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Mitochondrial DNA Biomarkers of Breast Cancer in Core-Needle Biopsy for Predicting Response to Neoadjuvant Chemotherapy

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ABSTRACT

Core-needle biopsy is employed to determine histological biomarkers for selecting neoadjuvant chemotherapy (NAC) in breast cancer (BC). Mitochondrial metabolism is correlated with tumor progression in BC. Three mitochondrial DNA (mtDNA) biomarkers in core-needle biopsy tissues were implicated in predicting NAC response and survival in BC patients. One-hundred newly-diagnosed patients with BC were recruited and followed-up for at least three years. Core-needle biopsy was applied for sampling BC to evaluate mtDNA copy number, mtDNA oxidation, D-loop mutations, and TP53 mutations. Histologically-related biomarkers, including tumor grade and the immunochemical status of estrogen, progesterone, and human epidermal growth factor 2 receptors, were surveyed. Tumor size was measured through ultrasound before and after NAC, and the pathology of the residual tumor was evaluated after the operation. The results revealed that only 40 patients with BC completed the 3 to 6-year follow-up. A greater number of mtDNA D-loop mutations (β = 46.131, p = 0.001) and lower mtDNA oxidation (β = −10.821, p = 0.001) in core-needle biopsy significantly predicted poor tumor reduction after NAC. However, the absence of mtDNA D-loop mutations in core-needle biopsy samples indicated long survival in BC patients (p = 0.021). Interestingly, a higher copy number (> 580/cell), lower oxidation (< 0.25), and no D-loop mutations in mtDNA

can be identified as reliable surrogates to predict good survival in patients with triple-negative BC. In conclusion, mtDNA biomarkers from core-needle biopsy can provide information to predict the response to NAC and survival in patients with BC.

Introduction

Mitochondria have emerged as key players in tumorigenicity; alterations in mitochondrial DNA (mtDNA) correlate with breast cancer (BC) pathogenesis. Germline and somatic mtDNA mutations are the most commonly mutated genes in BC [1]. Tseng et al. [2] reported that significant changes in mtDNA copy number, mtDNA D-loop mutations, and mtDNA common deletions were present in BC tissue as compared with adjacent healthy tissue. Moreover, mitochondrial dysfunction contributes to the etiology of cancer-related fatigue after chemotherapy (CT) [3]. In BC, the efficacy of CT depends on changes in mitochondrial biogenesis, including alterations in mtDNA copy number and mtDNA D-loop mutations [4-8]. Oxidative stress in mtDNA, indirectly measured by detecting 8-hydroxy-2′-deoxyguanosine (8-OHdG) accumulation in mtDNA, can predict the response to CT [9,10].

Core-needle biopsy, in addition to surgical pathology, provides critical information on histological biomarkers in BC before neoadjuvant chemotherapy (NAC) to reduce the tumor burden [11]. In the present study, biological information of mtDNA biomarkers, namely, mtDNA oxidation, mtDNA copy number, and mtDNA D-loop mutation, was retrieved from BC tissue via core-needle biopsy to evaluate the power of outcome prediction in BC patients after NAC.

Materials and Methods

Patients

One-hundred newly-diagnosed patients with BC who were not treated with CT or radiotherapy were recruited from the BC clinic at Changhua Christian Hospital, Taiwan, from 2014 to 2018. The demographic data, including average age; clinical subtype (luminal A, luminal HER2, HER2 overexpression, luminal B, and triple-negative BC [TNBC]); clinical stage; TNM stage; histological grade of the tumor; and immunohistochemical staining of estrogen receptor (ER), progesterone receptor (PR) ,and human epidermal growth factor receptor 2 (HER2) are shown in Table 1. Clinical subtypes were classified on the basis of the St. Gallen Consensus 2011 [12]. All patients underwent mastectomy or lumpectomy to evaluate the effect of NAC and were clinically followed-up after at least three years. The study was approved by the Institutional Review Board of Changhua Christian Hospital (No. 130810) and was conducted according to the principles of the Declaration of Helsinki.

Staining core-needle biopsy tissue from patients with BC

Core-needle biopsy tissues obtained from patients with BC before NAC were fixed in formaldehyde and embedded in paraffin blocks. The expressions of ER, PR, and HER2 were determined using immunohistochemical staining of 4-μm sections of paraffin-embedded tissue, as described previously [13]. Briefly, the tissue sections were deparaffinized, and antigen retrieval was performed in citrate buffer. The sections were covered with cling film and heated at 100°C for 10 min. Endogenous peroxides were inactivated with 3% H_2O_2 in methanol for 15 min. The slides were blocked with blocking buffer (3% bovine serum albumin in phosphate-buffered saline) for 30 min, incubated with primary monoclonal antibodies against ER, PR, and HER2 (Thermo Fisher Scientific, Pittsburgh, PA) overnight at 4°C, washed with phosphate-buffered saline with Tween-20, and incubated with a peroxidase-conjugated goat anti-mouse antibody for one hour. After 3,3'-Diaminobenzidine (DAB) staining, the slides were counterstained with hematoxylin and mounted with glass coverslips. Three specimens were prepared and examined for each sample. We randomly selected four areas on each slide to

Table 1. Demographic data of patients with breast cancer

Demographic	Values[a]
Number	40
Age (years)	56 ± 9
Clinical subtype, n (%)	
Luminal A	18 (45)
Luminal HER2	3 (8)
HER2 over-expression	5 (12)
Luminal B	4 (10)
TNBC	10 (25)
Stage, n (%)	
1	1 (3)
2	25 (62)
3	12 (30)
4	2 (5)
TNM (range)	
Tumor size (1–4)	2.4 ± 0.7
Lymph nodes (0–3)	0.8 ± 0.8
Metastasis (0–1)	0.1 ± 0.2
Grade (range)	
1–3	2.4 ± 0.6
Histochemical stain (range)	
ER (0–3)	1.8 ± 1.3
PR (0–3)	1.4 ± 1.3
HER2 (0–3)	1.6 ± 1.2
TP53 mutation (range)	
0–2	0.2 ± 0.4
Chemotherapy regime	
CT-A	FEC/D[b]
CT-B	DE/DH[c]
CT-C	DP/FDL/CMF[d]
Cycle number of chemotherapy mtDNA biomarkers	6.9 ± 1.6
mtDNA copy number (/cell)	580 (186–2,693)
mtDNA oxidation	0.25 (0.03–1.38)
mtDNA D-loop mutation	< 1 (0–8)

[a]Values were presented as mean ± standard deviation, number (%), or median (min–max).
[b]FEC/D: 5-fluorouracil, epirubicin, cyclophosphamide followed by docetaxel.
[c]DE/DH: docetaxel, epirubicin followed by docetaxel, Herceptin.
[d]DP/FDL/CMF: docetaxel, perjeta followed by 5-fluorouracil, docetaxel, leucovorin and cyclophosphamide, methotrexate, and 5-fluorouracil.
CT-A: chemotherapy-A; CT-B: chemotherapy-B; CT-C: chemotherapy-C; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; mtDNA: mitochondrial DNA;; PR, progesterone receptor; TNBC, triple-negative breast cancer.

evaluate staining intensity using a semiquantitative scoring system based on the percentage of positively-stained cells as follows: grade 0 for no expression, grade 1 for low expression (< 10% positive cells), grade 2 for median expression (10%–75% positive cells), and grade 3 for high expression (> 75% positive cells). Histological grading of BC was performed using hematoxylin and eosin staining and assessed using the Nottingham grading system [14].

Measurement of mtDNA copy number in leukocytes and BC tissue obtained through core-needle biopsy

The mtDNA copy number was analyzed in tissue samples from core-needle biopsy. DNA was extracted using a Gentra Puregene DNA Kit (Qiagen, Germany). In brief, the *ND1* gene (primers F 5′-AACATACCCATGGCCAACCT-3′ and R 5′-AGCGAAGGGTTGTAGTAGCCC-3′) in mtDNA and the β-globin gene (F 5′-GAAGAGCCAAGGACAGGTAC-3′ and R 5′-CAACTTCATCCACGT TCACC-3′) in nuclear DNA were amplified using a LightCycler 480 Instrument (Roche, Mannheim, Germany). The analysis was conducted using a previously-described protocol with some modifications [15,16]. The mtDNA content was calculated using the formula $2 \times 2^{(\beta\text{-globin cycle threshold (Ct) -ND1 Ct})}$ [16].

Measuring mtDNA oxidation in core-needle biopsy tissue

DNA was treated with 1 U of human 8-oxoguanine DNA glycosylase (hOGG1) at 37°C for 2 hr, after which 8-OHdG was removed to form an abasic site. The mitochondrial *ND1* gene primers 5′-AACATACCCATGGCCAACCT-3′ and 5′-AGCGAAGGGTTGTAGTAGCCC-3′ were subsequently used to detect mtDNA damage by LightCycler 480 Real-Time PCR (Roche, Mannheim, Germany). Oxidative mtDNA damage was determined by calculating the difference between Ct values (ΔCt) before and after treatment with hOGG1. The greater the ΔCt, the greater the abundance of 8-OHdG and the greater the degree of oxidative mtDNA damage [17].

Analysis of somatic mutations in the mtDNA D-loop

Primers *mtL16190* (5′-CCCCATGCTTACAAGCAAGT-3′) and *mtH602* (5′- GCTTTGAGGAGGTAAGCTAC-3′) were used to amplify mtDNA D-loop fragments. PCR was performed using a MJ Research Thermal Cycler. Somatic mutations were confirmed through the sequencing of PCR products using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3130xl genetic analyzer. Somatic mutations were identified through comparison with the mtDNA D-loop sequences of leukocytes and core-needle biopsy tissue.

Somatic mutation analyses of the exons of *TP53*

Mutations in exons 5–8 of *TP53* constitute 94.2% of all somatic mutations in the IARC database, version R11 [18]. Two primer pairs, TP53-5F (5′-GTTTCTTTGCTGCCGTCTT-3′) and TP53-6R (5′-ACTGACAACCACCCTTAACC-3′) and TP53-7F (5′-ATCTTGGGCCTGTGTTAT-3′) and TP53-8R (5′-AGAGGCAAGGAAAGGTGATA-3′), were used to amplify exons 5–8 of *TP53*. The analysis methods were performed according to the aforementioned protocol in the MJ Research Thermal Cycler PCR and DNA sequencing.

Response to NAC

The clinical response was evaluated through physical examination and 2D ultrasonography on the basis of the Response Evaluation Criteria in Solid Tumors (RECIST 1.1). Four types of clinical responses were defined as follows: complete response (CR), for complete disappearance of all known lesions; partial response (PAR), for at least a 30% decrease in the sum of the longest diameter of the target lesion; progressive disease (PD), for at least a 20% increase in the sum of the longest width of the primary lesion; and stable disease (SD), for neither sufficient shrinkage to qualify for CR or PAR nor sufficient increase to qualify for PD [19]. Pathologic complete response (pCR) was defined as the absence of tumor cells from dissected BC tissue after NAC.

Statistical analyses

The chi-square, likelihood ratio, or Fisher's exact tests were used to identify potential mtDNA biomarkers for predicting the clinical and pathological responses to NAC matched with biomarkers in tumor pathology (Tables 2–4). To evaluate the potential of mtDNA biomarkers for predicting tumor reduction after CT, a generalized linear model (GLM) was used, with adjustment for confounding factors, including the neoadjuvant panel, cancer stage, TNM, tumor grade, *TP53* mutation, and immunohistochemical staining (Table 5). Survival analysis was performed using the Kaplan–Meier method, and differences among subgroups were evaluated using the log-rank test (Figure 1). All statistical analyses were performed using the SPSS software package (version 17; SPSS, Chicago, IL), and a p value < 0.05 was considered statistically significant.

Results

Only 40 patients with BC completed at least three years of follow-up without withdrawing from this clinical study. With respect to clinical classification, a higher prevalence was found for luminal A and TNBC, at 45% and 25%, respectively. Approximately 92% of the patients had clinical stage II–III BC. For most patients, the histological grade was moderate. The average intensity for ER, PR, and HER2 staining was 1.4 ± 1.3 to 1.8 ± 1.3, and the average *TP53* mutation intensity was 0.2 ± 0.4. We used three CT protocols, including the CT-A panel (FEC/D: 5-fluorouracil, epirubicin, cyclophosphamide followed by docetaxel), CT-B panel (DE/DH: docetaxel, epirubicin followed by docetaxel, herceptin), and CT-C panel (DP/FDL/CMF: docetaxel, perjeta followed by 5-fluorouracil, docetaxel, leucovorin and cyclophosphamide, methotrexate, and 5-fluorouracil). The average number of cycles was 6.9 ± 1.6. The median values for mtDNA copy number and mtDNA oxidation were 580/cell and 0.25, respectively (Table 1). The mtDNA copy number and D-loop mutations were not correlated with clinical or histological biomarkers; however, increased mtDNA oxidation was correlated with increased tumor grade ($p = 0.038$) (Table 2). Unfortunately, we failed to reveal any interaction of mtDNA biomarkers with other recognized prognostic factors, including *TP53* mutations, ER, PR, and HER2 status.

Clinical response analysis revealed that clinical or histological biomarkers did not predict the impact of the clinical response after NAC. However, pathological response analysis revealed that patients aged less than 50 years ($p = 0.042$) or who chose the CT-B panel of NAC (DE/DH: docetaxel, epirubicin/docetaxel, herceptin) ($p = 0.001$) had a high prevalence of pCR in the pathology survey (Table 3). In a correlation study between pCR and three mtDNA biomarkers, BC patients without mtDNA D-loop mutations had 18% pCR after NAC; however, BC patients with mtDNA D-loop mutations had 0% pCR after NAC ($p = 0.023$, Table 4). No significant difference was found between mtDNA copy number/mtDNA oxidation and clinical/pathological responses (Table 4). However, using quantitative GLM, clinical stage, tumor size, mtDNA oxidation ($p = 0.001$), and mtDNA D-loop mutations ($p = 0.001$) were found to significantly predict the response of BC patients after NAC. According to β, an increase of one unit of mtDNA oxidation predicts 46.1% tumor reduction after NAC; an increase of one mtDNA D-loop mutation predicts 10.8% tumor growth after NAC (Table 5). Sequencing of *TP53* revealed 19 reported loci of mutations, and c.524G>A/c.818G>A mutations appeared repetitively (Table 6). A total of 13 homoplasmic or heteroplasmic mtDNA D-loop mutations were detected, of which homoplasmic c.441C>G has been reported for the first time in BC (Table 7). Interestingly, 4/13 mtDNA D-loop mutations had a poly-C cluster at np 303–309.

In the survival study, mtDNA D-loop mutations, but not copy number or oxidation, could predict a reduced survival rate ($p = 0.021$) (Figure 1A–1C). Interestingly, patients with TNBC, with a higher mtDNA copy number (> 580), lower mtDNA oxidation (< 0.25), and no mtDNA D-loop mutations, had a 100% survival rate within three years after NAC (Figure 1D–1F).

Table 2. Correlation between clinical and histological biomarkers and mitochondrial DNA biomarkers in core-needle biopsy

Item	mtDNA copy number(/cell)				mtDNA oxidation				mtDNA D-Loop mutation			
	< 580[a]	≥ 580[a]	χ^2	p value[b]	< 0.25[a]	≥ 0.25[a]	χ^2	p value[b]	0[a]	> 0[a]	χ^2	p value[b]
Age												
< 50	6	4	0.533	0.716	3	7	2.707	0.148	5	5	0.135	0.714
> 50	14	16			18	12			17	13		
Tumor size												
T1/T2	14	13	0.114	0.736	15	12	0.311	0.577	15	12	0.010	0.919
T3/T4	6	7			6	7			7	6		
Lymph nodes												
N0	7	6	0.114	0.736	7	6	0.014	0.906	8	5	0.333	0.564
N1-3	13	14			14	13			14	13		
Metastasis												
0	18	20	2.105	0.487	20	18	0.005	1.000	20	18	1.722	0.492
1	2	0			1	1			2	0		
Clinical stage												
I, II	13	13	0.000	1.000	13	13	0.186	0.666	15	11	0.218	0.641
III, IV	7	7			8	6			7	7		
ER												
–	7	6	0.114	0.736	8	5	0.631	0.427	8	5	0.333	0.564
+	13	14			13	14			14	13		
PR												
–	7	8	0.107	0.744	7	8	0.327	0.567	10	5	1.320	0.251
+	13	12			14	11			12	13		
HER2												
–	3	7	2.133	0.273	4	6	0.835	0.473	4	6	1.212	0.300
+	17	13			17	13			18	12		
Grade												
I, II	15	13	0.476	0.490	18	10	5.199	0.038*	15	13	0.077	0.781
III	5	7			3	9			7	5		
TP53												

Table 2. Correlation between clinical and histological biomarkers and mitochondrial DNA biomarkers in core-needle biopsy (continued)

Item	mtDNA copy number(/cell)				mtDNA oxidation				mtDNA D-Loop mutation			
	< 580[a]	≥ 580[a]	χ^2	p value[b]	< 0.25[a]	≥ 0.25[a]	χ^2	p value[b]	0[a]	> 0[a]	χ^2	p value[b]
–	18	15	1.558	0.407	17	16	0.073	1.000	19	14	0.505	0.680
+	2	5			4	3			3	4		

[*]p < 0.05 is considered a statistically significant difference.
[a]Values were presented as median.
[b]p value calculated using chi-square, likelihood ratio, or Fisher's exact tests.
ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; mtDNA: mitochondrial DNA; PR: progesterone receptor.

Table 3. Correlation between clinical and histological biomarkers in core-needle biopsy and clinical and pathological response

Item	Clinical response				Pathological response			
	CR+PAR (n)	PD+SD (n)	χ^2	p value[e]	No-pCR (n)	pCR (n)	χ^2	p value[e]
Age								
< 50	3	7	0.853	0.471	7	3	5.926	0.042[*]
≥ 50	14	16			29	1		
Tumor size								
T1, T2	11	16	0.105	1.000	25	2	0.620	0.584
T3, T4	6	7			11	2		
Lymph nodes								
N0	5	8	0.129	1.000	12	1	0.114	1.000
N1-3	12	15			24	3		
Metastasis								
0	16	22	0.048	1.000	35	3	3.743	0.192
1	1	1			1	1		
Clinical stage								
I, II	11	15	0.001	0.973	24	2	0.440	0.602
III, IV	6	8			12	2		
ER								
–	6	7	0.105	0.746	12	1	0.114	1.000

Table 3. Correlation between clinical and histological biomarkers in core-needle biopsy and clinical and pathological response (continued)

Item	Clinical response				Pathological response			
	CR+PAR (n)	PD+SD (n)	χ^2	p value[e]	No-pCR (n)	pCR (n)	χ^2	p value[e]
+	11	16			24	3		
PR								
–	6	9	0.061	0.804	12	3	2.667	0.139
+	11	14			24	1		
HER2								
–	5	5	0.307	0.580	10	0	1.481	0.556
+	12	18			26	4		
Grade								
I, II	11	17	0.395	0.530	26	2	0.847	0.570
III	6	6			10	2		
TP53								
–	14	19	0.000	1.000	29	4	0.943	1.000
+	3	4			7	0		
CT								
CT-A: FEC/D[a]	12	12	1.466	0.480	23	1	21.019	0.000*
CT-B: DE/DH[b]	1	3			1	3		
CT-C: DP/FDL/CMF[c]	4	8			12	0		
Cycle of CT[d,e]								
< 8	8	12	0.102	0.749	17	3	1.111	0.605
≥ 8	9	11			19	1		

*$p < 0.05$ is considered a statistically significant difference.

[a]FEC/D: 5-fluorouracil, epirubicin, cyclophosphamide followed by docetaxel.

[b]DE/DH: docetaxel, epirubicin followed by docetaxel, Herceptin.

[c]DP/FDL/CMF: docetaxel, perjeta followed by 5-fluorouracil, docetaxel, leucovorin and cyclophosphamide, methotrexate, and 5-fluorouracil.

[d]Values were presented as median.

[e]The p-value calculated using chi-square, likelihood ratio, or Fisher's exact tests.

CR: complete response; CT: chemotherapy; CT-A, chemotherapy-A; CT-B: chemotherapy-B; CT-C: chemotherapy-C; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; pCR, pathologic complete response; PAR: partial response; PD: progressive disease; PR: progesterone receptor; SD: stable disease.

Table 4. Relationships between mitochondrial DNA biomarkers in core-needle biopsy and clinical and pathological responses after neoadjuvant chemotherapy

mtDNA biomarkers	Clinical response				Pathological response			
	CR+PAR (n)	PD+SD (n)	χ^2	p value[a]	No-pCR (n)	pCR (n)	χ^2	p value[a]
mtDNA copy number								
< 580	8	12	0.102	0.749	16	4	4.444	0.106
≥ 580	9	11			20	0		
mtDNA oxidation								
< 0.25	8	13	0.351	0.554	19	2	0.111	1.000
≥ 0.25	9	10			17	2		
mtDNA D-Loop mutation								
0	10	12	0.175	0.676	18	4	5.145	0.023*
> 0	7	11			18	0		

*p < 0.05 is considered a statistically significant difference.
[a]The p value calculated using chi-square, likelihood ratio, or Fisher's exact tests.
CR: complete response; mtDNA: mitochondrial DNA; pCR: pathologic complete response; PD: progressive disease; PAR: partial response; SD: stable disease.

Table 5. Predictive power of clinical, histological, and mtDNA biomarkers for tumor reduction (%) after neoadjuvant chemotherapy

Factors	Tumor reduction (%)			
	β	SE	95% CI	p
Chemotherapy regime				
CT-A	–9.466	9.625	–28.330 to 9.398	0.325
CT-B	14.862	16.470	–17.418 to 47.141	0.367
CT-C	0			
Cycles of CT	4.895	2.528	–0.060 to 9.850	0.053
Clinical/histologic biomarkers				
Age	–0.699	0.539	–1.756 to 0.358	0.195
Stage (1–4)	39.768	18.428	3.649–75.887	0.031*
Tumor size (1–4)	–37.011	11.204	–58.971 to –15.051	0.001
Lymph node (0–3)	–7.071	9.075	–24.859 to 10.716	0.436
Metastasis (0–1)	47.245	20.403	7.255–87.235	0.021*
Grade (1–3)	7.828	9.996	–11.765 to 27.420	0.434
TP53 (0–2)	–12.740	12.686	–37.603 to 12.124	0.315
ER (0–3)	–8.898	5.065	–18.825 to 1.030	0.079
PR (0–3)	6.430	5.232	–3.824 to 16.684	0.219
HER2 (0–3)	8.888	5.079	–1.067 to 18.843	0.080
mtDNA biomarkers				
mtDNA copy number	–0.002	0.012	–0.024 to 0.021	0.882
mtDNA oxidation	46.131	14.121	18.454–73.809	0.001*
mtDNA D-loop mutation	–10.821	3.256	–17.201 to –4.440	0.001*

*p < 0.05 is considered a statistically significant difference.
CI: confidence interval; CT: chemotherapy; CT-A: chemotherapy-A; CT-B: chemotherapy-B; CT-C: chemotherapy-C; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; mtDNA: mitochondrial DNA; PR: progesterone receptor; SE: standard error.

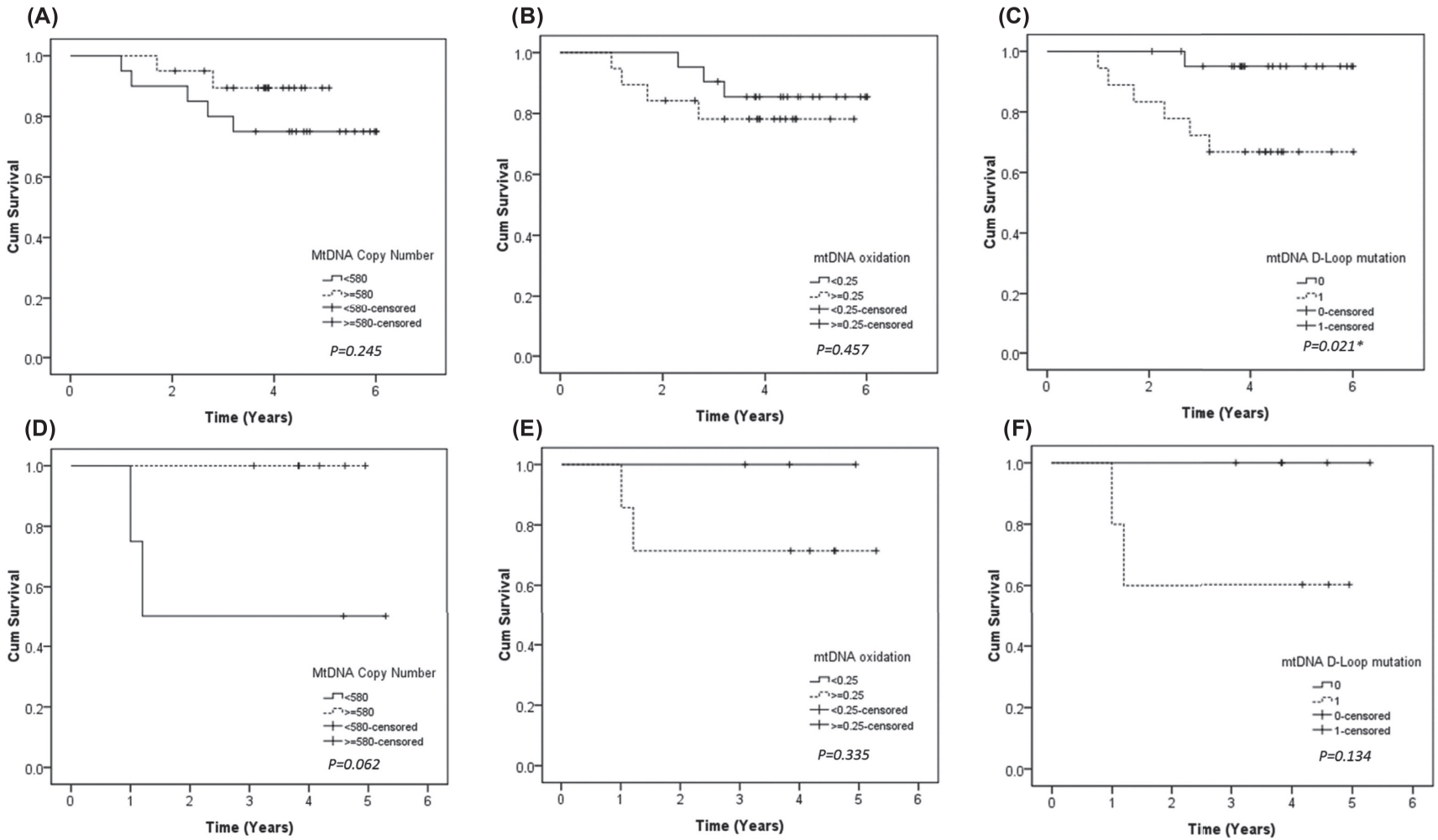

Figure 1. Survival curves of patients with breast cancer for three mitochondrial biomarkers. Time curves arranged by mitochondrial DNA (mtDNA) (A) copy number (p = 0.245), (B) oxidation (p = 0.457), and (C) D-loop mutations (p = 0.021) in all patients (N = 40). Time curves arranged by mtDNA (D) copy number (p = 0.062), (E) oxidation (p = 0.335), and (F) D-loop mutations (p = 0.134) in patients with triple-negative breast cancer (TNBC) (n = 10).

Discussion

This study contributes to our understanding of mtDNA biomarkers in tissues obtained through core-needle biopsy from patients with BC. Tseng et al. [20] reported that mtDNA copy number, oxidative stress in mtDNA, and somatic mtDNA D-loop mutations in BC tissue can be correlated with patient outcomes and survival but poorly-controlled in a panel of NACs and pathologic biomarkers from needle biopsy. In our previous study without information on NAC or biomarkers from needle biopsy, the number of somatic mtDNA D-loop mutations indicated poor prognosis through a mechanism independent of *TP53* [21]. In the present study, with the addition of information from panels of NACs and biomarkers from needle biopsy, the number of mtDNA D-loop mutations could predict not only the patient's survival but also the response to NAC. Moreover, we revealed a new marker, mtDNA oxidation (8-OHdG-related index), which is positively correlated with the histological grade of BC and accurately predicts tumor reduction after NAC. However, alterations in mtDNA oxidation fail to predict short-term survival outcomes in patients with BC. Notably, a high mtDNA copy number (> 580/cell), low mtDNA oxidation (< 0.25), and no mtDNA D-loop mutations are good surrogates for long-term survival in patients with TNBC.

MtDNA D-loop somatic mutations as biomarkers

The D-loop is a noncoding region in mtDNA that controls mtDNA replication [22]. Germline and somatic mutations are risk factors involved in the tumorigenesis and distal metastasis of BC [23, 24]. A report in 2020 on Malaysian women with BC revealed somatic mutations detected in the mtDNA D-loop, which correlated with genomic instability, tumor initiation, and metastasis [25]. In a study of familial BC, 72.7% of all mtDNA mutations were in the D-loop where genomic instability was also detected, predisposing individuals to cancer initiation and progression [26]. In another study, somatic mutations in the D-loop of mtDNA, especially heteroplasmy in the poly-C tract located between nucleotides 303 and 315, known as D310, correlated with mtDNA instability and early progression of BC [27]. Our findings revealed that mtDNA D-loop mutations, especially mutations in the D310 region, can reliably predict poor survival in patients with BC and indicate a lower pCR after NAC. In stage III colon cancer, fluorouracil-based NAC was only beneficial for patients without D-loop mutations [28]. However, in head and neck squamous cell carcinoma, D-loop mutations do not predict patient survival or response to NAC [29,30]. Thus, although D-loop mutations are an index of tumor progression, the tumor specificity of the biomarker determines the response to NAC.

Biomarkers of mtDNA oxidation

The role of oxidative stress in predicting BC initiation and progression is ambiguous [31]. Yuzefovych et al. [32] demonstrated that reduced mtDNA oxidation or overexpression of *hOGG-1* suppresses tumorigenesis. In the present study, BC patients with higher tumor grades (e.g., Grade III) presented higher mtDNA oxidation levels in tumor tissue than patients with lower grades (e.g., Grades I and II). Our findings are consistent with those of previous studies indicating that elevated oxidative stress in mitochondria promotes tumor growth and metastasis. NAC-associated oxidative stress in tumor tissue was found to affect the response to NAC [33]. Atukeren et al. [34] reported that patients with BC experienced greater oxidative stress during CT and increased serum levels of thiobarbituric acid-reactive substances, total nitrite/nitrate, and 8-OHdG. Increasing oxidative stress through *UCP2* inhibition sensitized BC cells to cisplatin or tamoxifen [35]. In this study, an increase in mtDNA oxidation predicted a good response to NAC, probably due to the synergism between endogenous oxidative stress produced in the mitochondria and exogenous oxidative stress from NAC. Thus, mtDNA oxidation robustly predicts the response to NAC but cannot predict survival outcomes in BC patients or rapid tumor growth after NAC.

MtDNA copy number as a biomarker

The mtDNA copy number is a sensitive marker of the cellular redox status, genomic modification, and oxidant/antioxidant balance [15]. TNBC cell lines demonstrate a high degree of variability in mitochondrial dysfunction, including the metabolic switch to glycolysis and reduced mtDNA content [36,37]. Weerts et al. revealed that fewer than 350 mtDNA copies per cell predict poor prognosis for metastasis in patients with BC [38]. In recent years, we developed a mitochondrial transplantation technique to increase the number of mitochondria to suppress chemoresistance in BC. Transplanting healthy mitochondria, including healthy mtDNA, into BC cell lines, including MCF-7 and MDA-MB-231, increased the susceptibility of both cell lines to doxorubicin and paclitaxel by suppressing Drp-1 phosphorylation and inducing the nuclear translocation of apoptosis-inducing factor. However, transplantation of dysfunctional mitochondria, including mutant mtDNA (minic mtDNA D-loop mutation), promoted glycolysis, reduced mitochondrial respiration, and increased resistance to CT [39]. In this study, alterations in mtDNA copy number did not predict the response to NAC, but high mtDNA content, low mtDNA oxidation, and the absence of mtDNA D-loop mutation predicted good prognosis, especially in patients with TNBC. These results are consistent with reports indicating that the functional or genetic heterogeneity of mitochondria in BC can predict the risk of metastasis and prognosis prediction and risk stratification [36, 40].

Limitations of the study

This study is based on a relatively small sample size (N = 40) of patients with long-term follow-up, which may limit the statistical power of the findings and affect the robustness of the data. This limitation could influence the thorough analysis of the relationship between mtDNA biomarkers and other pathogenic biomarkers (e.g., TP53, ER, PR, and HER2), as well as the potential impact of various CT regimens (e.g., CT-A, CT-B, and CT-C). Furthermore, the data were derived from a single institution, which may limit the generalizability of the results. Larger, multi-center cohorts are needed to provide more robust conclusions regarding the reliability of BC biomarkers.

In conclusion, we found that three mtDNA biomarkers identified using core-needle biopsy samples from patients with BC before NAC could predict clinical and pathological responses and survival. MtDNA D-loop mutations are predictors of the response to NAC and tumor progression in patients with BC. Moreover, we identified that greater mtDNA oxidation in BC is associated with greater severity of tumorgenicity and is positively correlated with tumor shrinkage after NAC but fails to predict survival outcomes in patients with BC. However, although the mtDNA copy number in BC tissue does not predict the response to NAC, it appears to be valuable in predicting survival outcomes in patients with TNBC, especially those with a high mtDNA copy number, low mtDNA oxidation, and no mutations in the mtDNA D-loop. Finally, these three mtDNA biomarkers can guide physicians in predicting the response to NAC for early monitoring of BC progression regardless of the choice of CT.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Table 6. *TP53* mutations in core needle biopsy tissue from patients with breast cancer

Nucleotide change	Amino acid	Exon	Mutation type	N	Other mutations at same codon	Reference
c.380C>T	p. S127F	5	missense	1	—	Kato S et al., 2003 [41]
c.476C>A	p. A159D	5	missense	1	—	Guinee et al., 1995 [42]
c.489C>T	p. Y163Y	5	silent	1	—	McKenzie al., 1997 [43]
c.524G>A	p. R175H	5	missense	2	—	Hollstein et al., 1991 [44]
c.578A>T	p. H193L	6	missense	1	—	Neri et al., 1993 [45]
c.590T>A	p. V197E	6	missense	1	—	Konishi et al., 1993 [46]
c.600T>A	p. N200K	6	missense	1		Lin Y et al., 1996 [47]
c.645T>G	p. S215R	6	missense	1	—	Taguchi et al., 1994 [48]
c.659A>G	p. R175H	6	missense	1	—	Bennett et al., 1991 [49]
c.733G>A	p. G245S	7	missense	1	—	Portier et al., 1992 [50]
c.743G>A	p. R248Q	7	missense	1	—	Hollstein et al., 1990 [51]
c.783T>A	p. S261R	8	missense	1	—	Kringen et al., 2005 [52]
c.817C>T	p. R273C	8	missense	1	—	Ishioka et al., 1991 [53]
c.818G>A	p. R273H	8	missense	2	—	Chiba et al., 1990 [54]
c.832C>T	p. P278S	8	missense	1	—	Hollstein et al., 1990 [51]
c.838A>G	p. R280G	8	missense	1	—	Sheu et al., 1992 [55]
c.839G>A	p. R280K	8	missense	1		Ziegler et al., 1993 [56]
c.853G>A	p. E285K	8	missense	1	—	Osborne et al., 1991 [57]
c.913A>T	p. K305*	8	nonsense	1	—	Hollstein et al., 1991 [44]

Table 7. Somatic mitochondrial DNA D-loop mutations in core-needle biopsy samples from patients with breast cancer

np	CRS	Somatic mutation	normal→tumor	function	N	Previously reported tumor	References
249	A	del A	homo→hetero	CR/HV2	1	Ovarian	Liu et al., 2001 [58]
251	G	G→A	homo→homo	CR/HV2	1	POLG/PEO muscle	Del Bo et al., 2003 [59]
303-309	7C	7C→8C	homo→hetero	CR/CSB2	1	Multiple tumor types	Tan et al., 2002 [60]
	7C	8C→9C	homo→hetero		2		Wu et al., 2005 [61]
	7C	9C→7C	homo→hetero		1		Sanchez-Cespedes et al., 2001 [62]
	7C	9C→8C	homo→hetero		4		Legras et al., 2008 [63]
324	C	C→G	homo→hetero	CR/HV2	2	POLG/MNGIE muscle	Del Bo et al., 2003 [59]
386	C	C→A	homo→homo	CR/OHR	1	Bladder	Brandon et al., 2006 [64]
441	C	C→G	homo→homo	CR/mtF1	1	None	Present study
456	C	C→T	homo→homo	CR/HV3	1	Thyroid	Máximo et al., 2002 [65]
16304	T	T→C	homo→homo	CR/HV1	1	Esophageal, breast & prostate	Brandon et al., 2006 [64]
16390	G	G→A	homo→homo	CR/HV1	1	Breast, ovarian	Bragoszewski et al., 2008 [66]
16519	T	T→C	homo→hetero	CR/7SDNA	1	Glioblastoma, gastric, lung, ovarian, prostate	Wu et al., 2005 [61]

CRS: Cambridge reference sequence; CSB2: conserved sequence block 2; hetero: heteroplasmic; homo: homoplasmic; HV: hypervariable; MNGIE: mitochondrial neurogastrointestinal encephalomyopathy; mtTF1: mitochondrial TF1 binding site; PEO: progressive external ophthalmoplegia; POLG: progressive external ophthalmoplegia OHR: H-strand origin.

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