RESEARCH ARTICLE

Plasma Circulating Cell-free Nuclear and Mitochondrial DNA as Potential Biomarkers in the Peripheral Blood of Breast Cancer Patients

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Abstract

**Background:** In Egypt, breast cancer is estimated to be the most common cancer among females. It is also a leading cause of cancer-related mortality. Use of circulating cell-free DNA (ccf-DNA) as non-invasive biomarkers is a promising tool for diagnosis and follow-up of breast cancer (BC) patients. **Objective:** To assess the role of circulating cell free DNA (nuclear and mitochondrial) in diagnosing BC. **Materials and Methods:** Multiplex real time PCR was used to detect the level of ccf nuclear and mitochondrial DNA in the peripheral blood of 50 breast cancer patients together with 30 patients with benign lesions and 20 healthy controls. Laboratory investigations, histopathological staging and receptor studies were carried out for the cancer group. Receiver operating characteristic curves were used to evaluate the performance of ccf-nDNA and mtDNA. **Results:** The levels of both nDNA and mtDNA in the cancer group were significantly higher in comparison to the benign and the healthy control group. There was a statistically significant association between nDNA and mtDNA levels and well established prognostic parameters; namely, histological grade, tumour stage, lymph node status and hormonal receptor status. **Conclusions:** Our data suggests that nuclear and mitochondrial ccf-DNA may be used as non-invasive biomarkers in BC.

Keywords: Breast cancer - ccf nuclear DNA - ccf mitochondrial DNA - diagnostic markers

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**Introduction**

Incidence rates of breast cancer are rising and mortality rates are proportionally high in Arab countries compared to rates in developed countries. The common late diagnosis among Arab women has been related to the low participation rates of Arab women in breast cancer screening activities (Donnelly et al., 2012).

In Egypt, breast cancer is still considered number one killing cancer in females despite increased general awareness of the disease. Dubey et al. reported that the incidence of breast cancer in Egypt at 2012 was 18,660 newly diagnosed cases and out of this the mortality rates was estimated at 7,161. This indicated the fact that the situation of breast cancer in Egypt is almost worst like India (Dubey et al., 2015).

In breast cancer, the role of serum markers is less well established. The most widely used serum markers are CA 15-3 and carcinoembryonic antigen (CEA) (Banin et al., 2014). CA 15-3 is elevated in 69% of advanced breast cancer cases; its role in cancer screening is limited, being elevated in other malignancies including pancreatic (Hampton, 2007), ovarian (Zachariah et al., 2008), lung (Uchida et al., 2015) and colorectal cancers (Diehl et al., 2008). Also, it is elevated in other benign conditions as previously reported (Chiu et al., 2006; Rainer et al., 2007 and Rani et al., 2007).

Therefore, the need for diagnostic and prognostic markers is critical. The presence of ccf nucleic acid within the plasma was first reported in 1948, attracting the interest of scientists in investigating the role of this new marker in the diagnosis of different cancers. Studies on DNA in plasma and serum of cancer patients were predominantly pioneered by Leon et al. and Shapiro et al. who in 1977 and in 1983, respectively, revealed higher levels of plasma and serum DNA in patients with cancer compared to those with benign disease (Leon et al., 1977; Shapiro et al., 1983). Changes in the level of ccf-nDNA and mtDNA were reported in different cancers (Yu et al., 2007 and Zhong et al., 2007; Board et al., 2010; Tanaka et al., 2012; Elshimali et al., 2013).

In breast cancer patients, many authors have reported increased levels of serum and plasma ccf-DNA when compared to benign and control groups (Zhong et al., 2007; Beaver et al., 2014; Olsson et al., 2015).

Mitochondria play an important role in cellular...
energy metabolism, reactive oxygen species (ROS) generation and apoptosis (Elshimagi et al., 2013). Altered mitochondrial function in cancer cells has been recognised and postulated to be the fundamental cause of cancer (Kim et al., 2004).

There is a discrepancy in the results of mtDNA as the actual mtDNA copy number in certain cancers might depend upon the specific site of mutation associated with that cancer. For example, mutations in the D loop region, which controls mtDNA replication, would be expected to result in a decrease in copy number. On the other hand, mtDNA mutations in genes encoding oxidative phosphorylation proteins might be expected to result in an increase in mtDNA copy number; it has been hypothesized that this might occur as a compensatory response to mitochondrial dysfunction (Kim et al., 2004).

In breast cancer, hepatocellular carcinoma, lung, bladder and kidney cancer, mtDNA mutations were detected with high frequency in blood plasma and other body fluids such as nipple aspirate fluid, bronchial lavage and urine (Jakupeciak et al., 2008).

In an extension of this research, we sought to investigate the possible association between circulating cell-free DNA (nuclear and mitochondrial) and breast cancer in a group of Egyptian patients and its relation with other well established prognostic factors including pathological parameters and hormonal status.

**Materials and Methods**

This study was conducted on 50 newly diagnosed breast cancer cases, aged from 31-75 years with a mean of 50.1 years, 30 patients with benign breast lesions aged from 33-52 years with a mean of 43.3 years and 20 apparently healthy age and sex matched donors aged from 33-51 years with a mean of 42.1 years. All patients presented to the outpatient clinic at the National Cancer Institute, Cairo University. Informed consent was taken from all participants prior to enrolment in this study and the study was approved by the ethical committee of NCI, Cairo University.

All cases and controls were subjected to full history taking and clinical examination.

**Laboratory investigations were carried out in the form of:**

i. Routine laboratory investigations (CBC, liver functions [AST and ALT] and kidney profile [serum creatinine and urea]); ii. Specific laboratory investigations for breast cancer cases in the form of tumour markers (CEA and CA 15-3), receptor study (ER and PR).

**Imaging techniques were performed for patients with cancer in the form of:**

i. Breast mammography for benign and malignant breast lesions; ii. Chest X-ray for cancer patients; iii. Liver and bone scan for cancer patients to exclude metastasis; iv. Histopathological study and grading for breast lesions; v. Immunohistological study (ER & PR receptors).

**Molecular study: genomic DNA analysis was performed for estimation of cell-free nuclear DNA and mitochondrial DNA by real-time PCR.**

From each patient, 5 mL venous blood was withdrawn; 1 mL was collected in an EDTA tube for CBC assay, 2 mL in plain tubes, sera were separated for assay of serum CA 15-3 level, and 2 mL in sterile EDTA vacutainers for DNA extraction and analysis for circulating cell free nuclear and mitochondrial DNA using real time PCR analysis. Samples were collected before surgical operation and processed within 2 h after venipuncture. To ensure cell-free plasma collection, all EDTA-blood samples were centrifuged in 2 steps (3,000 rpm for 10 min and then 12,000 rpm for 10 min). Samples were stored at -20°C until the time of assay.

**Methodology**

**Extraction and quantitation of plasma of plasma cell-free DNA:** Plasma DNA extraction was performed using a Qiagen DNA extraction Mini-kit (QIAGEN Hilden, Germany). A 50 μL of DNA was extracted from 200 μL of plasma according to the standardised protocol. DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific). Amplification of plasma DNA was performed using real-time PCR with Taqman assay (Applied Biosystems, Foster City, California).

For simultaneous quantification of ccf-nDNA and mtDNA from plasma, a multiplex qPCR was performed using glyceraldehyde (GAPDH) and mtDNA (MTATP8).

- The gene IDs, amplicon length, annealing temperature and sequence information of primer and probe for the GAPDH reference gene was as follows: forward primer 5’-CCC CAC ACA CAT GCA CTT ACC 3’, reverse primer 5’-CCT AGT CCC AGG GCT TTG ATT -3’, probe VIC 5’-TAG GAA GGA CAG GCA AC -3’. For the MTATP8 reference gene the forward primer sequence was 5’-ATT AAA CAC AAA ACTA CCA CTT ACC -3’, the reverse primer 5’-TGG TTC TCA GGG TTT GTT CTT -3’ and the probe FAM 5’-CCT CAC CAA AGC CCA TA -3’. The amplicon length of the GAPDH and MTATP8 genes was 79 bp and 78 bp, respectively.

**PCR was carried out in 25 μl of total reaction volume containing 12.5 μl of TaqMan universal PCR master mix (Applied Biosystems, USA: P/N4304437), 1 μl from each primer (10 pmol/μl) of both genes 1 μl from each probe (5pmol/μl), 2 μl from each of the extracted DNA was added and 4.5 μl nuclease free water. We performed qPCR using the Applied biosystems StepOne™ detection system (Applied Biosystems, Foster City, CA, USA) under the following conditions: an initiation step for 2 minutes at 50°C followed by a first denaturation for 10 minutes at 95°C and another step consisting of 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. For construction of the calibration curve a known DNA concentration of 10 ng/μl was used with a dilution factor of 5; the correlation coefficient was 0.999 for GAPDH and 0.998 for MTATP8, and slope of the curve was -3.2 for GAPDH and -3.1 for MTATP8. The relative amount of DNA in the target sample was calculated by interpolating the standard amplification curve of known DNA concentration with the amplification cycle threshold of the unknown target sample; data are expressed as copies/μl. To convert results into copies/ml, multiply the results in copies/μl by 1000.

**Statistical Methods**

**Specimen collection**

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Data was analysed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as median and range (for non-parametric data). Comparison between two groups was made using Mann-Whitney test (non-parametric t-test). Comparison between the three groups was made using Kruskal-Wallis test (non-parametric ANOVA) then a post-hoc test on a rank of variables was used for pair-wise comparison. The receiver operating characteristic (ROC) curve was used for prediction of cut-off values of the markers studied. P-value < 0.05 was considered significant.

Results

Age of the studied groups

The median age for cancer patients was 48.5 years with a range of 31-75 years, the median age of cases with benign breast lesions was 44 years with a range of 33 to 52 years and the median age of healthy control individuals was 42.5 years with a range of 33-51 years.

Comparison of plasma cellular cell-free nuclear DNA (ccf-nDNA) and mitochondrial DNA (mtDNA), CA 15-3 and CEA levels between the three study groups

The median level of ccf-nDNA in the malignant disease group was significantly higher than in the benign and control groups. No significant difference was observed in the median level of ccf-nDNA between the benign disease group and the control group, P=0.532.

The mitochondrial DNA level in the malignant disease group was significantly higher in comparison to the benign disease group (P=0.001) and the healthy group (P=0.001). Comparing the median level of mtDNA between the benign disease group and the healthy controls revealed no significant difference (2.2 vs. 2.15, P=0.827).

Tumour characteristics in breast cancer group

Tumour size: According to the TNM staging system, cases were categorised into three groups as follows: T1 (≤2 cm), T2 (>2-5 cm) and T3 tumours >5 cm (Connolly, 2006). As regards plasma ccf-nDNA level, in spite of the higher value observed in patients with tumour size >5 cm compared to the other groups, the difference is statistically insignificant, P=0.832. On the other hand, plasma mtDNA was lower in the group of patients with breast cancer lesions >5 cm compared with those of focal lesion from 2-5 cm. Data are presented in Table 2.

Pathological type: Histopathological study of the tumour mass revealed 34 cases (68%) with invasive duct carcinoma, nine cases (18%) with invasive lobular carcinoma, five cases (10%) with adenocarcinoma and

| nDNA (copies/μl) | Malignant group (N = 50) | benign group (N = 30) | Control group (N = 20) | P value |
|------------------|---------------------------|-----------------------|------------------------|---------|
| median (25%-75% percentile) | 3453.2\textsuperscript{a} | 371\textsuperscript{b} | 90\textsuperscript{c} | 0.001 |
| mtDNA (copies/μl) | 678\textsuperscript{b} | (151.9 - 110505.8) | (43.3 - 1040) | (18 - 152.3) |
| median (25%-75% percentile) | (3.6 - 5498.6) | (0 - 116.5) | (0 - 89.7) | 0.001 |

Groups bearing different initials are statistically significant, P<0.05
two cases (4%) with mixed invasive duct and lobular carcinoma. In spite of the small number of invasive lobular carcinomas, this revealed the highest level of ccf-nDNA but the difference between groups is statistically insignificant. Data are represented in Table 2.

**Tumour grade:** Most of the cases were grade 2, representing 52% of all cases, followed by grade 3 (26%) and grade 1 (22%). A statistically significant increase was observed in the level of both cellular cell-free nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) when compared to different histological grades of the tumour masses. Data are illustrated in Table 2.

**Tumour stage:** According to the American Joint Committee on Cancer (Connolly, 2006), most of the studied patients were of stage II constituting 42 %, while 34% were stage I, 16% were stage III and 8% were stage IV. Stage III tumours showed the highest level of both markers when compared to grades I and II (Table 2).

**Immunohistochemical study of breast specimens**

Immunohistochemical study of breast specimens showed that 34/50 (68%) were positive for oestrogen receptor, of which 17 cases were negative for progesterone receptors, while 17/50 (34%) were positive for progesterone receptor. Dual positivity for both estrogen and progesterone receptors were encountered in 17 (34%) cases, while dual negativity was obtained in 16 (32%) cases.

**Her-2/neu immunostaining**

In this study immunostaining of cancer breast tissue revealed that 23 (46.9%) cases were Her-2/neu positive and 26 (53.1%) were negative with one missed case. A significant difference was observed when comparing median level of nDNA between both groups (123027 vs. 415 respectively, p=0.0001), on the other hand no significant difference was observed in mtDNA level (1278 vs. 289 respectively, P=0.121). Out of the 23 cases of Her-2 positive cases, 16 (69.5%) were dual ER & PR positive and 7 (30.6%) were dual ER & PR negative.

**Lymph node status and metastasis**

As regards lymph node status, 17 (34%) cases were node negative, while 33 (66%) were node positive cases. Node positive cases were categorised by number of involved nodes according to the TNM staging system, into N1 (1-3), N2 (4-9) and N3 (≥ 10). Most of the node positive cases were of N2 category constituting 36% of all studied cases, followed by N1 (22%) and N3 (8%). A statistically significant increase in advanced cancer cases was observed in both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) when compared to lymph node status, data are presented in (Figure 3).

**Bone scan**

Bone scans were performed on all cancer breast patients at the time of diagnosis to detect the presence of metastases. We demonstrated that eight breast cancer patients (16%) out of 50 had developed metastases. A

### Table 2. Comparison of Ccf-nDNA&mtDNA and Tumor Characteristics

| Tumor size       | Nuclear DNAa (copies/µl) | Mitochondrial DNAa(copies/µl) | P value |
|------------------|--------------------------|-------------------------------|---------|
| <2 cm (N = 5)    | 884.4 (397.1-100898)     | 407.4 (1.9 - 5103)           | 0.9     |
| 2-5 cm (N = 30)  | 2273 (229.7 - 17113)     | 977 (7 - 3285)               |         |
| > 5 cm (N =15)   | 5466 (134.3-198872.8)    | 678 (0.001- 7486.3)          |         |
| Tumor pathology  |                          |                               |         |
| Invasive duct carcinoma | 616.7 (131.9 - 38715.9) | 678 (0.001 - 5226.3)        |         |
| Invasive lobular carcinoma | 71889 (485.4 - 332066)  | 239.9 (5.4 - 4532.2)         | 0.8     |
| Others           | 17113 (208.9 - 2273351)  | 1886 (167- 8381)             |         |
| Tumor grade      |                          |                               |         |
| Grade I          | 64.1 (18 - 86.4)         | 0.001 (0.001 - 7)            |         |
| Grade II         | 2717.7 (286 - 23631)     | 1127.7 (9.7 -4382.6)         | 0.001   |
| Grade III        | 194984 (76552.8 -1520147.5) | 7188 (493.7- 20189.9)      |         |
| Tumor stage      |                          |                               |         |
| Stage I          | 77 (26.5 -830.3)         | 0.001 (0.001 - 151)          |         |
| Stage II         | 6407 (286 - 27764)       | 1862.1 (171.8 - 6061.8)      |         |
| Stage III        | 194984 (84841-35923)     | 8381 (4138.5 - 15557)        | 0.001   |
| Stage IV         | 1520147.5(132383.8 - 4160321.8) | 2555.7(754.5 - 125116.7) |         |

*Data are expressed as median (25th-75th percentile)
Table 3. Diagnostic Performance of nDNA, mtDNA and CA 15.3 in Discrimination Between Malignant and Benign Groups

|                | nDNA | mt DNA | CA 15.3 |
|----------------|------|--------|---------|
| Sensitivity    | 76%  | 61%    | 60%     |
| Specificity    | 70%  | 64%    | 62%     |
| PPV            | 70%  | 75%    | 72%     |
| NPV            | 76%  | 63%    | 56%     |
| AUC            | 79%  | 69%    | 0.67    |
| cut off        | 2236 copy/µl | 376 copy/µl | 14U/ml   |

PPV: positive predictive value; NPV: Negative predictive value; AUC: area under the curve

With regard to lymph node involvement, the level of nDNA was significantly elevated in breast cancer patients with lymph node metastasis than in those without lymph node metastasis. This agreed with other studies (Roth et al., 2011 and Nicolini et al., 2013) which explained that the more lymph node involvement, the more necrosis and apoptosis and the more DNA released into the circulation (Thyagarajan et al., 2013). Plasma mtDNA was significantly higher in breast cancer patients with node positive involvement and distant metastasis than in patients without node involvement or distant metastasis, and to our knowledge no studies have proved this. This may help to recognise cases with early metastasis.

In the present study, there was a significant increase in the level of plasma nuclear DNA in advanced tumour stages when comparing stage III to stages I and II. This finding was in agreement with other studies (Nicolini et al., 2013).

The plasma level of mtDNA was lower in grades I and II compared to grade III. This finding agreed with other studies (Dawson et al., 2013, Olsson et al., 2015).
studies (Nicoloni et al., 2013), where it was argued that this high level of grade III may be due to the inhibition of expression of hypoxia inducing factor (HIF). Also, mtDNA biosynthesis is no longer inhibited. On the other hand, other authors have reported that low mtDNA level in high grade tumour may be explained by the fact that high grade tumours are less exposed to oestrogen and are characterised by a high rate of cell division which could in turn be responsible for the lower number of mitochondria per cell (Cormio et al., 2012).

The level of mtDNA increased as we proceeded from stage I to stage IV with p value < 0.001. Other researchers have observed higher mtDNA level in stages III and IV than in stages I and II (p value=0.073), and they have linked that elevation to the compensatory mechanism of the cells to respond to the decline in respiratory function (Bai et al., 2011). Another study showed that mtDNA level first decreased at stage I and subsequently increased to normal levels at stage II and stage III-IV (Xia et al., 2009).

In this study we classified the cancer groups according to the histopathological study of the breast specimen into three groups: patients with invasive lobular carcinoma display higher values for nuclear DNA when compared to the other groups in spite of the small sample size of this group, yet the difference was statistically insignificant. This may be due to the fact that the high cellular destruction that occurred in this type of tumour being an aggressive form; this observation may alert surgeons to the need for rapid decision making for these cases. On the other hand, plasma mitochondrial levels showed the lowest plasma level in this group compared to the other groups.

Immunohistochemical study of the breast cancer specimens revealed that specimens with a positive hormonal profile showed a significantly high level of plasma mtDNA compared to specimens with a negative hormonal profile. This may help select those patients who will respond well to hormonal therapy. Our finding for mtDNA agreed with other studies suggesting that expression of ER, PR may stimulate mitochondrial biogenesis (Bai et al., 2011).

In the present work, ccfDNA was associated with Her2/neu expression which was reported to be an independent predictor for early recurrence, thus nuclear and mt DNA may be considered as a prognostic marker as well as a predictor of treatment response. This may help to stratify those patients to put them under close follow up or help clinicians to personalize the follow-up program.

In our study, cancer patients with a tumour mass > 5 cm revealed high plasma ccf-nDNA and low mtDNA levels when compared to patients with tumour masses < 5 cm but the difference was statistically insignificant, which may be attributed to small sample size. Another study reported that the level of nDNA was significantly higher in patients with breast cancer with a tumour size from 2 to 5 cm and more than 5 cm compared to those with a tumour size less than 2 cm (Board et al., 2010). In agreement with our findings, a study reported that breast tumours > 5 cm produced lower plasma mtDNA than smaller tumours, referring the reduction in mtDNA level to the hypoxia that occurred when tumours grew in size, resulting in reduction of mitochondrial biogenesis (Bai et al., 2011). An alternative explanation for the decreased mtDNA content in tumour tissues with increased tumour size could be that the unchanged mtDNA biosynthesis rate is unable to catch up with the accelerated cellular proliferation in tumors.

CA 15-3 is the most widely used marker for diagnosis and follow-up of cancer patients, yet it is elevated in a group of benign tumours and other malignant tumours. In this study we chose the value 14 U/L as the best cut-off value to differentiate patients with benign tumours from those with malignant tumours but still the sensitivity and specificity was low, so we decided to determine nuclear and mitochondrial DNA in parallel with CA 15-3, which increased the sensitivity and specificity of CA15-3 with both markers but was higher with nDNA than with mtDNA.

This study demonstrated that plasma ccfDNA was significant elevated in breast cancer patients and was correlated with other routinely used tumor characteristics such as tumor type and tumor grade in primary and metastatic breast carcinomas, Hormonal receptors and Her-2/neu expression. Our study has some limitations, it was conducted in a cross-section design, and follow up prospective study might elaborate more on the prognostic role of these markers. Also, the present study included all molecular subtypes, a more homogeneous group as regard molecular subtypes and conducted treatment could have elaborate more prognostic and predictive values.

In conclusion, these results highlight the potential value of ccf-DNA as a non-invasive marker for diagnosing cancer breast and differentiating between its different histopathological types and to stratify cancer breast patients to put them under close follow up or help clinicians to personalize the follow-up program .we prove the superior role of these markers over CA 15-3 in the diagnosis of those patients.

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