Published in final edited form as:

Mitochondrion. 2016 January ; 26: 104–112. doi:10.1016/j.mito.2015.12.001.

Mitochondrial common deletion is elevated in blood of breast cancer patients mediated by oxidative stress

Hezhongrong Nie¹, Guorong Chen², Jing He¹, Fengjiao Zhang¹, Ming Li¹, Qiufeng Wang¹, Huaibing Zhou¹, Jianxin Lyu^{1,*}, and Yidong Bai^{1,*}

¹Key Laboratory of Laboratory Medicine, Ministry of Education, Zhejiang Provincial Key Laboratory of Medical Genetics, College of Laboratory Medicine and Life sciences, Wenzhou Medical University, Wenzhou, Zhejiang, China

²Department of pathology of the First Affiliated Hospital, Wenzhou Medical University, Wenzhou, Zhejiang, China

Abstract

The 4,977 bp common deletion is one of the most frequently observed mitochondrial DNA (mtDNA) mutations in human tissues and has been implicated in various human cancer types. It is generally believed that continuous generation of intracellular reactive oxygen species (ROS) during oxidative phosphorylation (OXPHOS) is a major underlying mechanism for generation of such mtDNA deletions while antioxidant systems, including Manganese superoxide dismutase (MnSOD), mitigating the deleterious effects of ROS. However, the clinical significance of this common deletion remains to be explored. A comprehensive investigation on occurrence and accumulation of the common deletion and mtDNA copy number was carried out in breast carcinoma (BC) patients, benign breast disease (BBD) patients and age-matched healthy donors in our study. Meanwhile, the representative oxidative (ROS production, mtDNA and lipid oxidative damage) and anti-oxidative features (MnSOD expression level and variation) in blood samples from these groups were also analyzed. We found that the mtDNA common deletion is much more likely to be detected in BC patients at relatively high levels while the mtDNA content is lower. This alteration has been associated with a higher MnSOD level and higher oxidative damages in both BC and BBD patients. Our results indicate that the mtDNA common deletion in blood may serve a biomarker for the breast cancer.

Keywords

breast cancer; mtDNA mutations; 4,977 bp common deletion; MnSOD; oxidative stress

*Corresponding author: Wenzhou Medical University, Chashan, Wenzhou 325035, China. jxlu313@163.com (J. Lyu); baiy@uthscsa.edu (Y. Bai). Fax: 86-577-86689771; Tel: 86-577-86689805.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Breast cancer is one of the most frequently diagnosed cancers in women worldwide (Kim et al., 2014), and it is also one of the leading causes of cancer-related deaths in Chinese women (Fan et al., 2014). The progression of breast cancer involves multiple genetic events.

Tremendous progress has been made as a result of extensive research focusing on the nuclear genome. For example, mutations in the BRCA1 and BRCA2 genes encoded by the nuclear DNA (nDNA) are associated with a high risk of developing early onset breast cancer (Kwong et al., 2015). Similarly, mutations in TP53, PTEN (Suda et al., 2012) and CHEK1 and CHEK2 are also associated with increased susceptibility to breast cancer development (Sokolenko et al., 2015). In addition, increasing evidence suggests that mitochondrial function is severely impaired in various cancers including breast cancer (Deus et al., 2014; Neuzil and Moreno-Sanchez, 2013) due to genetic defects in the OXPHOS system (Chandra and Singh, 2011). Consequently, mtDNA mutations have been recognized as an important aspect of breast cancer occurrence and development (Nie et al., 2013; Shen et al., 2010; Shen et al., 2011; Yu et al., 2009).

Mitochondria are ubiquitous organelles and play important roles, including generating ATP through OXPHOS, producing ROS and initiating apoptosis (Desler et al., 2011). Mitochondria are the major intracellular source and also the most vulnerable targets of free radicals (Cui et al., 2012). Several studies have suggested that mitochondrial electron transport chain byproducts, i.e., free radicals (including ROS and RNS), may play multiple roles in tumor initiation, progression and maintenance (Brandon et al., 2006; Desouki et al., 2005; Li et al., 2012). Low levels of free radicals regulate cellular signaling and are essential in normal cell physiology (Matsubara et al., 2003). However, overproduction of free radicals may cause oxidation of a diverse array of cellular macromolecules, leading to alterations in normal cellular metabolism, signaling and function. Oxidative stress may occur if there is an imbalance between free radical production and antioxidant capacity (mainly comprising SOD, GPx and CAT) (Bresciani et al., 2015). The imbalance between oxidative species generation and antioxidant systems induced by both exogenous (diet and smoking) and endogenous estrogen factors have been implicated during the initiation, promotion and progression of breast cancer (Jerby et al., 2012; Vera-Ramirez et al., 2011; Yeon et al., 2011). The initial consequences of increased production of free radicals are changes in the structure of [the main/primary] biomolecules, including changes in nucleic acids (such as 8-OHdG) and lipids (such as MDA), which could lead to their dysfunction, while at the same time products derived from this damage are used as biomarkers of oxidative stress (Dayem et al., 2010; Gonenc et al., 2005; Sosa et al., 2013).

The mtDNA 4,977 bp or “common” deletion deletes between nucleotides 8,470 and 13,447 of the human mtDNA. This mutation removes all or part of the genes encoding four complex I subunits, one complex IV subunit, two complex V subunits and five tRNA genes, which are indispensable for maintaining normal mitochondrial function. It has been suggested that continuous generation of intracellular free radicals during OXPHOS is a major underlying mechanism for generating this deletion (Mandavilli et al., 2002; Santos et al., 2013), and such a deletion has been reported in several types of cancers (Chen et al., 2011; Dani et al., 2004; Tseng et al., 2006; Ye et al., 2008; Zhu et al., 2004). The common deletion in blood

was first detected using the nest PCR method (Gattermann et al., 1995), and has since been found in intravital and postmortem blood (von Wurmb et al., 1998) and in different human blood cells (Meissner et al., 2000; Mohamed et al., 2004).

Somatic mtDNA deletions have been used as an indication of mtDNA oxidative damage (Meissner et al., 2008; Shen et al., 2010). In response to oxidative stress, an elaborate antioxidant system arises to mitigate the deleterious effects of ROS. MnSOD, a superoxide radical-scavenging mitochondrial enzyme, is crucial for protecting cells and tissues from oxidant injury and hypoxia by converting O_2^- to H_2O_2 (Candas and Li, 2014). Furthermore, recent studies have suggested that MnSOD may function as a tumor suppressor (Zhang et al., 2006). Human MnSOD is an 88.6-kDa tetrameric protein containing a Mn^{2+} ion associated with each subunit. Low MnSOD expression has been reported in several tumor cell lines compared to their noncancerous counterparts (Dhar and St Clair, 2012). Furthermore, MnSOD levels positively correlate with the in vivo tumor grade in breast cancers, particularly with the invasive and metastatic phenotypes of advanced breast cancers (Tsanou et al., 2004). However, reports that thyroid tumors, central nervous system tumors, gastric and colorectal carcinomas, and acute leukemia exhibited high MnSOD levels are conflicting (Park et al., 2013; Pica et al., 2015; Termini et al., 2015).

In this study, we aim to comprehensively evaluate the clinical relevance of the mtDNA common deletion, MnSOD, and oxidative damage in breast cancer, and try to identify novel mitochondrial biomarkers reflecting oxidative stress and cancer progression.

2. Materials and Methods

2.1 Clinical samples and DNA extraction

Blood and tissues of 107 patients with BC and 118 patients with BBD were collected at the Department of Pathology, First Affiliated Hospital, Wenzhou Medical University, Wenzhou, PR China, between May, 2007 and December, 2008. The internal controls were adjacent normal tissue (BC patients) and blood from the same subject before any chemotherapy, radiotherapy or pharmacotherapy. These patients were confirmed as BC or BBD by at least 2 senior pathologists. A total of 113 peripheral blood samples from age-matched healthy donors who had a cancer-free history, and had no other known diseases which could be associated with mitochondrial defects, were also collected at the Physical Examination Center of the same hospital from October, 2008 to December, 2008. All subjects gave their written informed consent, which was approved by the Wenzhou Medical University Ethics Committee, before enrollment. The carcinoma and paracarcinoma normal frozen tissues were dissected under microscopes and then DNA was extracted as described previously (Chen et al., 2011).

2.2 PCR amplification for mtDNA common deletion

Nest PCR was used for detecting the common deletion: the outer primers were used for amplification of a 495 bp product, while the inner ones were for a 358 bp product (Table S1). The amplification conditions for the outer primers were as follows: initial denaturation was at 95 °C for 5 min, followed by 35 cycles with denaturing at 94 °C for 20 s, annealing at

55 °C for 20 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 5 min. The same amplification conditions were used for the inner primers, and 1 µl PCR product of the previous reaction was utilized as a DNA template while other conditions were the same except for the primers. The final PCR products were electrophoresed on a 2% agarose gel, and a 358 bp band was expected for patients exhibiting the common deletion (Figure S1A).

2.3 Quantification of common deletion level and mtDNA content

The common deletion level was measured by quantitative PCR (QPCR) on deletion products (Figure S1C) (Chen et al., 2011) and normalized by simultaneous measurement of overall mtDNA represented by *COX-I* fragment (Table S1) (Chen et al., 2011). Primers for construction of plasmids to generate templates for standard curves are also shown in Table S1. The probe for each PCR reaction was labeled with 5-FAM at the 5' end and ECLIPSE at the 3' end. The common deletion level was calculated using the ratio of the copy number of the common deletion to that of the *COX-I* fragment, while the mtDNA content was calculated from the ratio of *COX-I* fragment to the *β-actin* gene. DNA from HeLa cells was used as a control. The mtDNA content of breast tumor cells was presented relative to HeLa cells mtDNA content.

QPCR was carried out using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 20 µl reaction containing 0.5 µM each of the forward and reverse primers, 0.1 pM for each probe (*Cox-I* and *β-actin* genes) and 10 ng total DNA sample. The amplification conditions were as follows: 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 58 °C for 30 s. Each sample was tested in triplicate and serial concentrations of standard plasmid were included for the standard curve.

2.4 Measurement of ROS production

For quantitation of ROS levels, fresh peripheral blood samples from 50 BBD patients, 46 BC patients and 45 healthy controls were collected, and plasma was separated, centrifuged and assayed with the Peroxide Assay Kit (Biomedical Research Service). This kit is based on the peroxide-mediated oxidation of Fe^{+2} to Fe^{+3} in the presence of xylenol orange dye, which can be conveniently quantified by spectroscopy at 595nm absorbance. The molar extinction coefficient of the xylenol orange- Fe^{+3} complex is $1.5 \times 10^4 \text{cm}^{-1} \text{M}^{-1}$. Each sample was tested in triplicate, and the mean value was adopted for data presentation.

2.5 Determining the degree of oxidative mtDNA damage and lipid damage

The content of 8-hydroxy 2-deoxyguanosine (8-OHdG) in mtDNA was determined by a QPCR based technique as described in a previous report (Lin et al., 2008). One µl of sample DNA was treated with or without 1 U of hOGG1 in 1xNE buffer solution (20 mM Tris chloride, pH 8.0, 1 mM EDTA, 1 mM DDT, and 100 µg/ml of BSA) to a total volume of 10 µl at 37 °C for 1 h followed by 65 °C for 20 min (Lin et al., 2008). Then, the treated and non-treated DNAs were subjected to QPCR simultaneously. The Ct would increase from Ct1 to Ct2 if the mtDNA was harboring 8-OHdG (Lin et al., 2008). Using the equation for mtDNA quantification, we could calculate the total mtDNA templates under Ct1 and mtDNA templates without 8-OHdG formation under Ct2 to determine the degree of mtDNA integrity. The degree of oxidative mtDNA damage is defined as $\text{Ct}_2 - \text{Ct}_1$, which is the difference

between Ct1 and Ct2. The larger the Ct value, the more 8-OHdG the sample contains. Each reaction was performed in triplicate, and the mean value was adopted for data presentation.

For quantitation of oxidative lipid damage, plasma was de-proteinized by trichloroacetic acid precipitation. We mixed 0.1ml plasma with an equal volume of ice-cold 10% TCA, then incubated it on ice for 30 min. The samples were subjected to refrigerated centrifugation at 13,000g for 5 min, then the supernatant was recovered and combined with 0.1 ml of 2-thiobarbituric acid, and the mixture was incubated for 30 min at 95 °C. Samples were then cooled to room temperature, 0.2ml of n-butanol was added and the mixture was centrifuged for 3 min at 13,000g. The upper layer of each sample was removed, the absorbance at 532nm measured, and lipid peroxidation assays were performed as recommended by the manufacturer (Biomedical Research Service). MDA concentrations were calculated using a molar extinction coefficient of $1.56 \times 10^5 \text{ cm}^{-1}\text{M}^{-1}$. Each reaction was performed in triplicate, and the mean value was adopted for data presentation.

2.6 Determination of MnSOD variation and expression

Eight pairs of primers were designed to amplify the 5' flanking region, promoter region and 4 exons of the *MnSOD* gene (Table S2). The PCR conditions were set as: initial denaturation at 95 °C for 5 min, followed by 35 to 40 cycles of 94 °C, denaturation for 1 min, annealing at 51 °C to 63 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR products were then electrophoresed on a 2% agarose gel (Figure S1B). All PCR experiments included a negative control with no template DNA (double-distilled water). Then the products were purified using the Agarose Gel DNA Fragment Recovery Kit Ver.2.0 (TaKaRa, Japan) and subsequently sequenced on an ABI Prism 3730 sequence analyzer. These sequence results were compared with the updated consensus GenBank database.

Leukomonocytes were isolated from fresh peripheral blood with lymphocyte separation medium (Beyotime Biotech, Nantong, China) according to the manufacturer's instructions. Cell lysis was performed by vigorous shaking for 10 min in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40 and 0.1% SDS, supplemented with protease and phosphatase inhibitor cocktails I and II (Sigma, St. Louis, MO). Cell proteins were collected by centrifuging at 12,000 g at 4 °C for 10 min. Then, protein concentration was measured by the Bradford assay (Bio-Rad Laboratory, Hercules, CA). Lysates were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 50 µg aliquots were electrotransferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratory, Hercules, CA). The membranes were then blocked in blocking buffer (5% milk in Tris-buffered saline containing 0.05% Tween 20) for 1 h at room temperature. The membranes were incubated overnight at 4 °C with different primary antibodies. The antibodies used for immunoblotting included anti-MnSOD and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were then detected by incubating with secondary antibody conjugated with horseradish peroxidase and visualizing using enhanced chemiluminescence reagents (Bio-Rad, Hercules, CA). Protein amounts were analyzed using ImageJ analysis software version 1.38e and normalized with their respective control. Experiments were performed at least three times and in duplicate.

2.7 Statistical analysis

All statistical analyses were performed using SPSS software (version 16.0) (SPSS Inc., Chicago, IL, USA). The frequency of the common deletion and *MnSOD* variation in all patients was tested by the Chi-square test or Fisher's exact test. The mtDNA relative content and common deletion level were analyzed by Independence-Samples *t-test*. Hazard ratios (OR) of each parameter were presented with their 95% confidence intervals (95% CI) and all statistics were 2 sides. $p < 0.05$ was considered significantly between groups.

3. Results

3.1 Detection of the common deletion in all patients

Nested PCR is a highly sensitive method for detecting large scale deletions even at low levels. To investigate clinical implications of the mitochondrial common deletion, we first examined cancer tissue, matched with adjacent normal tissue and blood samples from 107 BC patients, tissues and blood samples of 118 BBD patients and blood samples of 113 age- and sex-matched healthy controls, for this deletion using this method as described in the previous section. As shown in Figure 1, no significant differences were detected for the occurrence of the common deletion among cancer tissues, adjacent normal tissue and blood of BC patients. Similarly, we did not find differences between the tissues of BC and BBD patients for the occurrence of this deletion. However, interestingly, 51 out of 107 BC patients (47.66%) harbored the common deletion in their peripheral blood, which was significantly higher than that of BBD patients (10.17%) and healthy controls (8.85%) ($p < 0.001$ for both). We can conclude that the common deletion occurring in blood was related to the progress of breast cancer development and thus may be a useful biomarker for diagnosis of breast tumors.

Since the common deletion has been reported to accumulate during aging, and aging is also the biggest risk factor for cancer, we first divided our patients into 4 age groups to study the implications of aging for mtDNA defects. As shown in Table 1, within each age group, the BC patients had a higher occurrence of the common deletion in blood cells than the BBD patients and normal people. To explore what factors contribute to the increased occurrence of the common deletion in breast cancer, we further examined some clinical characteristics and other risk factors in these patients including body mass index (BMI), number of children, age, tumor stage, metastasis and expression of representative proteins that have been associated with breast cancer. We did not find any significant associations between the occurrence of the common deletion with BMI, number of children (Table S3), tumor stage, metastasis status and expression levels of ER, PR and Her-2 genes (Table 2). Interestingly, in BC patients, negative expression of TP53 and positive expression of Bcl-2 in the tumor tissue were associated with increased likelihood of detecting the common deletion (Table 2).

3.2 The common deletion levels in BC and BBD patients

To further investigate the significance of the common deletion in breast cancer, we then measured its levels using QPCR in the patients carrying it. As shown in Table 3, although there was no significant difference between BC patients' carcinoma tissues and BBD patients' tissues ($p = 0.696$), or between BC patients' blood and adjacent non-tumor tissue

($p=0.135$), we found that the common deletion in blood of BC patients was markedly higher than in BBD patients ($p<0.001$). We also found that the 4,977 bp deletion level in the bloods was significantly higher than the carcinoma tissues of BC ($p=0.032$), while this common deletion level in the bloods was lower than that in the tissues of BBD patients ($p=0.006$). Moreover, in BC patients who carried the common deletion in both carcinoma and paracarcinoma tissues, the deletion level was always significantly lower in the carcinoma tissues ($p=0.037$).

Interestingly, the average and mean levels of deletion were negative correlated with age in BC ($r=-0.3409$, $R^2=0.0057$) (Figure 2A) and BBD ($r=-0.0216$, $R^2=0.0008$) (Figure 2B) patients' blood and in BC ($r=-7.4838$, $R^2=0.1439$) patients' paracarcinoma tissues (Figure 2A), but were positive correlated with age in carcinoma tissues of BC patients ($r=0.5606$, $R^2=0.0408$) (Figure 2A) and in BBD patients' tissues ($r=0.1378$, $R^2=0.0034$) (Figure 2B).

3.3 Common deletion and mtDNA content

Alterations of mtDNA content have been widely reported in cancer patients (Chen et al., 2011; Mohideen et al., 2015; Shen et al., 2015), and mtDNA biogenesis can be induced upon stress (Dai et al., 2014). To investigate whether there was a correlation between mtDNA content and the common deletion in breast cancer, we plotted mtDNA copy numbers against common deletion levels in BC and BBD patients carrying this deletion. We found that with increased frequency of the mtDNA common deletion, there was no significant relationship with the mtDNA content in blood cells of BC ($r=-8.8596$, $R^2=0.0239$) (Figure 3A) or BBD patients ($r=-0.6165$, $R^2=0.0097$) (Figure 3B), or with the increased level of the common deletion in tissues of BBD patients ($r=0.1288$, $R^2=0.0006$) (Figure 3B), in carcinoma tissues of BC patients ($r=0.581$, $R^2=0.0017$) (Figure 3A) or in paracarcinoma tissues of BC patients ($r=12.432$, $R^2=0.0173$) (Figure 3A).

To examine the overall mtDNA content in BC and BBD patients, we measured mtDNA copy numbers in all patients, with and without the common deletion. We did not detect any significant difference in mtDNA content between cancer tissue and adjacent normal tissue ($p=0.528$). However, the mtDNA content in BC patients' blood was significantly lower ($p=0.028$) than that of BBD patients, while the mtDNA content in both paracarcinoma tissues ($p<0.001$) and the carcinoma tissues ($p=0.001$) of BC patients was also significantly lower than in BBD patients (Table 4).

3.4 Oxidative damage status in breast cancer patients

Since cancer cells are reported to be under increased oxidative stress (Deus et al., 2014; Neuzil and Moreno-Sanchez, 2013), and the mtDNA common deletion has also been suggested to result from oxidative damage (Santos et al., 2013), we first examined ROS production by direct biochemical measurements of cellular peroxide levels in representative patients with BC or with BBD and controls. As shown in Figure 4A, plasma peroxide levels were higher in both BBD and BC patients compared with healthy control subjects ($p=0.0259$ for BBD; $p=0.0352$ for BC).

We then analyzed oxidative damage in mtDNA as indicated by the presence of 8-OHdG (Carioca et al., 2015) which was determined by a QPCR-based technique as described in a

previous report (Lin et al., 2008). We detected significant increases in mtDNA damage in blood samples of both BC and BBD patients (Figure 4B).

Malondialdehyde (MDA) results from lipid peroxidation of polyunsaturated fatty acids, and the amount of MDA has been utilized to indicate the degree of lipid peroxidation (Gonenc et al., 2005). As shown in Figure 4C, the average and mean levels of MDA for BC patients were higher than those obtained for healthy subjects ($p=0.0460$).

3.5 Impaired MnSOD in BC patients

In response to oxidative stress, the antioxidant system is activated to mitigate the deleterious effects (Choudhari et al., 2014). MnSOD is a superoxide radical-scavenging mitochondrial enzyme and an important component of the cellular anti-oxidant system and altered levels have been implicated in breast cancer (Becuwe et al., 2014; Tsanou et al., 2004). We measured the expression of MnSOD in blood samples of BC and BBD patients (Figure 5), together with the control. We found that levels of MnSOD in both BC and BBD patients were significantly higher than in normal controls ($p=0.0059$ for BC and $p=0.0292$ for BBD).

Because polymorphism of the *MnSOD* gene has been associated with increased risk of breast cancer, we then analyzed the 6 common *MnSOD* variations (Egan et al., 2003; Kostyrykina et al., 2009) in our samples (Table 5). Among these 6 sites, we found that the C to G change at the -38 promoter region was significantly higher in BC patients compared with control individuals ($p=0.002$).

4. Discussion

The mitochondrial DNA common deletion is one of the first described and most-studied mtDNA mutations, and it has been implicated in many human diseases (Dimberg et al., 2015; Li et al., 2015; Zhang et al., 2015). Multiple reports have associated the occurrence of this mtDNA common deletion with a wide range of cancer types including breast cancer (Lu et al., 2009; Nie et al., 2013). Investigations involving cell lines further implicated the common deletion in apoptosis and tumorigenesis (Lee et al., 2007; Wang and Lu, 2009).

The common deletion is more likely to be present in post-mitotic tissues with high energy demands, and its frequency increased with aging (Markaryan et al., 2008; Zhong et al., 2011). However, the presence of the common deletion in blood is inconclusive or controversial (Abnet et al., 2004; Dani et al., 2004; Mohamed et al., 2004; Upadhyay et al., 2009). Using a more sensitive detection method, we found that the common deletion is more likely to be present in BC patients' blood compared with that of BBD patients and normal people (Figure 1, $p<0.001$ both).

As compared with deletion levels in the bloods, the carcinoma tissues from BC had lower level of 4977 bp deletion ($p<0.032$), but the tissues from BBD patients had higher common deletion level ($p<0.006$). For BC patients, the common deletion level in paracarcinoma tissues was significantly higher than that of the carcinoma tissues of BC patients ($p=0.037$). The high common deletion level in the blood of BC patients could be a consequence of the selective death of cancer cells, carrying higher common deletion levels, and the subsequent

release of their DNA content including mtDNA from tumor tissues into the blood, while the decreased level of the common deletion in cancer tissue is likely the result of the same selection process.

Alterations in mtDNA content have been reported in many types of cancer, and multiple mechanisms have been proposed including dysregulation of mtDNA replication (Mambo et al., 2005; Shen et al., 2010; Sun et al., 2009; Tseng et al., 2006). We found that, compared with BBD patients, mtDNA content was lower in both blood and tissues of BC patients, possibly due to increased oxidative damage to the replication machinery (Agaronyan et al., 2015; Copeland and Longley, 2014).

Because the mtDNA common deletion has been suggested as an indicator of oxidative damage, we analyzed both the generation of ROS and the levels of oxidative damage in blood samples at the DNA and lipid levels. Our data showed that the oxidative stress and damage levels in breast tumor patients (BC and BBD) are higher than those in matched control individuals. The reason why BBD patients exhibited increased oxidative damage but did not show increased levels of the common deletion in blood samples is not clear.

One important regulatory mechanism in controlling oxidative stress/damage is the antioxidant system, including MnSOD. Among the 6 variation sites we analyzed in this study, -38C/G locates in the promoter region. Transcription factor database searches indicated that the -38C/G is within a binding site for both Sp1 and AP-2 (Xu et al., 2008). In cancer cells, high levels of MnSOD correlated with high levels of Sp1 and low levels of AP-2, whereas in transformed cells, low levels of MnSOD were associated with high levels of AP-2 and low levels of Sp1 (Xu et al., 2008). It is likely that polymorphism at the -38 position plays a modulatory role in expression of MnSOD.

Previously, correlations between MnSOD level and the frequency of mtDNA deletions have been reported in human liver and skin fibroblast cultures (Pitkanen and Robinson, 1996; Yen et al., 1994). Our results indicated that MnSOD levels in blood samples of BC and BBD patients were higher than in those of healthy controls, possibly as a reaction to increased oxidative stress (Polat et al., 2002; Yeh et al., 2005). However, such up-regulation was not enough to mitigate the oxidative damage induced by the enhanced ROS production.

Altogether, as illustrated in Figure 6, we reason that breast cancer cells are subjected to increased oxidative stress which induces activation of cellular anti-oxidant systems including MnSOD. When the oxidative stress level overcomes the cellular defenses, oxidative signaling will further activate the tumorigenesis cascade, and at the same time causes oxidative damage, including generation and accumulation of the mtDNA common deletion. Within the tumor, cell death occurs when the common deletion level reaches a threshold, and the DNA content will be released into the blood. If our reasoning is correct, then the mtDNA common deletion level in blood could serve as a useful biomarker for breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Zhejiang Provincial Natural Science Foundation of China (Grant No. LZ12H12001).

References

- Abnet CC, Huppi K, Carrera A, Armistead D, McKenney K, Hu N, Tang ZZ, Taylor PR, Dawsey SM. Control region mutations and the ‘common deletion’ are frequent in the mitochondrial DNA of patients with esophageal squamous cell carcinoma. *BMC Cancer*. 2004; 4:30. [PubMed: 15230979]
- Agaronyan K, Morozov YI, Anikin M, Temiakov D. Mitochondrial biology. Replication-transcription switch in human mitochondria. *Science*. 2015; 347:548–551. [PubMed: 25635099]
- Becuwe P, Ennen M, Klotz R, Barbieux C, Grandemange S. Manganese superoxide dismutase in breast cancer: from molecular mechanisms of gene regulation to biological and clinical significance. *Free radical biology & medicine*. 2014; 77:139–151. [PubMed: 25224035]
- Brandon M, Baldi P, Wallace DC. Mitochondrial mutations in cancer. *Oncogene*. 2006; 25:4647–4662. [PubMed: 16892079]
- Bresciani G, da Cruz IB, Gonzalez-Gallego J. Manganese superoxide dismutase and oxidative stress modulation. *Advances in clinical chemistry*. 2015; 68:87–130. [PubMed: 25858870]
- Candas D, Li JJ. MnSOD in oxidative stress response-potential regulation via mitochondrial protein influx. *Antioxidants & redox signaling*. 2014; 20:1599–1617. [PubMed: 23581847]
- Carioca AA, Verde SM, Luzia LA, Rondo PH, Latorre MR, Ellery TH, Damasceno NR. Association of oxidative stress biomarkers with adiposity and clinical staging in women with breast cancer. *European journal of clinical nutrition*. 2015
- Chandra D, Singh KK. Genetic insights into OXPHOS defect and its role in cancer. *Biochim Biophys Acta*. 2011; 1807:620–625. [PubMed: 21074512]
- Chen T, He J, Shen L, Fang H, Nie H, Jin T, Wei X, Xin Y, Jiang Y, Li H, Chen G, Lu J, Bai Y. The mitochondrial DNA 4, 977-bp deletion and its implication in copy number alteration in colorectal cancer. *BMC Med Genet*. 2011; 12:8. [PubMed: 21232124]
- Choudhari SK, Chaudhary M, Gadgil AR, Sharma A, Tekade S. Oxidative and antioxidative mechanisms in oral cancer and precancer: a review. *Oral Oncol*. 2014; 50:10–18. [PubMed: 24126222]
- Copeland WC, Longley MJ. Mitochondrial genome maintenance in health and disease. *DNA Repair (Amst)*. 2014; 19:190–198. [PubMed: 24780559]
- Cui H, Kong Y, Zhang H. Oxidative stress, mitochondrial dysfunction, and aging. *Journal of signal transduction*. 2012; 2012:646354. [PubMed: 21977319]
- Dai SH, Chen T, Wang YH, Zhu J, Luo P, Rao W, Yang YF, Fei Z, Jiang XF. Sirt3 protects cortical neurons against oxidative stress via regulating mitochondrial Ca²⁺ and mitochondrial biogenesis. *Int J Mol Sci*. 2014; 15:14591–14609. [PubMed: 25196599]
- Dani MA, Dani SU, Lima SP, Martinez A, Rossi BM, Soares F, Zago MA, Simpson AJ. Less DeltamtDNA4977 than normal in various types of tumors suggests that cancer cells are essentially free of this mutation. *Genetics and molecular research : GMR*. 2004; 3:395–409. [PubMed: 15614730]
- Dayem AA, Choi HY, Kim JH, Cho SG. Role of oxidative stress in stem, cancer, and cancer stem cells. *Cancers*. 2010; 2:859–884. [PubMed: 24281098]
- Desler C, Marcker ML, Singh KK, Rasmussen LJ. The importance of mitochondrial DNA in aging and cancer. *J Aging Res*. 2011; 2011:407536. [PubMed: 21584235]
- Desouki MM, Kulawiec M, Bansal S, Das GM, Singh KK. Cross talk between mitochondria and superoxide generating NADPH oxidase in breast and ovarian tumors. *Cancer Biol Ther*. 2005; 4:1367–1373. [PubMed: 16294028]
- Deus CM, Coelho AR, Serafim TL, Oliveira PJ. Targeting mitochondrial function for the treatment of breast cancer. *Future Med Chem*. 2014; 6:1499–1513. [PubMed: 25365234]
- Dhar SK, St Clair DK. Manganese superoxide dismutase regulation and cancer. *Free radical biology & medicine*. 2012; 52:2209–2222. [PubMed: 22561706]

- Dimberg J, Hong TT, Nguyen LT, Skarstedt M, Lofgren S, Matussek A. Common 4977 bp deletion and novel alterations in mitochondrial DNA in Vietnamese patients with breast cancer. *Springerplus*. 2015; 4:58. [PubMed: 25674508]
- Egan KM, Thompson PA, Titus-Ernstoff L, Moore JH, Ambrosone CB. MnSOD polymorphism and breast cancer in a population-based case-control study. *Cancer Lett*. 2003; 199:27–33. [PubMed: 12963120]
- Fan L, Strasser-Weippl K, Li JJ, St Louis J, Finkelstein DM, Yu KD, Chen WQ, Shao ZM, Goss PE. Breast cancer in China. *Lancet Oncol*. 2014; 15:e279–289. [PubMed: 24872111]
- Gattermann N, Berneburg M, Heinisch J, Aul C, Schneider W. Detection of the ageing-associated 5-Kb common deletion of mitochondrial DNA in blood and bone marrow of hematologically normal adults. Absence of the deletion in clonal bone marrow disorders. *Leukemia*. 1995; 9:1704–1710. [PubMed: 7564514]
- Gonenc A, Tokgoz D, Aslan S, Torun M. Oxidative stress in relation to lipid profiles in different stages of breast cancer. *Indian journal of biochemistry & biophysics*. 2005; 42:190–194. [PubMed: 23923564]
- Jerby L, Wolf L, Denkert C, Stein GY, Hilvo M, Oresic M, Geiger T, Ruppin E. Metabolic associations of reduced proliferation and oxidative stress in advanced breast cancer. *Cancer research*. 2012; 72:5712–5720. [PubMed: 22986741]
- Kim H, Shin MJ, Kim SJ, Kim IJ, Park I. The relation of visualization of internal mammary lymph nodes on lymphoscintigraphy to axillary lymph node metastases in breast cancer. *Lymphatic research and biology*. 2014; 12:295–300. [PubMed: 25144255]
- Kostyrykina NA, Pechkovskiy EA, Boyarskikh UA, Sushko AG, Voronina EN, Lazarev AF, Petrova VD, Zarubina NA, Selezneva IA, Sinkina TV, Terekhova SA, Filipenko ML. Associations of polymorphic variant of MnSOD gene with breast cancer in residents of the Altai Region. *Bull Exp Biol Med*. 2009; 147:84–87. [PubMed: 19526139]
- Kwong A, Shin VY, Ho JC, Kang E, Nakamura S, Teo SH, Lee AS, Sng JH, Ginsburg OM, Kurian AW, Weitzel JN, Siu MT, Law FB, Chan TL, Narod SA, Ford JM, Ma ES, Kim SW. Comprehensive spectrum of BRCA1 and BRCA2 deleterious mutations in breast cancer in Asian countries. *J Med Genet*. 2015
- Lee HC, Hsu LS, Yin PH, Lee LM, Chi CW. Heteroplasmic mutation of mitochondrial DNA D-loop and 4977-bp deletion in human cancer cells during mitochondrial DNA depletion. *Mitochondrion*. 2007; 7:157–163. [PubMed: 17280876]
- Li H, Liu H, Cao L, Shi A, Yang H, Cai W. The complete mitochondrial genome of the damselfly *Alloeorhynchus bakeri* (Hemiptera: Nabidae). *International journal of biological sciences*. 2012; 8:93–107. [PubMed: 22211108]
- Li T, Chen GL, Lan H, Mao L, Zeng B. Prevalence of the 4977-bp and 4408-bp mitochondrial DNA deletions in mesenteric arteries from patients with colorectal cancer. *Mitochondrial DNA*. 2015:1–3.
- Lin CS, Wang LS, Tsai CM, Wei YH. Low copy number and low oxidative damage of mitochondrial DNA are associated with tumor progression in lung cancer tissues after neoadjuvant chemotherapy. *Interactive cardiovascular and thoracic surgery*. 2008; 7:954–958. [PubMed: 18685121]
- Lu J, Sharma LK, Bai Y. Implications of mitochondrial DNA mutations and mitochondrial dysfunction in tumorigenesis. *Cell Res*. 2009; 19:802–815. [PubMed: 19532122]
- Mambo E, Chatterjee A, Xing M, Tallini G, Haugen BR, Yeung SC, Sukumar S, Sidransky D. Tumor-specific changes in mtDNA content in human cancer. *International journal of cancer. Journal international du cancer*. 2005; 116:920–924. [PubMed: 15856456]
- Mandavilli BS, Santos JH, Van Houten B. Mitochondrial DNA repair and aging. *Mutation research*. 2002; 509:127–151. [PubMed: 12427535]
- Markaryan A, Nelson EG, Hinojosa R. Detection of mitochondrial DNA deletions in the cochlea and its structural elements from archival human temporal bone tissue. *Mutation research*. 2008; 640:38–45. [PubMed: 18242646]
- Matsubara K, Komatsu S, Oka T, Kato N. Vitamin B6-mediated suppression of colon tumorigenesis, cell proliferation, and angiogenesis (review). *The Journal of nutritional biochemistry*. 2003; 14:246–250. [PubMed: 12832027]

- Meissner C, Bruse P, Mohamed SA, Schulz A, Warnk H, Storm T, Oehmichen M. The 4977 bp deletion of mitochondrial DNA in human skeletal muscle, heart and different areas of the brain: a useful biomarker or more? *Experimental gerontology*. 2008; 43:645–652. [PubMed: 18439778]
- Meissner C, Mohamed SA, Klueter H, Hamann K, von Wurmb N, Oehmichen M. Quantification of mitochondrial DNA in human blood cells using an automated detection system. *Forensic science international*. 2000; 113:109–112. [PubMed: 10978610]
- Mohamed SA, Wesch D, Blumenthal A, Bruse P, Windler K, Ernst M, Kabelitz D, Oehmichen M, Meissner C. Detection of the 4977 bp deletion of mitochondrial DNA in different human blood cells. *Experimental gerontology*. 2004; 39:181–188. [PubMed: 15036410]
- Mohideen AM, Dicks E, Parfrey P, Green R, Savas S. Mitochondrial DNA polymorphisms, its copy number change and outcome in colorectal cancer. *BMC Res Notes*. 2015; 8:272. [PubMed: 26116242]
- Neuzil J, Moreno-Sanchez R. The bioenergetics of cancer, the warburg hypothesis and the mitochondrial function. *Curr Pharm Biotechnol*. 2013; 14:249–250. [PubMed: 22201592]
- Nie H, Shu H, Vartak R, Milstein AC, Mo Y, Hu X, Fang H, Shen L, Ding Z, Lu J, Bai Y. Mitochondrial common deletion, a potential biomarker for cancer occurrence, is selected against in cancer background: a meta-analysis of 38 studies. *PloS one*. 2013; 8:e67953. [PubMed: 23861839]
- Park WS, Chung KW, Young MS, Kim SK, Lee YJ, Lee EK. Differential protein expression of lymph node metastases of papillary thyroid carcinoma harboring the BRAF mutation. *Anticancer research*. 2013; 33:4357–4364. [PubMed: 24123003]
- Pica A, Di Santi A, D'Angelo V, Iannotta A, Ramaglia M, Di Martino M, Pollio ML, Schiattarella A, Borrelli A, Mancini A, Indolfi P, Casale F. Effect of rMnSOD on survival signaling in pediatric high risk T-cell acute lymphoblastic leukaemia. *Journal of cellular physiology*. 2015; 230:1086–1093. [PubMed: 25294747]
- Pitkanen S, Robinson BH. Mitochondrial complex I deficiency leads to increased production of superoxide radicals and induction of superoxide dismutase. *The Journal of clinical investigation*. 1996; 98:345–351. [PubMed: 8755643]
- Polat MF, Taysi S, Gul M, Cikman O, Yilmaz I, Bakan E, Erdogan F. Oxidant/antioxidant status in blood of patients with malignant breast tumour and benign breast disease. *Cell biochemistry and function*. 2002; 20:327–331. [PubMed: 12415567]
- Santos RX, Correia SC, Zhu X, Smith MA, Moreira PI, Castellani RJ, Nunomura A, Perry G. Mitochondrial DNA oxidative damage and repair in aging and Alzheimer's disease. *Antioxidants & redox signaling*. 2013; 18:2444–2457. [PubMed: 23216311]
- Shen J, Wan J, Song R, Zhao H. Peripheral blood mitochondrial DNA copy number, length heteroplasmy, and breast cancer risk: a replication study. *Carcinogenesis*. 2015
- Shen L, Fang H, Chen T, He J, Zhang M, Wei X, Xin Y, Jiang Y, Ding Z, Ji J, Lu J, Bai Y. Evaluating mitochondrial DNA in cancer occurrence and development. *Annals of the New York Academy of Sciences*. 2010; 1201:26–33. [PubMed: 20649535]
- Shen L, Wei J, Chen T, He J, Qu J, He X, Jiang L, Qu Y, Fang H, Chen G, Lu J, Bai Y. Evaluating mitochondrial DNA in patients with breast cancer and benign breast disease. *J Cancer Res Clin Oncol*. 2011; 137:669–675. [PubMed: 20552226]
- Sokolenko AP, Preobrazhenskaya EV, Aleksakhina SN, Iyevleva AG, Mitiushkina NV, Zaitseva OA, Yatsuk OS, Tiurin VI, Strelkova TN, Togo AV, Imyaninov EN. Candidate gene analysis of BRCA1/2 mutation-negative high-risk Russian breast cancer patients. *Cancer Lett*. 2015; 359:259–261. [PubMed: 25619955]
- Sosa V, Moline T, Somoza R, Paciucci R, Kondoh H, MELL. Oxidative stress and cancer: an overview. *Ageing research reviews*. 2013; 12:376–390. [PubMed: 23123177]
- Suda T, Oba H, Takei H, Kurosumi M, Hayashi S, Yamaguchi Y. ER-activating ability of breast cancer stromal fibroblasts is regulated independently of alteration of TP53 and PTEN tumor suppressor genes. *Biochem Biophys Res Commun*. 2012; 428:259–263. [PubMed: 23068099]
- Sun MJ, Cheng WL, Wei YH, Kuo CL, Sun S, Tsai HD, Lin HM, Liu CS. Low copy number and high 4977 deletion of mitochondrial DNA in uterosacral ligaments are associated with pelvic organ prolapse progression. *International urogynecology journal and pelvic floor dysfunction*. 2009; 20:867–872. [PubMed: 19495551]

- Termini L, Fregnani JH, Boccardo E, da Costa WH, Longatto-Filho A, Andreoli MA, Costa MC, Lopes A, da Cunha IW, Soares FA, Villa LL, Guimaraes GC. SOD2 immunoexpression predicts lymph node metastasis in penile cancer. *BMC clinical pathology*. 2015; 15:3. [PubMed: 25745358]
- Tsanou E, Ioachim E, Briasoulis E, Damala K, Charchanti A, Karavasilis V, Pavlidis N, Agnantis NJ. Immunohistochemical expression of superoxide dismutase (MnSOD) anti-oxidant enzyme in invasive breast carcinoma. *Histology and histopathology*. 2004; 19:807–813. [PubMed: 15168344]
- Tseng LM, Yin PH, Chi CW, Hsu CY, Wu CW, Lee LM, Wei YH, Lee HC. Mitochondrial DNA mutations and mitochondrial DNA depletion in breast cancer. *Genes, chromosomes & cancer*. 2006; 45:629–638. [PubMed: 16568452]
- Upadhyay R, Jain M, Kumar S, ChandGhoshal U, Mittal B. Role of mitochondrial DNA 4977-bp deletions in esophageal cancer susceptibility and prognosis in a northern Indian population. *Cancer Genet Cytogenet*. 2009; 195:175–178. [PubMed: 19963120]
- Vera-Ramirez L, Sanchez-Rovira P, Ramirez-Tortosa MC, Ramirez-Tortosa CL, Granados-Principal S, Lorente JA, Quiles JL. Free radicals in breast carcinogenesis, breast cancer progression and cancer stem cells. Biological bases to develop oxidative-based therapies. *Critical reviews in oncology/hematology*. 2011; 80:347–368. [PubMed: 21288735]
- von Wurmb N, Oehmichen M, Meissner C. Demonstration of the 4977 bp deletion in human mitochondrial DNA from intravital and postmortem blood. *Mutation research*. 1998; 422:247–254. [PubMed: 9838148]
- Wang J, Lu YY. Mitochondrial DNA 4977-bp deletion correlated with reactive oxygen species production and manganese superoxidizedismutase expression in gastric tumor cells. *Chin Med J (Engl)*. 2009; 122:431–436. [PubMed: 19302750]
- Xu Y, Fang F, Dhar SK, Bosch A, St Clair WH, Kasarskis EJ, St Clair DK. Mutations in the SOD2 promoter reveal a molecular basis for an activating protein 2-dependent dysregulation of manganese superoxide dismutase expression in cancer cells. *Molecular cancer research : MCR*. 2008; 6:1881–1893. [PubMed: 19074833]
- Ye C, Shu XO, Wen W, Pierce L, Courtney R, Gao YT, Zheng W, Cai Q. Quantitative analysis of mitochondrial DNA 4977-bp deletion in sporadic breast cancer and benign breast diseases. *Breast cancer research and treatment*. 2008; 108:427–434. [PubMed: 17541740]
- Yeh CC, Hou MF, Tsai SM, Lin SK, Hsiao JK, Huang JC, Wang LH, Wu SH, Hou LA, Ma H, Tsai LY. Superoxide anion radical, lipid peroxides and antioxidant status in the blood of patients with breast cancer. *Clinica chimica acta; international journal of clinical chemistry*. 2005; 361:104–111.
- Yen TC, King KL, Lee HC, Yeh SH, Wei YH. Age-dependent increase of mitochondrial DNA deletions together with lipid peroxides and superoxide dismutase in human liver mitochondria. *Free radical biology & medicine*. 1994; 16:207–214. [PubMed: 8005516]
- Yeon JY, Suh YJ, Kim SW, Baik HW, Sung CJ, Kim HS, Sung MK. Evaluation of dietary factors in relation to the biomarkers of oxidative stress and inflammation in breast cancer risk. *Nutrition*. 2011; 27:912–918. [PubMed: 21353477]
- Yu M, Shi Y, Wei X, Yang Y, Zang F, Niu R. Mitochondrial DNA depletion promotes impaired oxidative status and adaptive resistance to apoptosis in T47D breast cancer cells. *Eur J Cancer Prev*. 2009; 18:445–457. [PubMed: 19609211]
- Zhang Y, Ma Y, Bu D, Liu H, Xia C, Zhu S, Pan H, Pei P, Zheng X, Wang S, Xu Y, Qi Y. Deletion of a 4977-bp Fragment in the Mitochondrial Genome Is Associated with Mitochondrial Disease Severity. *PloS one*. 2015; 10:e0128624. [PubMed: 26024530]
- Zhang Y, Smith BJ, Oberley LW. Enzymatic activity is necessary for the tumor-suppressive effects of MnSOD. *Antioxidants & redox signaling*. 2006; 8:1283–1293. [PubMed: 16910776]
- Zhong Y, Hu YJ, Yang Y, Peng W, Sun Y, Chen B, Huang X, Kong WJ. Contribution of common deletion to total deletion burden in mitochondrial DNA from inner ear of d-galactose-induced aging rats. *Mutation research*. 2011; 712:11–19. [PubMed: 21473872]
- Zhu W, Qin W, Sauter ER. Large-scale mitochondrial DNA deletion mutations and nuclear genome instability in human breast cancer. *Cancer detection and prevention*. 2004; 28:119–126. [PubMed: 15068836]

Fig. 1. Detection of the common deletion frequency in all patients

BC: breast carcinoma, BBD: benign breast disease, Normal: healthy control.

Fig. 2. Correlation analyses between the 4,977 bp deletion level and age for **A.** BC patients and **B.** BBD patients.

Fig. 3. Correlation analyses between the 4,977 bp deletion level and relative mtDNA content for **A.** BC patients and **B.** BBD patients. (b-blood, p-paracarcinoma, c-carcinoma, t-tumor.)

Fig. 4.

A. Peroxide levels measured by a biochemical assay based on xylenol orange-iron complex formation; **B.** The mtDNA oxidative damage was evaluated by qPCR and defined as a ΔC_t ; **C.** Mean plasma levels of malondialdehyde (MDA) in patients (BBD and BC) and controls. (Sample sizes: control=45, BBD=50, BC=46; measured in blood samples.)

Fig. 5. Normalized expression of MnSOD in blood of age-matched healthy donors (ctrl), BBD and BC patients. (Sample sizes: Ctrl=45, BBD=50, BC=46; measured in blood samples.)

Fig. 6.

A model illustrating how the mtDNA common deletion play a central role in breast cancer patients.

Table 1

Detection of the 4,977 bp common deletion in blood in relation to age.

Age (Yr)	Normal blood			BBD blood			BC blood			P-value	OR	P-value [#]	OR [#]	P-value ^{&}	OR ^{&}
	N	With CD	Without CD	N	With CD	Without CD	N	With CD	Without CD						
40	19	0	19	44	4	40	10	3	7		1.00			0.209	4.29
41–50	35	6	29	50	7	43	37	16	21	0.694	1.78	0.016	3.68	0.002	4.68
51–60	33	2	31	21	1	20	38	17	21	0.631	1.89	<0.001	12.55	0.001	16.19
>60	26	2	24	3	0	3	22	15	7	0.102	5.00	<0.001	25.71		
Total	113	10	103	118	12	106	107	51	56			<0.001	9.38	<0.001	8.05

P-value derived from Chi-square test for the detection of the 4,977 bp deletion in blood of BC patients when compared with the age factor.

P-value[#] derived from Chi-square between normal and BC patients in the same age group.

P-value[&] derived from Chi-square between BBD and BC patients in the same age group.

BBD: Breast benign disease; BC: Breast carcinoma; CD: Common deletion; OR: Hazard ratios.

Relationship between the frequency of the 4,977 bp deletion and clinicopathological parameters of BC.

Table 2

Characteristic	Blood				Paracarcinoma				Carcinoma			
	With CD		Without CD		With CD		Without CD		With CD		Without CD	
I	30	27			24	33			27	30		
Tumor Stage II	18	27			19	26			21	24		
III	3	2	0.381		1	4	0.617		2	3	0.612	
+	14	25			15	24			17	22		
Metastasis -	37	31	0.065	0.469	29	39	0.672	0.841	33	35	0.622	0.820
+	25	35			26	34			30	30		
ER [#] -	23	20	0.236	0.621	17	26	0.700	1.170	20	23	0.727	1.150
+	25	36			26	35			29	32		
PR [#] -	23	19	0.168	0.574	17	25	0.828	1.092	20	22	0.994	0.997
+	20	31			20	31			28	23		
Her-2 [#] -	23	20	0.166	0.561	19	24	0.626	0.815	17	26	0.137	1.862
+	24	26			11	27			12	26		
TP53 [#] -	19	19	0.853	0.923	24	26	0.070	2.266	28	22	0.023	2.758
+	30	30			30	30			36	24		
Bcl-2 [#] -	16	18	0.784	1.125	12	22	0.168	1.833	11	23	0.010	3.136

P-value derived from Chi-square test for detection rate of the 4,977 bp deletion in BC patients.

[#] Incomplete data due to the loss of original clinical data.

ER: Estrogen Receptor; PR: Progesterone Receptor; Her-2: Human epidermal growth factor receptor-2;

TP53: Tumor Protein 53; Bcl-2: B-cell lymphoma 2.

Table 3

mtDNA 4,977 bp deletion level in all breast tumor patients.

Characteristic	BBD (n=54)			BC (n=72)		
	Blood	Tissue	Blood	Paracarcinoma	Carcinoma	
With CD	12	51	51	44	50	
Minimum (E-05)	0.347	0.484	0.565	1.370	0.582	
Median (E-05)	1.592	5.963	19.630	25.330	6.086	
Maximum (E-05)	27.02	124.40	215.20	1126.03	182.10	
Mean±SD (E-05)	4.16±7.47 ^{c, e}	15.53±23.97 ^{a, e}	34.76±47.59 ^{b, c, d}	78.90±187.23 ^{b, f}	17.63±29.65 ^{a, d, f}	
95% CI (E-05)	-0.58–8.91	8.79–22.27	21.37–48.15	21.98–135.82	9.20–26.05	

^aBC-c; BBD-t, $p=0.696$;
^bBC-b; p, $p=0.135$;
^cBC-b; BBD-b, $p<0.001$;
^dBC-b; c, $p=0.032$;
^eBBD-b; t, $p=0.006$;
^fBC-c; p, $p=0.037$.

Table 4

Relative mtDNA content for all patients with the 4,977 bp deletion.

Characteristic	BBD (n=54)			BC (n=72)		
	Blood	Tissue	Blood	Paracarcinoma	Carcinoma	
With CD	12	51	51	44	50	
Mean±SD	1.43±1.19 ^b	4.72±4.73 ^{c, d}	0.77±0.83 ^b	1.90±1.98 ^{a, c}	2.17±2.12 ^{a, d}	
Median	1.22	3.54	0.55	1.10	1.43	
95% CI	0.67–2.19	3.39–6.05	0.54–1.01	1.30–2.51	1.57–2.77	
Without CD	42	3	21	28	22	
Mean±SD	1.18±1.13	2.21±1.76	0.52±0.29	1.50±1.79	3.09±4.82	
Median	0.63	2.01	0.50	1.09	1.65	
95% CI	0.83–1.53	–2.15–6.58	0.38–0.65	0.81–2.20	0.95–5.23	
<i>P</i> -value ^e	0.510	0.369	0.173	0.390	0.264	

^aBC-c; *p*=0.528;

^bBC-b; BBD-b, *p*=0.028;

^cBC-p; BBD-t, *p*<0.001;

^dBC-c; BBD-t, *p*=0.001;

^ebetween with CD and not.

Table 5

MnSOD variation sites.

site	Genbank	blood	character	BC (n=93)	BBD (n=96)	Control (n=197)	<i>P</i> ^a	<i>P</i> ^b
-2368	G	G/A	transition	13	13	31	0.697	0.622
-1402	G	G/C	transversion	27	25	46	0.298	0.614
-299	C	C/A	transversion	19	17	33	0.446	0.838
-38	C	C/G	transversion	64	59	98	0.002	0.059
106	A	A/G	transition	64	60	117	0.122	0.610
402	T	T/C	transition	29	26	44	0.105	0.371

^aBC versus Control

^bBBD versus Control