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Clinical Significance of Circulating Cell-Free Mitochondrial DNA in Breast Cancer Patients

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ABSTRACT

Background: Breast cancer is one of the most frequent cancers and the leading cause of death globally. In Egypt, it accounts for 18% of all cancer cases.

Objective: In order to assess its clinical value as an early diagnostic marker, the current study examined circulating cell-free mitochondrial DNA (Mt.DNA) detected by RT-PCR in breast cancer patient samples.

Subjects and Methods: 25 breast cancer patients and 15 patients with benign masses participated in this study. Patients were selected from Ain-Shams University Hospitals' outpatient clinics and surgery department. The results of these patients were compared with a control group of 10 age-matched healthy persons. For the purposes of determining CEA, CA 15.3, and Mt.DNA, blood samples were obtained.

Results: The combined use of Mt.DNA and CA15.3 or CEA, raised the diagnostic sensitivity in discriminating patients with breast cancer from non-cancer patients to 100%, with 96% specificity, 96% PPV, 100% NPV and 98% efficacy.

Conclusion: Our research showed that, as compared to women with benign breast illnesses and healthy controls, breast cancer patients have considerably reduced Mt. DNA levels. Mt. DNA levels were also much lower in stage I breast cancer than in benign conditions and healthy individuals, suggesting its potential for use as an early marker.

Keywords: Cancer breast, Mitochondrial DNA, Tumor marker.

INTRODUCTION

With more than 2 million cases in 2020, breast cancer will be the most frequently diagnosed malignancy in females around the world. 18% of cancer cases are in Egypt [(1)]. However, early diagnosis allows for better treatment options, more successful follow-up, and ultimately a better prognosis for the patients [(2)].

Serum biomarkers are used to diagnose breast cancer. However, they are unreliable for the detection of breast cancer in terms of sensitivity and negative predictive values [(3)]. Numerous studies have shown that tumor cells reduce mitochondrial activity to change their metabolism in response to their environment [(4)]. There have been reports of point mutations, substantial deletions, and changes in copy number in a number of cancer forms, among other Mt.DNA modifications [(5)]. Reduced Mt.DNA has been correlated to malignancies of the kidney, breast, ovary, and liver [(6)].

Scientists are interested in Mt.DNA because the alterations in the Mt.DNA could be used as a sensitive early biomarker for the non-invasive detection of a variety of solid cancers, including breast cancer [(7)].

This work was to study circulating cell-free mitochondrial DNA (Mt.DNA) analyzed by q rt-PCR in a group of breast cancer patients to evaluate its clinical importance as an early diagnostic marker. This might make early intervention possible and affect the therapy regimens.

SUBJECTS AND METHODS

Twenty-five (25) female patients with breast cancer and fifteen (15) female patients with benign breast masses participated in this study. Patients were gathered from Ain-Shams University Hospitals' outpatient clinics and surgery department. The outcomes of these patients were contrasted with a control group of ten (10) age-matched, seemingly healthy persons. After receiving oral consent, everyone agreed to participate voluntarily.

A) Group I (Breast Cancer Cases):

Group I consisted of twenty-five (25) female patients with breast cancer. They were diagnosed by histopathology. They ranged between 39 and 67 years (median age 53 years, Interquartile range (IQR) 43.5-58.5 years). According to TNM staging, they were subdivided into:

1. **Stage I: (n=7).** This group included 7 cancer cases. They ranged between 39 and 53 years (median age 43 years, IQR 40-51 years).
2. **Stage II: (n=12).** This group included 12 cancer cases. They ranged between 45 and 67 years (median age 50 years, IQR 47-60 years).
3. **Stage III: (n=6).** This group included 6 cancer cases. They ranged between 48 and 52 years (median age 50 years, IQR 48-51 years).

B) Group II (Pathological Control Patients): This group included fifteen 15 age-matched female patients serving as a pathological control group. They ranged between 35 and 51 years (median age 43 years, IQR 37-48 years). These patients had benign diseases as fibroadenoma, ductal epithelial hyperplasia and fibrocystic disease of the breast.

C) Group III (Healthy Controls): This group included ten 10 healthy females serving as a healthy control group. They ranged between 40 and 65 years (median age of 52.5 years, IQR 47-56 years).

Ethical Consideration:

Each participant provided written informed consent, which is collected after the project was given ethical board approval at Ain Shams

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University. The Declaration of Helsinki, the World Medical Association's code of ethics for studies that involve humans, regulated the implementation of this work.

Analytical Methods

Assay of plasma cell-free mitochondrial DNA was carried out by real-time PCR technique by QIAamp DNA Blood Mini Kit supplied from (Qiagen Incorporations, 28159 Avenue, Stanford Valencia. CA91355, USA). The Detection of the gene was carried out by STATAGENE (Mx3005P) supplied by (Applied Biosystem, 850 Lincoln Centre Drive, Foster City, California, 94404, USA).

Results were reported in relative amplification. It is based on the expression level of a target gene; Mt.DNA sequence of ATP 8 gene versus the expression level of an internal control gene which is considered a reference gene (GAPDH). This expression was detected through determination of TC for both genes at a constant level of fluorescence. Then, ΔCT value for each sample was calculated by the difference between the CT value of the target gene and the CT of the reference gene. Finally, the level of the target gene was calculated using the formula of 2 ΔCT.

Statistical Methods

In order to conduct the statistical analysis, the IBM SPSS software package, version (V. 20.0, IBM Corp., USA, 2011) was used. For quantitatively skewed data, the median and interquartile range were used. Mann-Whitney U test for skewed data was used to compare each pair of groups. When comparing statistically more than two sets of data, the Kruskal-Wallis test was used if one or both of the data sets have skewed distributions. The receiver operating characteristic curve analysis was used to assess the diagnostic performance of the studied parameters. P value < 0.05 was considered significant.

RESULTS

Tables (1-9) and Figures (1-3) show the results of the study.

Descriptive data of various studied parameters in breast cancer patients (Group A), pathological controls (Group B) and healthy controls (Group C) are shown in **Table (1).** In addition, levels of Mt.DNA in different studied groups are shown in **Figure (1).**

Table (1): Descriptive Statistics of Various Studied Parameters in Breast Cancer Patients (Group A), Pathological Controls (Group B) and Healthy Controls (Group C)

Parameters	Group A (n= 25) M (Q1-Q3)	Group B (n= 15) M (Q1-Q3)	Group C (n= 10) M (Q1-Q3)
Age (Years)	50 (44.5-56.5)	46 (43-50)	51 (47-56)
Positive mammogram findings (n%)	80%	20%	-
CA 15.3 (U/mL)	18 (11.5-36.9)	11 (6.5-15.9)	9.3 (7.0-12.3)
CEA(ng/mL)	22.7 (14.4-35.4)	3.3 (2.5-5.2)	3.3 (2.5-5.2)
Mt. DNA ($2^{\Delta CT}$)	0.11 (0.06-0.28)	0.39 (0.18-0.97)	0.72 (0.18-2.84)

M: Median; Q1:25th percentile; Q3:75thpercentile, Group A: Breast cancer patients, Group B: Pathological controls, Group C: Healthy controls.

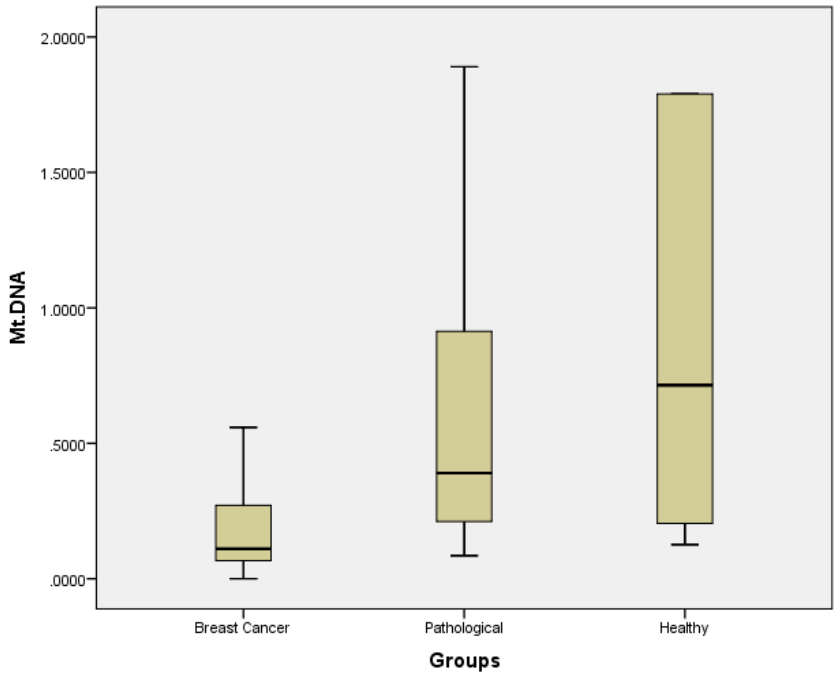


Figure (1): Levels of Mt. DNA in the various studied groups

A significant difference in CA15.3, CEA, and Mt.DNA among different groups was found using the Kruskall-Wallis Test, as shown in **Table (2)** (H= 10.7, 26.6, and 15.3, respectively, P 0.01). Between the several study groups, there was no significant difference in age (H= 1.7, P > 0.05).

Table (2): Comparative Statistics of the Various Studied Parameters among the Various Studied Groups using Kruskall-Wallis Test

Parameters	Group A (n= 25) M (Q1-Q3)	Group B (n= 15) M (Q1-Q3)	Group C (n= 10) M (Q1-Q3)	H	P
Age (Years)	53 (47.5-50)	46(43-50)	51(47-56)	1.7	> 0.05
CA15.3(U/mL)	18 (11.5-36.9)	11 (6.5-15.9)	9.3 (7.0-12.3)	10.7	< 0.01
CEA(ng/mL)	22.7 (14.4-35.4)	3.3 (2.5-5.2)	3.3 (2.5-5.2)	26.6	< 0.01
Mt.DNA($2^{\Delta CT}$)	0.11 (0.06-0.28)	0.39 (0.18-0.97)	0.72 (0.19-2.84)	15.3	< 0.01

M: Median; Q1:25th percentile; Q3:75th percentile, Group A: Breast cancer patients, Group B: Pathological controls, Group C: Healthy controls. P < 0.01: Highly significant difference, P>0.05: Non-significant difference.

Comparative statistics of the various researched parameters in the various studied groups are shown in **Table 3** using the Wilcoxon Rank Sum Test. When compared to both pathological and healthy control groups, breast cancer patients had significantly higher levels of both CA15.3 and CEA (Z= 2.6, 2.7, 2.8, and 4.4, respectively; P 0.01). However, when compared to both pathological and healthy control participants, breast cancer patients had significantly lower amounts of Mt.DNA (Z=-3.1, -3.2, respectively, P 0.01).

However, there was no difference in the values of the various parameters under investigation between the diseased control group and the healthy control groups (Z= 0.5, 2.8, and -0.3, respectively, P > 0.05).

TNM staging was used to group breast cancer patients into three stages: stage I (n = 7), stage II (n = 12), and stage III (n = 6). In **Figure (2)**, the median values of Mt.DNA at various phases are displayed.

Table (3): Comparative Statistics of the Various Studied Parameters in the Various Studied Groups as compared to each other using Wilcoxon Rank Sum Test

Parameters	Group A Versus Group B		Group A Versus Group C		Group B Versus Group C	
	Z	p	Z	p	Z	p
CA15.3(U/mL)	2.6	< 0.01	2.7	< 0.01	0.5	>0.05
CEA(ng/mL)	2.8	< 0.01	4.4	< 0.01	2.8	>0.05
Mt.DNA ($2^{\Delta CT}$)	-3.1	< 0.01	-3.2	< 0.01	-0.3	>0.05

Group A: Breast cancer patients, Group B: Pathological controls, Group C: Healthy controls.
P < 0.01: Highly significant difference. P>0.05: Non-significant difference

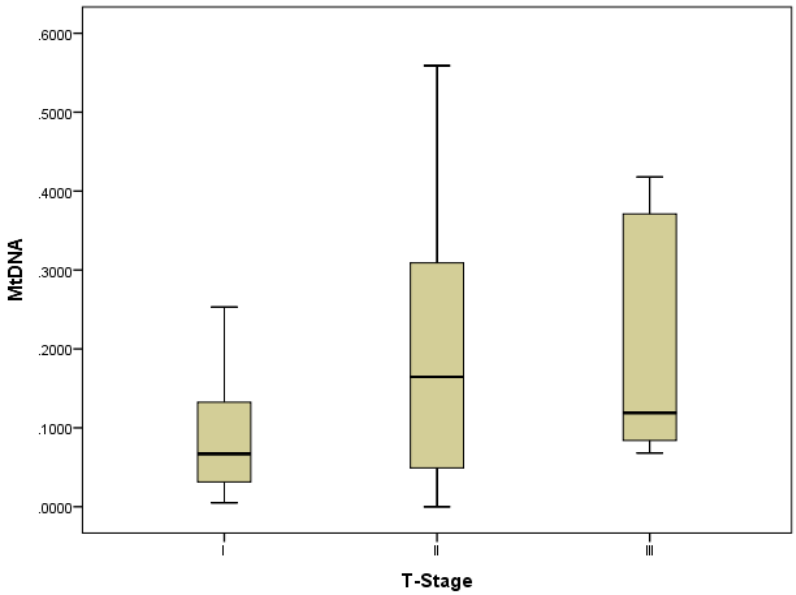


Figure (2): Mt. DNA levels in various stages of breast cancer

Statistical comparison between the different studied parameters in different stages of breast cancer using Kruskal-Wallis are shown in **Table (4).** This revealed a highly significant difference in both CA15.3 and CEA in all stages of breast cancer (H= 17.9, 9.8, P < 0.001, respectively) and no statistically significant difference in Mt.DNA was recorded between different stages of breast cancer (H= 1.7, P > 0.05).

Table (4): Statistical Comparison of CA15.3, CEA and Mt. DNA among the Different Stages of Breast Cancer Patients using Kruskall-Wallis Test

Parameters	Stage I (n=7) M(Q1-Q3)	Stage II (n=12) M(Q1-Q3)	Stage III (n=6) M(Q1-Q3)	H	P
CA15.3(U/mL)	9.6 (7.2-12)	18.6 (14.3-21.1)	47 (42.1-51.1)	17.9	<0.001
CEA(ng/mL)	16.9 (7.9-19.3)	23.8 (18.2-31.4)	69 (31.8-75.5)	9.8	<0.001
Mt.DNA($2^{\Delta CT}$)	0.067 (0.006-0.14)	0.16 (0.03- 0.32)	0.119 (0.08-0.38)	1.7	>0.05

M: Median; Q1:25th percentile, Q3:75th percentile
P>0.05: Non-significant difference, p<0.001: Highly significant difference.

Table (5) provides a statistical comparison between CA15.3 and CEA at various phases using the Wilcoxon Rank Sum Test. According to this, there was a highly significant difference in the levels of both CA15.3 and CEA between stage I and stage II as well as between stage I and stage III (Z= -2, -2.1, -3, and -2.4, respectively, P 0.01). In contrast, neither parameter nor the comparison of stage II to stage III revealed a significant difference (Z= -3.2, -2.2, respectively, P > 0.05).

Table (5): Comparative Statistics of CA15.3 and CEA in Different Stages using Wilcoxon Rank Sum Test

Parameters	Stage I Versus Stage II		Stage I Versus Stage III		Stage II Versus Stage III	
	Z	**p**	**Z**	**p**	**Z**	**p**
CA15.3(U/mL)	-2	< 0.01	-3	< 0.01	-3.2	>0.05
CEA (ng/mL)	-2.1	< 0.01	-2.4	< 0.01	-2.2	>0.05

P>0.05: Non-significant difference, p<0.01: Highly significant difference.

Statistical comparison of different studied parameters in stage I breast cancer versus pathological and healthy controls showed that the only parameter which is significantly different in breast cancer patients from both control groups is Mt.DNA (Z= -2.89, -2.92, respectively, P < 0.01) **(Table 6).**

Table (6): Statistical Comparison between Levels of CA15.3, CEA and Mt. DNA in Stage I Breast Cancer Patients Versus Pathological and Healthy Controls using Wilcoxon Rank Sum Test

Parameter	Stage I versus Group B		Stage I versus Group C	
	z	**P**	**z**	**P**
CA15.3(U/mL)	-0.63	> 0.05	0.88	>0.05
CEA(ng/mL)	0.24	> 0.05	3.53	>0.05
Mt. DNA($2^{\Delta CT}$)	-2.89	<0.01	-2.92	<0.01

Group B: Pathological controls, Group C: Healthy controls.
P>0.05: Non-significant difference, p<0.01: Highly significant difference.

Table (7) compares statistical data for Mt. DNA values based on receptor (PR) and estrogen receptor (ER) status. Breast cancer patients' Mt. DNA readings did not significantly.
differ based on their ER or PR status (Z= 0.76, 1.68, and P > 0.05, respectively).

Table (7): Comparative statistics between Mt. DNA regarding Estrogen Receptors and Progesterone Receptors Status in Breast Cancer patients using Wilcoxon Rank Sum Test

Parameters		Group A Median(Q1-Q3)	z	P
Mt. DNA	ER +ve (n=14)	0.12(0.08-0.33)	0.76	>0.05
	ER -ve (n=11)	0.11(0.006-0.25)		
Mt. DNA	PR +ve (n=8)	0.23(0.88-0.41)	1.68	>0.05
	PR –ve (n=17)	0.11(0.005-0.24)		

Q1:25th percentile, Q3:75th percentile. Group A: Breast cancer patients, P>0.05: Non-significant difference.

Table (8) displays the results of a correlation analysis utilising Spearman's rank correlation of several examined factors in breast cancer patients. Mt. DNA and CA 15.3 and CEA did not significantly correlate (rs= 0.212, 0.293, P > 0.05).

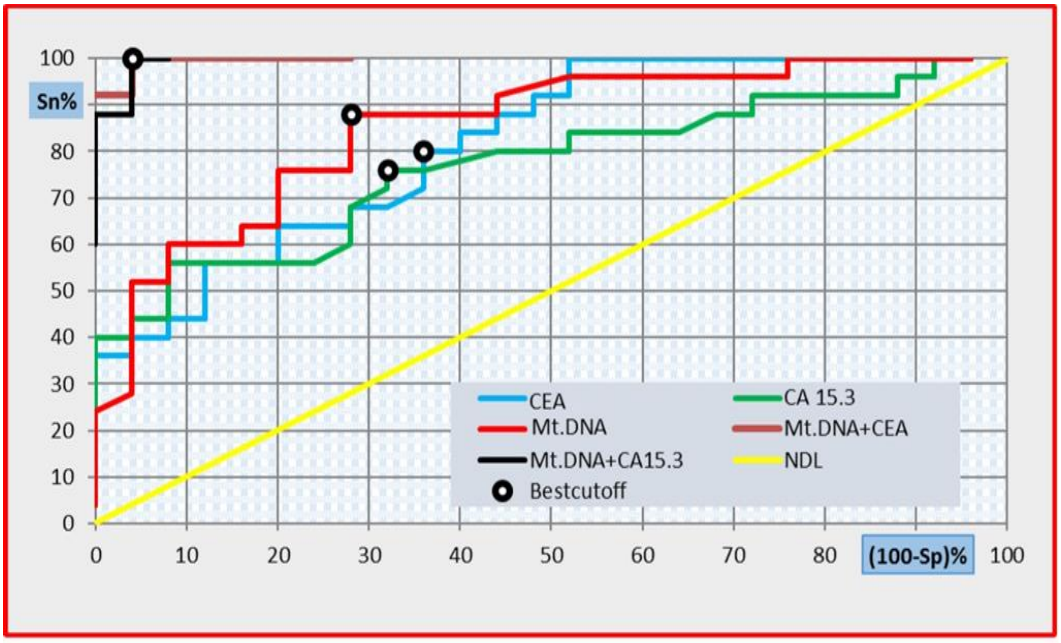
Table (8): Correlation Study between the Different Studied Parameters in Breast Cancer Patients' Group using Spearman's Rank Correlation Analysis

Parameter	CA15.3(U/mL)		CEA(ng/mL)	
	R	p	r	p
CA15.3(U/mL)	-	-	0.632	<0.01
Mt.DNA($2^{\Delta CT}$)	0.212	>0.05	0.293	>0.05

p>0.05: Non-significant correlation, p<0.01: Highly significant correlation.

Receiver operating characteristic (ROC) curve analysis was applied to assess the diagnostic performance of CA15.3, CEA and Mt.DNA in discriminating patients with breast cancer from non-cancer patients as shown in **Table (9) and Figure (3).** At the optimum cut-off level of 12 U/mL for CA 15.3, the diagnostic sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and total efficacy were 72%, 68%, 71%, 69% and 70%, respectively. Area under the curve (AUC) was 0.753. At the optimum cut-off level of 10.6 ng/mL for CEA, the diagnostic sensitivity, specificity, PPV, NPV and total efficacy were 80%, 64%, 69%, 76% and 70%, respectively. AUC was 0.753. As regards Mt.DNA, at the optimum cut-off level of 0.297 ($2^{\Delta CT}$). The diagnostic sensitivity was 88%, exceeding that of mammography (80%) with 72% specificity, 76% PPV, 86% NPV and 80% of diagnostic efficacy. AUC was 0.767.

When Mt.DNA and CA15.3 or CEA were used together, the diagnostic sensitivity for separating breast cancer patients from non-cancer patients increased to 100%, with 96% specificity, 96% PPV, 100% NPV, and 98% effectiveness. AUC values were 0.952 and 0.987. **Table (9)** and **Figure (3).**



AUC	
Mt.DNA	0.767
CA 15.3	0.753
CEA	0.753
Mt.DNA+CA 15.3	0.952
Mt.DNA+CEA	0.987

Figure (3): ROC curve analysis showing the diagnostic performance of CA15.3, CEA and Mt. DNA and their combination in discrimination between breast cancer patients from control groups

Table (9): Diagnostic Performance of Different Studied Parameters in Discrimination between Breast Cancer Patients from Control Groups

Parameter	Cutoff	Diagnostic Sensitivity (%)	Diagnostic Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Diagnostic Efficacy (%)
CA15.3(U/mL)	12	72	68	71	69	70
CEA(ng/mL)	10.6	80	64	69	76	70
Mt.DNA($2^{\Delta CT}$)	0.297	88	72	76	86	80
Mt.DNA($2^{\Delta CT}$)+ CEA(ng/mL)	0.14 10.6	100	96	96	100	98
Mt.DNA($2^{\Delta CT}$)+ CA15.3(U/mL)	0.297 17	100	96	96	100	98

DISCUSSION

Breast cancer remains the most frequent type of cancer worldwide, with nearly 1.7 million new cases diagnosed in 2012. This represents about 12% of all new cancer cases and 25% of all cancers in women, **Torre *et al.*** [8] reported. With early diagnosis of breast cancer, the mortality rates decrease. Many studies reported that on early diagnosis of breast cancer, 5-year relative survival is over 93% for localized breast cancer. Therefore, it is of great importance to diagnose cancer in time for immediate treatment **Chong and Jin** [9]**.**

Unfortunately, the traditional imaging techniques such as mammography and ultrasonography, as well as the currently accepted markers such as serum CEA and serum CA 15.3 cannot adequately identify early-stage patients. Therefore, there is an urgent need to search for better markers with higher sensitivity to vastly improve breast cancer diagnosis, staging and treatment **Buas *et al.*** [10]**.** Several attempts were held to develop non-invasive tests for early cancer diagnosis based on analysis of tumor-derived genetic material. The discovery of mitochondrial DNA (Mt.DNA) has sparked the interest of scientists as it opens up a new possibility for a non-invasive method for the early diagnosis of breast cancer **Siegel *et al.*** [11]**.**

Some evidence suggests that mitochondrial functions especially oxidative phosphorylation process is severely impaired in various cancers including breast cancer. Proteins that participate in the proper functioning of the mitochondria are encoded by both nuclear DNA (n.DNA) and mitochondrial DNA (Mt.DNA) **Yadav and Chandria** [12]**.**

In view of previous observations, the aim of the present work was to assess the clinical utility of circulating cell-free mitochondrial DNA as an early diagnostic marker for breast cancer patients and to correlate its level with that obtained by routinely used markers, CEA and CA15.5.

This study was conducted on 40 female patients attending to Surgery Department, Ain Shams University Hospitals. Patients were classified into two groups according to their diagnosis. Group I, included 25 newly diagnosed breast cancer female patients and Group II included 15 female patients with benign breast diseases such as fibroadenoma, ductal epithelial hyperplasia and fibrocystic disease of the breast. In addition, 10 apparently healthy female subjects served as a healthy control group. All studied patient groups were submitted to routine mammography, CT scan, serum CEA and CA15.3 assay in addition to, steroid receptors study. Definitive diagnosis was performed.

Our study revealed that Mt.DNA were significantly lower in breast cancer patients when compared to both pathological and healthy control subjects. These finding are in agreement with **Neelu and Dhyan** [13] and **Xia *et al.*** [14]**.** who reported that Mt.DNA was significantly down-regulated in breast cancer patients as compared to diseased and healthy controls. Therefore, decreased mitochondrial content in breast cancer may have diagnostic value.

These findings were explained by **Kohler *et al.*** [15] and **Naviaux** [16] who reported that decreased Mt.DNA levels might be due to mutation or deletion occurring to it as a consequence of exposure to ROS. Such mutation and deletion especially in the D-Loop region; which controls replication and transcription of Mt.DNA; may lead to changes in transcription and replication rate which finally result in a decrease of Mt.DNA levels in breast cancer patients.

In addition, **Fan *et al.*** [17] used animals and cell culture models. They showed that reduced Mt.DNA content could lead to cell resistance to apoptosis, and favor cancer cell growth.

In our study, serum CEA and CA15.3 levels showed a significant increase with tumor stage. This was supported by the published data by **Park *et al.*** [18] who confirmed their significant higher values with higher tumor stage. So, assessment of CEA and CA15.3 may be used as prognostic markers of breast cancer.

As regards Mt.DNA, inspite of down-regulation of its expression with disease progression, no significant difference was observed. This was in agreement with **Fan *et al.*** [17] who revealed that there is no correlation between Mt.DNA level and tumor stage. However, **Xia *et al.*** [14] reported that Mt.DNA levels were positively associated with TNM stage.

Worthy to note, on comparing CEA, CA15.3 and Mt.DNA in stage I breast cancer patients versus diseased and healthy control subjects, no significant difference was found as regard CEA or CA15.3. Meanwhile, there was a highly significant decrease of Mt.DNA in stage I breast cancer patients. **Xia *et al.*** [14]

also proved that there is a significant decrease of Mt.DNA level in stage I breast cancer patients than other stages.

In addition, in our study, no significant difference in Mt.DNA levels were found regarding the hormonal receptor's status (ER and PR). Our results are in accordance with **Xia *et al.*** [14] who conducted his study on 60 patients, that found that there is no significant difference in Mt.DNA level regarding the hormonal receptors status in breast cancer patients.

This is in contrast to results by **Bai *et al.*** [19] who conducted his study on 302 patients, which showed that ER+/PR+ tumors have a significantly higher Mt. DNA content compared to the tumors of ER−/PR−, ER+/PR−, or ER−/PR+. They suggest by these results that expression of ER and PR may stimulate mitochondrial biogenesis.

The diagnostic performance of Mt.DNA was assessed to discriminate between breast cancer patients versus non-cancer patients using ROC curve analysis. This revealed that the best diagnostic cut-off level of Mt.DNA was 0.297 ($2^{\Delta CT}$). At this cut-off, the diagnostic sensitivity was 88%, exceeding that of mammography (80%), specificity was 72%, positive predictive value (PPV) was 76 %, negative predictive value (NPV) was 86 % and efficacy was 80%, AUC was 0.767. Furthermore, the combination of Mt. DNA at cut-off 0.14 ($2^{\Delta CT}$) simultaneously with CA15.3 at cut-off 10.6 ng/mL or CEA at the cut-off 17 U/mL raised the sensitivity to 100%, specificity to 96% positive predictive value to 96%, negative predictive value to 100% and diagnostic efficacy to 88%. AUC was 0.952, 0.987, respectively.

Thus, the results of our study indicated that Mt.DNA levels are significantly lower in breast cancer patients compared to benign breast diseases and healthy subjects. Furthermore, Mt.DNA levels were significantly lower in stage I breast cancer than in benign diseases and healthy subjects, which suggested its value as a promising marker for early diagnosis of breast cancer. Moreover, the diagnostic sensitivity of Mt.DNA is enhanced by its combination with CEA or CA15.3 for the early diagnosis of breast cancer.

CONCLUSION

This study found a significant decrease in Mt. DNA level in breast cancer patients when compared to both diseased and healthy control groups.

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Competing interests: Nil.

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