients with benign breast lesions and healthy controls. Furthermore CFDNA levels were observed to increase as breast cancer progressed to later disease stages thus the quantitative detection of CFDNA may possess value for early detection of breast cancer.

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Keywords: CCFDNA, breast cancer, Real Time PCR, Tumor marker; Detection; Cell-Free; DNA; Blood; Breast; Cancer; Patient; Egyptian; Hospital;

Abbreviations: Circulating cell free DNA (CCFDNA), Polymerase chain reaction (PCR), Carcinoembryonic antigen (CEA), Cancerantigen15-3 (CA15-3), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

1. Introduction

In females the most common cancer is Breast cancer and it is considered the second most common cancer worldwide (1). And also The International Agency for Research on Cancer reported the same finding of (1), (2) and (3) reported that cancer breast is the most common cause of cancer death in females, the rate of breast cancer is higher In Egypt than the records of the worldwide representing about 32.04% of cancers in females (3).

Early breast cancer detection by many diagnostic tools such as breast mammography or ultrasound may be useful in detection of breast cancer (4). But, several limitations for these tools are present such as radiation exposure (5).

Two of the most widely investigated tumor markers in breast cancer were Serum carcinoembryonic antigen (CEA) and cancer antigen 15-3(CA15-3), however CEA and CA15-3 were of limited use in the early breast cancer diagnosis due to a lack of their sensitivity and specificity (6).

Circulating cell free DNA (CCFDNA) molecules were first identified in 1948, subsequent investigations revealed CCFDNA to be present in higher levels among patients with autoimmune diseases and cancer as compared with healthy individuals (7).

CCFDNA is formed of extracellular nucleic acids present in humans plasma or serum, it has several other names such as circulating acids, extracellular nucleic acids or cell-free nucleic acids (8), the concentration of CCFDNA is very low In healthy Individuals since most of them removed efficiently from circulation by phagocytes (9). However apoptotic tumor cells may release CCFDNA into the peripheral blood and detection of this released CCFDNA in blood of cancer patient may be useful in early cancer diagnosis. (10).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most common housekeeping genes commonly used in comparisons of gene expression data and in molecular biology. (11).

It is a member of the single-copy gene family in the human genome and is characterized by low gene

amplification or deletion mutation probability in various types of cancer occurrence. Thus, GAPDH is a good candidate to quantify the free DNA content released from cancers and to reflect the tumor burden. (12).

2. Subjects and Methods

Our study was carried out at Microbiology and Immunology Department, Clinical Pathology Department, Department of Medical Biochemistry and Molecular Biology and Clinical Oncology Department Faculty of Medicine, Tanta University. The patients were selected from the Out-patient Clinics of oncology in Tanta University Hospital. This study was conducted on 80 female patients in addition to 20 apparently healthy female as a control group. They were divided into three groups: Group (1) was included 60 newly diagnosed breast cancer females, their ages ranged from 36 to 70 years, group (2) was included 20 females patients with benign breast lesions, their ages ranged from 33 to 56 years. And group (3) was included 20 apparently healthy females as a control group, their age ranged between 32-65 years. Written informed consents were obtained from all subjects before enrollment into the study. Inclusion criteria included were newly diagnosed breast cancer patients with no prior surgery; no chemotherapy had to be initiated after diagnosis. Exclusion criteria were autoimmune diseases, acute or severe chronic liver disease, acute inflammatory diseases, hematologic diseases, other malignancy, connective tissue diseases, Myocardial infarction, and Pregnancy. For all included subjects the following were fulfilled: complete history was tacked and were examined clinically, imaging techniques for patients in the form of (Breast mammography for benign & malignant breast lesions, Chest X-ray for cancer patients, Liver and bone scan for cancer patients to exclude metastasis), histopathological study and grading for breast lesions, Laboratory investigation were done for all cases in the form of Routine laboratory investigations in the form of CBC, Liver functions (AST & ALT, Kidney functions (Serum creatinine & Urea) and Specific laboratory investigations in the form of Tumor markets (CEA & CA 15-3), and Estimation of cell free DNA in plasma using real time PCR.

- Sample collection and Preparation:

10 ml venous blood were collected from all subjects included first part in plain vacutainer tube left to clot at 37o C. Sera were separated by centrifugation and used for immediate assay of liver, kidney functions & tumor markers (CEA & CA15-3). The second part was collected on EDTA tube for CBC. The third part was transferred into another (EDTA) tube, and then centrifuged for 10 minutes at 4000 r.p.m. The plasma was transferred to new eppendorf tubes and centrifuged again at maximum speed (16.000g) for 10 minutes to remove cellular DNA completely from the plasma fraction. Then DNA was extracted for estimation of cell frees DNA & kept at-20C until the time of analysis.

-Analysis of cell frees DNA by real time PCR:

a) DNA was extracted from Peripheral blood by DNA extraction kits (QIAamp DNA Blood Mini Kits from Clinilab co.).

b) DNA was Amplified and Detectedby Quanti Tect Probe PCR Kit from Clinilab co.:

GAPDH gene in CFDNA was determined by real-time detection polymerase chain reaction, the Quanti Tect Probe PCR Kit and the ABI 7500 Real Time PCR System (Applied Biosystems, USA) BY fluorescent labeled probes were used.

Principle:

Quanti Tect Probe PCR Kits have master mix which was an optimized, ready-to-use for highly specific and sensitive real-time Quantification of gDNA and cDNA targets by sequence- specific probes. The kits were designed for use with all types of sequence-specific probes, included hydrolysis probe selection (e.g., taqMan® and other dual-labeled probes), FRET probes, and Molecular Beacons. Quanti Tect Probe PCR Kits contained a unique PCR buffer that contained a balanced combination of K⁺ and NH₄⁺ions, which promote specific primer annealing, enabling high PCR specificity and sensitivity. In addition, Hot Star TaqDNA Polymerase provides a stringent hot start, prevented the formation of nonspecific products. Quanti Tect probe PCR Master Mix also contained dUTP, starting PCR, enabled pretreatment with uracil-N-glycosylase (UNG) prior to starting PCR, which ensured any contaminating PCR products do not affect subsequent PCR reactions.

Table (1): Sequences of primer and probe in GAPDH.

Gene	Sequences of Primers and probes (5' - 3')		Length of Primer/Probe	Amplicon Lengths (bp)
<i>GAPDH</i>	Forward	5-GGAAGGTGAAGGTCCGAGTC-3	20	97
	Reverse	5-GAAGATGGTGTATGGGATTTC-3	20	
	Probe	5-FAM CAAGCTTCCCGTTCTCAGCC TAMRA-3	20	

The results were expressed by cycle threshold which was inversely proportional to CFDNA concentration.

3. Results

This study was conducted on 80 female patients in addition to 20 apparently healthy female as a control group. They were divided into three groups: Group (1) was included 60 newly diagnosed breast cancer females, their ages ranged from 36 to 70 years, group (2) was included 20 females patients with benign breast lesions, their ages ranged from 33 to 56 years, and group (3) was included 20 apparently healthy females as a control group, their age ranged between 32-65 years. According to histopathological examination of the study population included 60 patients with breast cancer were included 42 cases of invasive duct carcinoma, 8 cases of invasive lobular carcinoma, 7 cases of adenocarcinoma and 3 cases of

mixed invasive duct and lobular carcinoma. The benign breast group included 20 cases with lesions 16 fibroadenomas and 4 granulomatous mastitis. As shown in table 2 there was statistically significant difference between breast cancer group and control group and between breast cancer group and benign group (p values were significant) but not statistically significant between benign group and control group as regard CFDNA (cycle threshold) p value was non-significant. As shown in table 3 and 4, figure 1 and 2 there was significant correlation between CFDNA and breast cancer grade and stage in which it was high early from the first stage and grade of breast cancer and increase by the progress of the cancer. As shown in table 5 and figure 3 detection of CFDNA by real time PCR was have the highest sensitivity and specificity when compared to CEA and CA.15.3, in which the sensitivity was 88% and specificity was 90%.

Table (2): Statistical comparison of CEA, CA15.3 and CFDNA between studied groups.

		Group1 (n=60)	Group2 (n=20)	Group3 (n20)	P value	
CEA (ng/ml)	Range	1.15 – 112.3	1.1 – 22.3	1.12 – 4.85	0.001*	P1: 0.129
	Median	8.61	7.28	1.45		P2: 0.001*
CA15.3(u/ml)	Range	12.2 – 400	11.5 – 41	3.5 – 20	0.001*	P1: 0.005*
	Median	72.25	29.40	17.51		P2: 0.001*
CFDNA (cycle threshold)	Range	24.7 – 38.4	40.10 – 43	40.52 – 45.5	0.001*	P1: 0.001*
	Mean ± S. D	33.58 ± 3.04	41.49 ± 0.91	43.05 ± 1.75		P2: 0.001*

P1: p value comparing between breast cancer and benign P2: p value comparing between breast cancer and control P3: p value comparing between benign and control *statistically significant p value less than or equal to 0.05

Table (3): correlation between CFDNA (cycle threshold) and grade, stage of breast cancer.

	CFDNA	
	r.	p
Breast cancer grade	- 0.760	0.001*
Breast cancer stage	- 0.746	0.001*

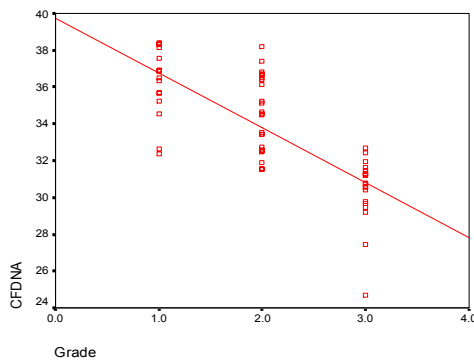


Figure (1): correlation between CFDNA (cycle threshold) and grade of breast cancer.

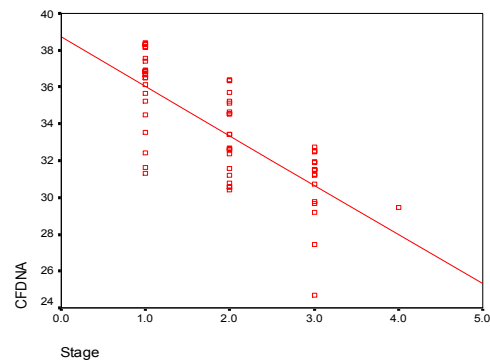


Figure (2): correlation between CFDNA (cycle threshold) and stage of breast cancer.

Table (4): CFDNA in different breast cancer grades and stages.

	N	Range	Mean	± S. D	f. test	p. value
Tumor Grade	I	16 32.35 – 38.4	36.27	± 1.87	44.346	0.001*
	II	24 31.5 – 38.21	34.43	± 2.07		
	III	20 24.7 – 32.68	30.42	± 1.81		
Tumor Stage	I	23 31.29 – 38.4	36.12	± 2.10	23.868	0.001*
	II	20 30.4 – 36.4	33.24	± 1.99		
	III	16 24.7 – 32.75	30.63	± 2.11		
	IV	1 29.45	29.45	± 0		

N= Number

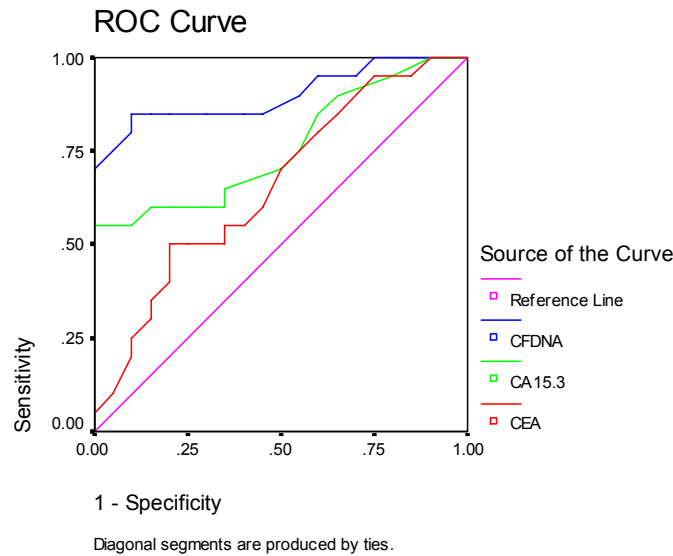


Figure (3): ROC curve for plasma CEA, CA15.3 and CFDNA

Table (5): comparison between CEA, CA15.3 and CFDNA as regard sensitivity and specificity.

	Sensitivity	Specificity	PPV	NPV	Accuracy
CEA	63%	57%	74%	43%	61%
CA15.3	72%	53%	75%	48%	65%
CFDNA	88%	90%	94%	80%	89%

4. Discussion

A few non-invasive biomarkers are present for screening, diagnosis and follow up of patients with breast cancer (13). So there is a strong need for new noninvasive method for early detection and monitoring of breast cancer patients. Our study aimed to detect CFDNA in breast cancer patient blood to be used as noninvasive method for breast cancer diagnosis and follow up. Our study showed that there was statistically significant difference between breast cancer group and control group and between breast cancer group and benign group but not statistically significant between benign group and control group as regard CFDNA (cycle threshold) and this was in

agreement with (14) and (15) a possible reason may be duo to CFDNA was released from cancer cells only and was not found in hyperplasia samples and control samples and this was reported by (16), moreover in the study of (10) and (17) they found that elevated CFDNA levels in patients with breast cancer before surgery and return to normal levels in post-surgery patients. also our study revealed that there was significant correlation between CFDNA and breast cancer grade and stage in which it was high early from the first stage and grade of breast cancer and increase by the progress of the cancer and this was in agreement with (18) and (19) and they referred this elevation to more necrosis occurring in late tumor

stages than early stages. Our study showed that detection of CFDNA by real time PCR was have the highest sensitivity and specificity when compared to CEA and CA.15.3, and this was in agreement with (20) and (2).

Conclusion

The blood levels of CFDNA were significantly increased in patients with breast cancer compared with those of patients with benign breast lesions and healthy controls. Furthermore CFDNA levels were observed to increase as breast cancer progressed to later disease stages thus the quantitative detection of CFDNA may possess value for early detection of breast cancer.

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