RESEARCH ARTICLE

Mitochondrial DNA Levels in Blood and Tissue Samples from Breast Cancer Patients of Different Stages

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Abstract

Aims: Alterations in mitochondrial DNA (mtDNA) have been implicated in carcinogenesis and tumor progression. We here evaluated the diagnostic and prognostic potential of mtDNA as a biomarker for breast cancer. Methods: Using multiplex real-time polymerase chain reaction, nuclear DNA (nDNA) and mtDNA levels in serum, buffy coat, tumor, and tumor-adjacent tissue samples from 50 breast cancer patients were determined and assessed for associations with clinicopathological features. To evaluate mtDNA as a biomarker for distinguishing between the four sample types, we created receiver operating characteristic (ROC) curves. Results: The mtDNA levels in buffy coat were significantly lower than in other sample types. Relative to tumor-adjacent tissue, reduced levels of mtDNA were identified in buffy coat and tumor tissue but not in serum. According to ROC curve analysis, mtDNA levels could be used to distinguish between buffy coat and tumor-adjacent tissue samples with good sensitivity (77%) and specificity (83%). Moreover, mtDNA levels in serum and tumor tissue were positively associated with cancer TMN stage. Conclusions: The mtDNA levels in blood samples may represent a promising, non-invasive biomarker in breast cancer patients. Additional, large-scale validation studies are required to establish the potential use of mtDNA levels in the early diagnosis and monitoring of breast cancer.

Keywords: Mitochondrial DNA (mtDNA) - quantitative alterations - blood - tissue - breast cancer

Asian Pac J Cancer Prev, **15 (3)**, 1339-1344

Introduction

Breast cancer is one of the most common cancers and a leading cause of cancer death in women. Prognosis relies primarily on early diagnosis, selection of appropriate therapeutic strategies, and efficient follow-up. In the past two decades, established and emerging tumor-specific biomarkers and molecular diagnostics have played an important role in the diagnosis, prognosis, and tailored treatment of cancers (Weigel et al., 2010). However, tumor biomarkers for the early diagnosis of breast cancer have not yet been validated or incorporated into oncology practice (Radpour et al., 2009).

Nuclear and mitochondrial genomes coexist in eukaryotic cells. Compared to the nuclear genome, the mitochondrial genome is shorter and more simply organized, which makes genome-wide screening of mtDNA easier and more cost-effective. In addition, the higher abundance of mtDNA molecules in the cell makes mtDNA amplification a much more sensitive method for use with trace samples, single cells, and bodily fluids. Due in part to these advantages over nDNA-based methods, the detection of aberrant changes in mtDNA brings a lot attention for its usage in the early diagnosis of cancer. In light of the promise of using circulating DNA as a non-invasive marker for cancer assessment, changes in blood mtDNA may serve as a particularly sensitive, early biomarker for the non-invasive detection of tumors (Fliss et al., 2000; Radpour et al., 2009; Yu, 2011; Gonzalez-Masia et al., 2013). Mutated mtDNA is readily detected in the tissue of many solid tumors (Copeland et al., 2002) as well as bodily fluids sampled in early-stage patients (Hibi et al., 2001; Jeronimo et al., 2001; Nomoto et al., 2002; Okochi et al., 2002; Yu, 2011). In addition, alterations in mtDNA levels are associated with various types of cancer. An increase in the amount of mtDNA is seen in prostate cancer (tissue and plasma) (Mehra et al., 2007; Mizumachi et al., 2008), untreated head and neck cancer (saliva) (Jiang et al., 2005), thyroid cancer (tissue) (Mambo et al., 2005), pancreatic cancer (cell lines) (Jones et al., 2001), ovarian cancer (tissue) (Wang et al., 2006), and endometrial cancer (tissue) (Wang et al., 2005), while a decrease is seen in gastric cancer (tissus) (Wu et al., 2005), previously treated head and neck cancer (saliva) (Jiang et al., 2006), renal cancer (cell lines and blood) (Selvanayagam et al., 1996; Xing et al., 2008), lung cancer (Dai et al., 2013), and

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Table 1. Clinical Characteristics of the 50 Breast Cancer Patients That Participated in This Study

Variables	N (%)
Age (years)	
Mean	52.6
Median	53
Range	30–76
Sex	
Female	49 (98)
Male	1 (2)
Tumor histological grade	
I	1 (2)
II	16 (32)
III	19 (38)
Lymph-node invasion	
Positive	22 (44)
Negative	26 (52)
TNM stage	
I	9 (18)
II	17 (34)
III	9 (18)
IV	5 (10)
Distant metastasis	
Yes	2 (4)
No	47 (94)

hepatic cancer (tissue) (Lee et al., 2004; Yin et al., 2004; Morten et al., 2007). Thus, previous research indicates that alterations in mtDNA may play an important role in tumorigenesis, and mtDNA levels may be regulated in a tumor-specific manner.

Several studies have identified somatic mtDNA mutations and deletions in breast tumor tissue, suggesting an association between mtDNA alterations and tumor progression and prognosis (Dani et al., 2004; Zhu et al., 2004; Tseng et al., 2006). Moreover, previous research has shown that the level of mtDNA is lower in breast tumor tissue than in adjacent tissue (Mambo et al., 2005; Yu et al., 2007; Fan et al., 2009). Kohler et al. (2009) found that the level of circulating mtDNA in plasma was significantly lower in patients with malignant and benign breast tumors than in healthy controls.

Our previous study further demonstrated that patients with stage I breast cancer had lower levels of circulating mtDNA in plasma than those those with more advanced cancer (stages II–IV) (Xia et al., 2009a). However, two epidemiological studies that investigated whole blood and buffy coat specimens demonstrated a positive association between mtDNA levels and breast cancer risk (Shen et al., 2010; Thyagarajan et al., 2013). Associations between mtDNA alterations and clinicopathological features of breast cancer have also been inconsistent across studies (Yu et al., 2007; Fan et al., 2009; Xia et al., 2009a). For example, Yu et al. showed that reduced mtDNA copy number was associated with patients'age (Yu et al., 2007), but other studies have not observed this association (Fan et al., 2009; Xia et al., 2009a). Due to these discrepancies, additional studies involving a larger number of cases are needed to assess the predictive value of mtDNA levels for early cancer diagnosis.

Although previous studies have examined mtDNA levels in tissue and blood samples from breast cancer patients, to our knowledge, no data exist regarding mtDNA content in paired blood and tissue samples. Therefore, in the present study, we collected serum, buffy coat, tumor tissue, and adjacent tissue samples from 50 breast cancer patients. We measured mtDNA levels in these samples and investigated associations with clinicopathological characteristics. These findings extend our understanding of the role of mtDNA alterations in breast cancer pathogenesis.

Materials and Methods

Study cohort and sampling procedures

Blood samples from 50 patients with breast cancer were taken before primary surgery. Serum and buffy coat specimens were prepared from the blood samples according to standard protocols, as described previously (Wang et al., 2012). In addition, formalin-fixed paraffin-embedded (FFPE) breast tumor and normal adjacent tissue samples were prepared after surgical resection, which were histologically reviewed by 2 independent expert pathologists. None of the patients had received neoadjuvant treatment prior to the primary surgery. Breast cancer characteristics, including gendar, age, histological grade, lymph node status, and presence of distant metastasis) are listed in Table 1. All tumors were histologically diagnosed as invasive ductal carcinomas. Tumors were staged according to TNM classification (Union for International Cancer Control, UICC) : 9 cases were classified as stage I, 17 cases as stage II, and 14 cases as stage III and IV. Almost all of the patients are females except one patient. The median age of the patients was 53 years (range, 30-76 years) . All patients gave their informed consent for sample retention and analysis for research purposes according to institutional guidelines. This study was approved by the research ethics committee of the Medical School of Xi'an Jiao Tong University, China.

DNA isolation and multiplex quantitative real-time polymerase chain reaction (PCR)

DNA was extracted from 200 µl buffy coat and serum samples using a QIAamp Blood Kit (Qiagen, Frederick, MD, USA) and from FFPE tissues using a Mag-Bind® FFPE DNA Kit (Omega Bio-Tek, Norcross, GA, USA), based on the manufacturers' instructions. DNA was quantified using a Nanodrop spectrophotometer (Thermo scientific, Fitchburg, WI, USA).

For the simultaneous quantification of circulating cell-free (ccf) nDNA and mtDNA from the DNA samples, a multiplex quantitative PCR (qPCR) targeting the nDNA-encoded glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and the mtDNA-encoded ATPase8 (MTATP8) gene was performed. Primers and amplification conditions have been described previously (Xia et al., 2009a; Xia et al., 2009b). To determine the quantities of mtDNA and nDNA present in the tested samples, the average threshold cycle (Ct) values for nDNA and mtDNA were obtained from each reaction. The amount of mtDNA was calculated using the ΔCt, a function of the difference in average Ct for nDNA and mtDNA ($\Delta Ct = Ct_{nDNA} - Ct_{mtDNA}$) for a single reaction, as an exponent of 2 ($2^{\Delta Ct}$).

Table 2. mtDNA Levels in the Four Types of Sample Obtained from Breast Cancer Patients*

Sample type	Minimum	Median	Maximum	Mean	SD	N
Serum						
ΔCt	5.69	7.83	10.22	7.757	0.7683	50
$2^{\Delta Ct}$	51.52	227.3	1194	250.4	168.3	50
Buffy coat						
ΔCt	0.27	5.13	12.35	5.234	2.381	48
$2^{\Delta Ct}$	1.206	35.02	5221	241.3	846.1	48
Tumor tissue						
ΔCt	6.44	7.33	8.95	7.421	0.5318	48
$2^{\Delta Ct}$	86.82	160.9	494.6	184.7	83.6	48
Tumor-adjacent tissue						
ΔCt	2.85	8.88	12.98	8.379	2.525	48
$2^{\Delta Ct}$	7.198	468.8	8088	1007	1565	48

*ΔCt, Ct_{nDNA}-Ct_{mtDNA}; SD, standard deviation

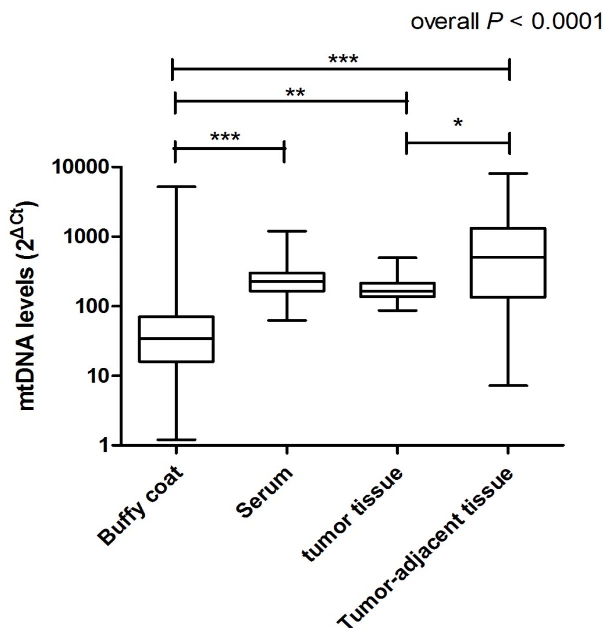

Figure 1. mtDNA Levels in Four Sample Types (n=44) Obtained from Breast Cancer Patients. The P values shown were obtained from Friedman test (upper right corner) and Dunn's Rank test: *P < 0.05; ***P < 0.001. Horizontal lines: group medians; boxes: 25--75% quartiles; range, peak and minimum

Statistical analysis

Statistical analyses were conducted with GraphPad Prism software (Version 4.0, San Diego, CA, USA). The distribution of data was evaluated using the Shapiro-Wilk normality test, and nonparametric statistical methods were utilized for non-normally distributed variables. Unless otherwise indicated, differences with P < 0.05 were considered statistically significant. The relationship between nDNA and mtDNA content in tested samples was examined using Spearman correlation coefficients (rs). Friedman nonparametric test (for more than two related samples) was used to compare mtDNA levels amongst the four types of samples (plasma, buffy coat, tumor tissue, and adjacent tissue) (Bewick et al., 2004). When the Friedman test results indicated a significant difference (P < 0.05), post-hoc, multiple pairwise comparisons of the sample types were performed using Dunn's method. The associations between clinicopathological variables and

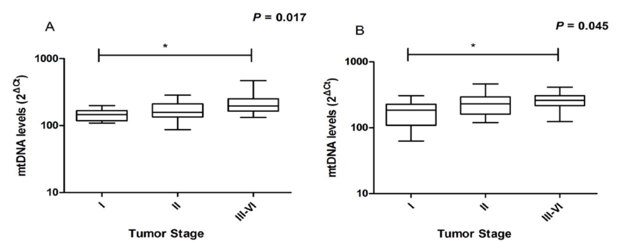

Figure 2. Association between mtDNA Levels and the Clinical Stage of Tumors in (A) Tumor Tissue and (B) Serum Samples. The P values shown were obtained from the Kruskal-Wallis test (upper right corners) and Dunn's rank test: *P < 0.05. Horizontal lines: group medians; boxes: 25--75% quartiles; range, peak and minimum

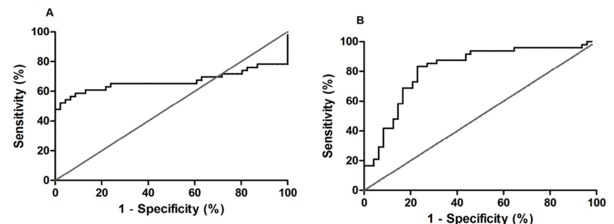

Figure 3. Receiver Operating Characteristic Curves Using mtDNA Levels to Discriminate between Sample Types. (A) Curve for discriminating between paired tumor and adjacent tissue (Sensitivity = 98%; Specificity = 52%). (B) Curve for discriminating between buffy coat and tumor-adjacent tissue (Sensitivity = 77%; Specificity = 83%)

100.0
6
75.0
56
50.0
25.0
31
0

mtDNA levels were analyzed using the Mann-Whitney test (for two unrelated samples) or the nonparametric Kruskal-Wallis test (for more than two unrelated samples) (Bewick et al., 2004). To evaluate the sensitivity and specificity of mtDNA level as a marker for distinguishing between serum, buffy coat, tumor tissue, and adjacent tissue, we performed ROC curve analysis.

Results

nDNA and mtDNA levels in serum, buffy coat, and tissue samples from breast cancer patients

Using multiplex real-time PCR, we measured mtDNA levels (relative to nDNA levels) in serum, buffy coat, tumor tissue, and adjacent breast tissue from 50 patients with breast cancer (Table 2). A small number of values are missing due to aberrant amplification signals for GAPDH relative to the external control employed. Values with a positive ΔCt indicate a greater amount of mtDNA than nDNA. Thus, our data showed that the mtDNA content in the four types of samples are all higher than nDNA. Wide variability in mtDNA levels was observed in our study, and the buffy coat and tumor-adjacent tissues showed an extremely larger variability in mtDNA levels than those in serum and tumor tissues ($max/min_{Buffy\ coat}$=4329 vs $max/min_{adjacent\ tissues}$=1124 vs max/min_{serum}=23 vs $max/min_{tumor\ tissues}$ =5.7). In addition, in buffy coat and tumor-adjacent tissue, the average Ct values for the nuclear gene GAPDH were significantly correlated with those for the mtDNA gene MTATP8 (rs = 0.709 and 0.845, respectively, with Spearman's rho test P < 0.001). By contrast, no significant or poor correlations between Ct values for the two genes were detected in serum and tumor tissue (r_s=0.263, P =

0.077 and rs = 0.342, $P = 0.017$, respectively). These results suggest that the relationship between nDNA and mtDNA in serum and tumor tissue from breast cancer patients is different from that present in buffy coat and tumor-adjacent breast tissue.

Comparison of mtDNA levels in serum, buffy coat, tumor tissue, and adjacent tissue

Using Friedman test with multiple comparisons, we analyzed mtDNA levels amongst the four types of sample. As shown in Figure 1, mtDNA levels among the four types of sample were significantly different ($P < 0.0001$). The mtDNA levels in buffy coat were significantly lower than those in other sample types, with a median value that was 6.49-fold, 4.59-fold, and 13.39-fold lower than for serum, tumor tissue, and tumor-adjacent tissue, respectively. In addition, mtDNA levels in tumor tissue were significantly lower than those present in adjacent tissue ($P < 0.05$), with a 2.91-fold lower median value. However, no significant difference in mtDNA levels was observed between serum and tumor-adjacent tissue samples.

Association between mtDNA levels and clinicopathological characteristics

Associations between mtDNA levels in the four sample types and clinicopathological characteristics were analyzed. The results showed that mtDNA levels were not associated with age, histological grade, or lymph node status (data not shown). However, mtDNA levels in serum and tumor tissue, but not buffy coat or tumor-adjacent tissue, were significantly lower in stage I breast cancer patients than in patients of higher stages ($P = 0.045$ and 0.017, respectively; Figure 2).

Receiver operating characteristic curves: Using mtDNA levels to discriminate between tumor-adjacent breast tissue and serum, buffy coat, and tumor tissue

Relative to tumor-adjacent breast tissue, decreased mtDNA levels were found in tumor-tissue and buffy coat samples. Using a receiver operating characteristic (ROC) curve, an optimal cut-off point of 470 for mtDNA level was indicated to discriminate between tumor and adjacent tissue, with a sensitivity of 98% and a specificity of 52% (AUC = 0.67, $P = 0.0056$, 95% CI: 0.5421 -0.7934; Figure 3). To discriminate between tumor-adjacent tissue and buffy coat, an optimal cut-off point of 116 for mtDNA content was indicated, with a sensitivity of 77% and a specificity of 83% (AUC = 0.82, $P < 0.0001$, 95% CI: 0.7285 -0.9052; Figure 3). Based on sensitivity and specificity, assessing mtDNA levels in buffy coat relative to those in tumor-adjacent tissue in patients with breast cancer could be more effective than analyzing mtDNA levels in tumor tissue.

Discussion

To the best of our knowledge, this is the first study to identify alterations in mtDNA levels in paired blood and tissue samples from breast cancer patients. Our data revealed that (1) mtDNA levels in matched serum, buffy coat, tumor tissue, and tumor-adjacent tissue were significantly different and (2) mtDNA levels in serum and tumor tissue were associated with the clinical stages of breast cancer. These findings indicate that alterations in mtDNA are associated with the development and progression of breast cancer, suggesting a clinical application for measuring mtDNA levels as a non-invasive biomarker for breast cancer.

In the present study, we found that nDNA and mtDNA levels were poorly correlated in serum and tumor tissue samples, although high correlations were observed in buffy coat and tumor-adjacent tissues. We also found no correlation between nDNA and mtDNA levels in peripheral blood from breast cancer patients in a previous study (Xia et al., 2009a). In addition, another previous study found significant correlation between nDNA and mtDNA levels in adjacent normal tissues but not in tumor tissues from breast cancer patients (Fan et al., 2009). Together, these findings suggest an altered relationship between nDNA and mtDNA in serum and tumor tissues from breast cancer patients, probably due to a cancer-related difference in nDNA and mtDNA compartmentalization and degradation (Mehra et al., 2007).

By comparing mtDNA content among the four sample types, we discovered that tumor tissue contains significantly less mtDNA relative to nDNA than tumor-adjacent tissue. This is consistent with previous breast cancer studies (Mambo et al., 2005; Tseng et al., 2006; Yu et al., 2007; Fan et al., 2009), in which reduced mtDNA levels were identified in tumor tissue relative to adjacent tissue. Moreover, we found that buffy coat exhibited significantly lower mtDNA levels than other sample types. However, no significant difference in mtDNA levels between serum and tumor-adjacent tissue. ROC curve analysis demonstrated that mtDNA levels can be used to discriminate between buffy coat and tumor-adjacent tissue with better specificity and sensitivity than obtained when comparing tumor and tumor-adjacent tissue. This indicate that identifying reduced mtDNA levels in buffy coat samples from patients with breast cancer may represent a promising approach for the development of diagnostic biomarkers. However, due to few reports have been exploited buffy coat to investigate the role of mtDNA alteration in tumor biology, and lack of data to compare the mtDNA levels between patients and healthy subjects, therefore, the utility of buffy coat mtDNA levels as a diagnostic marker needs further study.

Associations between mtDNA levels in the four sample types and clinicopathological features were examined in this study. No relationship between mtDNA levels and age, histological grade, or lymph node status of cancers was identified for any sample type. However, in serum and tumor tissue, mtDNA levels were positively associated with the clinical stages of breast cancer, consistent with our previous findings on peripheral blood samples (Xia et al., 2009a). Our current findings add additional support to the idea that mtDNA alterations play an important role in the progression of breast cancer. The increased mtDNA levels observed in the more advanced stages of breast cancer could be a compensatory response to a decline in respiratory function (Barrientos et al., 1997). Furthermore, since the clinical value of circulating

nucleic acids in serum or plasma has been shown as a non-invasive method for the diagnosis, prognosis, and management of cancer patients, therefore, the positive association between serum mtDNA levels and breast cancer stage demonstrates the potential of circulating mtDNA as a non-invasive biomarker for breast cancer. While some previous studies have reported associations between mtDNA content and age, estrogen receptor (ER) positive or progesterone receptor (PR) positive status, and menopausal status of breast cancer patients, the results have not been consistent (Mambo et al., 2005; Yu et al., 2007; Fan et al., 2009; Xia et al., 2009a). For example, Yu et al. showed that reduced mtDNA copy number was more frequent in patients older than 50 years of age (Yu et al., 2007), but other studies, including the current one and another performed previously in our lab, have not observed this association (Fan et al., 2009; Xia et al., 2009a). In addition, Yu et al. (2007) found an inverse association between mtDNA levels and histological tumor grade, which was not replicated in the present study or several previously published studies (Mambo et al., 2005; Fan et al., 2009). Limited by our study's relatively small sample size and the absence of some clinical information, it was difficult to evaluate comprehensively the influence of all clinicopathological factors that have been examined in previous studies on mtDNA levels. Therefore, in the future, a large-scale study is needed to verify and further investigate the relationship between mtDNA levels and breast cancer clinicopathological characteristics.

In conclusion, in this study we demonstrated that mtDNA levels in buffy coat were significantly lower than those in serum, tumor tissue, or tumor-adjacent tissue from breast cancer patients. In addition, we (1) identified an association between mtDNA levels in serum and TMN stage and (2) demonstrated the sensitivity and specificity of using mtDNA levels to discriminate between buffy coat and tumor-adjacent tissue. These findings suggest that mtDNA levels in the blood sample may be a promising non-invasive biomarker for the diagnosis and prognosis of breast cancer. Yu et al. (2007) have shown that somatic mutations in the D-loop region of mtDNA are likely a key contributing factor to the decreased mtDNA levels found in breast tumor tissue. Additional case-control studies are needed to further explore the relationship between qualitative and quantitative mtDNA alterations and breast cancer and reveal the mechanisms underlying mtDNA alterations between paired blood and tissue samples from breast cancer patients.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81102026/H1622) and the Science and technology project of Shaanxi Province (No. S2011SF1851).

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