Mitochondrial DNA alterations correlate with the pathological status and the immunological ER, PR, HER-2/neu, p53 and Ki-67 expression in breast invasive ductal carcinoma

CHEN-SUNG LIN[1,2,6*], SHI-CHUAN CHANG[3,10*], LIANG-HUNG OU[2,7], CHIEN-MING CHEN[8], SOPHIE SWEN-WAN HSIEH[9], YU-PING CHUNG[9], KUANG-LIANG KING[2,11], SHOEI-LOONG LIN[2,7,12] and YAU-HUEI WEI[1,2,4,5]

[1]Institute of Clinical Medicine, [2]Faculty of Medicine, [3]Institute of Emergency and Critical Care Medicine, and [4]Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei 112; [5]Department of Medicine, Mackay Medical College, New Taipei City 252; [6]Division of Thoracic Surgery and [7]Division of General Surgery, Department of Surgery, [8]Division of Hematology and Oncology, Department of Internal Medicine, and [9]Department of Pathology, Taipei Hospital, Ministry of Health and Welfare, New Taipei City 242; [10]Division of General Chest Medicine, Department of Chest, and [11]Division of General Surgery, Department of Surgery, Taipei Veterans General Hospital, Taipei 112; [12]Chon-inn Hospital, Chon-inn Healthcare Corp. Aggregate, New Taipei City 220, Taiwan, R.O.C.

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Abstract. We analyzed the changes in mitochondrial DNA (mtDNA) copy numbers and the shifting of mtDNA D310 sequence variations (D310 mutation) with their relationships to pathological status and the expression levels of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER-2/neu), tumor-suppressor protein p53 and cellular proliferation protein Ki-67 in breast invasive ductal carcinoma (BIDC), respectively. Fifty-one paraffin-embedded BIDCs and their paired non-cancerous breast tissues were dissected for DNA extraction. The mtDNA copy number and mtDNA D310 sequence variations were determined by quantitative real-time polymerase chain reaction (q-PCR) and PCR-based direct sequencing, respectively. The expression levels of ER, PR, HER-2/neu, p53 and Ki-67 were determined by immunohistochemical (IHC) staining. Compared to the paired non-cancerous breast tissues, 24 (47.1%) BIDCs had elevated mtDNA copy numbers and 29 (56.9%) harbored mtDNA D310 mutations. Advanced T-status (p=0.056), negative-ER (p=0.005), negative-PR (p=0.007), positive-p53 (p=0.050) and higher Ki-67 (p=0.004) expressions were related to a higher mtDNA copy ratio. In addition, advanced T-status (p=0.019) and negative-HER-2/neu expression (p=0.061) were associated with mtDNA D310 mutations. In conclusion, higher mtDNA copy ratio and D310 mutations may be relevant biomarkers correlated with pathological T-status and the expression levels of ER, PR, HER-2/neu, p53 and Ki-67 in BIDCs.

Correspondence to: Professor Shoei-Loong Lin, Chon-inn Hospital, Chon-inn Healthcare Corp. Aggregate, 196 Section 1, Wen-Hua Road, Banqiao, New Taipei City 220, Taiwan, R.O.C.
E-mail: drslin705@gmail.com

Professor Yau-Huei Wei, Department of Medicine, Mackay Medical College, 46 Section 3, Zhong-Zheng Road, San-Zhi, New Taipei City 252, Taiwan, R.O.C.
E-mail: joeman@mmc.edu.tw

*Contributed equally

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Introduction

Mitochondria are the intracellular organelles responsible for ATP production to meet the energy demands (1). Generally, there are several hundred to 1,000 mitochondria in a human cell and 2-10 copies of mitochondrial DNA (mtDNA) in a human mitochondrion to compose the mitochondrial network (2). The amount of mtDNA copies in a human cell is highly variable, depending on the cell type and the surrounding pathophysiological conditions, and usually is positively correlated to the energy demands (3). Different from the heterozygotic nature of nuclear DNA (nDNA), the mtDNA is exclusively transmitted through maternal lineage with a single origin and the majority of mtDNA copies in the post-mitotic tissues are assumed identical after birth. This specific feature is termed as homoplasmy (4). Due to the lack of introns, naked DNA exposure without adequate histone protection, impaired DNA repair system and an environment with high reactive oxygen species (ROS) concentration in the inner membrane of mitochondria, human mtDNA is far more susceptible to oxidative damages or mutations than nDNA (5-7). When the damaged or the

mutated mtDNA variants coexist with the wild-type inborn mtDNA molecules, the homoplasmy is disrupted and shifted to a condition termed as heteroplasmy (8).

Human mtDNA, containing a coding and a non-coding region, is a circular and double-stranded DNA structure 16.6-kb in size. The inner strand is called the light strand (L) and the outer strand is called the heavy strand (H). Based on the L strand, the entire mtDNA has been sequenced completely as the revised Cambridge Reference Sequence (rCRS) (9). The coding region of mtDNA codes for 13 polypeptides that are required for the composition of respiratory chain complexes, and 2 rRNAs plus a set of 22 tRNAs necessary for protein synthesis in the mitochondria. All the other ~90 polypeptides constituting the respiratory chain complexes are encoded in nDNA. The non-coding region 1.1-kb in size, also called the displacement loop (D-loop), is the regulatory region for specific protein binding to trigger mtDNA replication and transcription (10,11).

Although damages can occur anywhere throughout the entire mtDNA, they are frequently found in the D-loop, particularly in the D310 region (12). Between nucleotide position (np) 303 and np 316 of the D-loop, there is a poly-cytidine (C) tract with a thymidine (T) interrupted at np 310 (-C_{303}CCCCC CT_{310}CCCCCC$_{316}$- = C_7TC_6). The C number remains constant as 6 after T, however, it is highly variable before T with a range from 6 to 12 (C_6, C_7, C_8, C_9, C_{10}, C_{11} and C_{12}), and 7 (C_7, wild-type) being the most common one. These variations with a T shifting over np 310 of the D-loop in mtDNA are termed as D310 polymorphism or D310 sequence variations (9).

Breast invasive ductal carcinoma (BIDC) is the most commonly diagnosed malignancy worldwide among women (13). Due to the multidisciplinary treatment modalities, including surgical resection and combinations of perioperative chemotherapy, radiotherapy, hormone-ablation or targeted therapies, and the advance in the understanding of its underlying molecular pathogenesis, the associated morbidity and mortality associated with BIDC have been reduced gradually during the past decades (14). In order to achieve an optimal therapeutic result, surgical-pathological T-, N- and M-status and the cancer stage, and the immunological expression levels of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER-2/neu), protein p53 and protein Ki-67 are routinely assessed in BIDC patients, at present (15-18). However, the roles of mtDNA alterations in BIDC remain speculative. In this retrospective study, we aimed to appraise the associations among mtDNA alterations, including the change in mtDNA copy number and the shifting of mtDNA D310 sequence variations (D310 mutation), the pathological status, and the immunological ER, PR, HER-2/neu, p53 and Ki-67 expression levels in BICDs, respectively.

Materials and methods

Patient selection, tissue preparation and DNA extraction. A total of 51 women diagnosed with BIDC, without obvious distant organ metastasis on preoperative assessments, who underwent curative modified radical mastectomy plus axillary lymph node dissection as the primary treatment modality between January 2009 and June 2011 were enrolled. None of the patients received preoperative neoadjuvant chemotherapy, radiotherapy or both. Their pathologic status (TNM and cancer stage according to the American Joint Committee on Cancer; AJCC, 7th edition) and ER, PR, HER-2/neu, p53 and Ki-67 expression levels, and clinical data were recorded in detail for systemic analysis. Approval from the Institutional Review Board was obtained to conduct the present study.

As reviewed by an experienced pathologist, representative areas harboring BIDC and paired non-cancerous breast tissue on pathologic slides were located, and thin sections (~5-μm) from matched formalin-fixed and paraffin-embedded tissue blocks were prepared for DNA extraction. These tissue samples were stored in 1.5-ml Eppendorf vials and mixed with 500 μl xylene (Merck KGaA, Darmstadt, Germany) at room temperature for 16 h. After centrifuging at 10,000 x g for 10 min at room temperature and discarding the supernatants, these tissue samples were re-hydrated with 100, 80, 60 and 40% alcohol aqueous solution and then pure distilled water for 5 min in steps. Finally, the hydrated tissue samples were mixed with 200 μl QuickExtract DNA extraction solution (Epicenter, Madison, WI, USA) plus 3 μl of 5% butylated hydroxytoluene in methanol to extract total cellular DNA at 65˚C for 3 h as previously described (19,20). The DNA sample was kept at -20˚C until use.

Standard curves for mtDNA and nDNA quantifications. Quantitative real-time polymerase chain reaction (q-PCR) using LightCycler® FastStart DNA Master SYBR-Green I (Roche Applied Science, Mannheim, Germany) to detect threshold cycle (Ct) was applied for mtDNA and nDNA standard curve establishment and subsequent quantification. Briefly, genomic DNA of 143B osteosarcoma cells were 4-fold diluted from 20 to 0.0048828 ng/μl and then subjected to q-PCR for Ct value determination. The sequences of the primers used for mtDNA amplification (near the ND1 region, mainly coding for tRNA leucine 1) were: mtF3212, 5'-CACCC AAGAACAGGGTTTGT-3'; and mtR3319, 5'-TGGCCATGGG ATTGTTGTTAA-3'. The sequences of primers used for nDNA amplification (18S rRNA region) were: 18SF1546, 5'-T AGAGGGACAAGTGGCGTTC-3'; and 18SR1650, 5'-CGCT GAGCCAGTCAGTGT-3' (21). The equations of standard curves set for mtDNA and nDNA quantification were set as previously described (19,20,22). Then the mtDNA and nDNA copies of the clinical samples relative to mtDNA and nDNA copies of the 143B osteosarcoma cells were calculated.

Determination of mtDNA copy number and mtDNA copy ratio. The mtDNA copy number was defined as the total mtDNA copies divided by the total nDNA copies for each clinical sample. q-PCR was applied for sample mtDNA and nDNA quantification. For each reaction, 1 μl (10 ng/μl) of sample DNA was amplified in a 10 μl mixture that containing 0.25 μl of each primer (20 μM, mtF3212 and mtR3319 for mtDNA quantification; 18SF1546 and 18SR1650 for nDNA quantification), 1.2 μl of 3 mM $MgCl_2$, 1 μl of LightCycler® FastStart DNA Master SYBR-Green I and 6.3 μl of PCR grade H_2O. Simultaneously, 1 μl of DNA from 143B cells (1 ng/μl) and PCR grade H_2O were included as positive and negative controls, respectively. The PCR conditions were set as: hot start at 95˚C for 10 min followed by 40 cycles of 95˚C for 20 sec,

62˚C for 20 sec and 72˚C for 20 sec. Fluorescence intensity for Ct value detection was measured at the end of every extension phase at 79˚C. Using the equations of the established standard curves, the mtDNA copies and nDNA copies of sample DNA relative to those of 143B cells (1 ng/μl) were determined. The mtDNA copy number (total mtDNA copies/total nDNA copies; i.e. relative mtDNA copies to 143B/relative nDNA copies to 143B) of each clinical sample was determined after adjusting the mtDNA copy number of the 143B cells to 1.000. Each reaction was carried out in duplicate and the mean value was used for data presentation. To evaluate the change in mtDNA copy number between the BIDC and paired non-cancerous breast tissues, we defined mtDNA copy ratio as the mtDNA copy number of the BIDC divided by mtDNA copy number of the paired non-cancerous breast tissue.

Sequencing of the D310 region. The D310 region of mtDNA was amplified by PCR and then subjected to direct sequencing as previously described (20,23,24). Each 50 μl PCR reaction contained 25 μl of RBC SensiZyme® Hotstart *Taq* Premix (RBC Bioscience, New Taipei City, Taiwan), 22 μl of PCR-grade H_2O, 1 μl of each primer (H76-1, 5'-CACGCGATA GCATTGCGA-3'; and L335, 5'-TAAGTGCTGTGGCCAGA AGC-3'), and 1 μl of sample DNA (10 ng/μl). The PCR procedures included a hot start at 95˚C for 10 min, 40 cycles of 95˚C for 15 sec, 58˚C for 15 sec and 72˚C for 30 sec, and a final extension at 72˚C for 7 min. After confirmation by 3% agarose gel electrophoresis, the PCR products were subjected to direct sequencing (MB Mission Biotech, Taipei, Taiwan). The D310 sequence variations, including patterns of homoplasmy or heteroplasmy, number of detected D310 variants, and the predominant D310 variant were determined as previously described (20,23,24). Compared to the D310 sequences of the paired non-cancerous breast tissue, any sequence alterations detected in the BIDC were defined as D310 mutations (20).

Immunohistochemical staining. The expression levels of ER, PR, HER-2/neu, p53 and Ki-67 were determined by immunohistochemical (IHC) staining, and these parameters are routinely determined in the pathological examinations in our hospital. Thin sections (~4-μm) from the formalin-fixed and paraffin-embedded tissues blocks were cut for IHC staining. Characteristics of the primary antibodies used for IHC and their criteria for positive/high expression are illustrated in Table I. Briefly, the sections were de-paraffinized with xylene (Merck KGaA) and then rehydrated with 100% alcohol, 95% alcohol solution and pure distilled running water in steps. After reacting with target retrieval solution (EnVision™ FLEX, pH 9.0, 50X, K8004; pH 6.0, 10X, S2369; both from Dako, Carpinteria, CA, USA) at 99˚C for 20 min, they were cooled at room temperature for 20 min and incubated in TBS buffer (EnVision™ FLEX Wash Buffer, 20X, K8007; Dako) for 5 min. Endogenous peroxidase was blocked with peroxidase-blocking reagent (DM821; Dako) for 5 min. After washing with TBS, the primary antibodies were applied at room temperature for 20 min (HER-2/neu, 30 min). After washing with TBS, the sections were incubated with horseradish peroxidase (HRP; DM822; Dako) at room temperature for 20 min, and the sections were again washed in TBS. The color was developed by 10 min of incubation with 3,3-diaminobenzidine tetrahydrochloride

Table I. Details of the antibodies, dilutions, antigen retrieval methods and expression criteria used in this study.

Primary antibody	Dilution/incubation time	Antigen retrival	Specificity	Source	Product code	High expression criteria
ER	Ready to use/20 min	pH 9.0/heat at 99˚C	Monoclonal rabbit anti-human ERα	Dako[a]	IS084	>10% cancer cells with strong nuclear staining
PR	Ready to use/20 min	pH 9.0/heat at 99˚C	Monoclonal mouse anti-human PR	Dako[a]	IS068	>10% cancer cells with strong nuclear staining
HER-2/neu	1:800/30 min	pH 6.0/heat at 99˚C	Polyclonal rabbit anti-human c-erbB-2	Dako[a]	A0485	HercepTest™ criteria
Ki-67	Ready to use/20 min	pH 6.0/heat at 99˚C	Monoclonal mouse anti-Ki-67	Dako[a]	IS626	>20% cancer cells with strong nuclear staining
p53	Ready to use/20 min	pH 9.0/heat at 99˚C	Monoclonal mouse anti-p53	Dako[a]	IS616	>50% cancer cells with strong nuclear staining

[a]Carpinteria, CA, USA. ER, estrogen receptor; PR, progesterone receptor; HER-2/neu, human epidermal growth factor receptor-2.

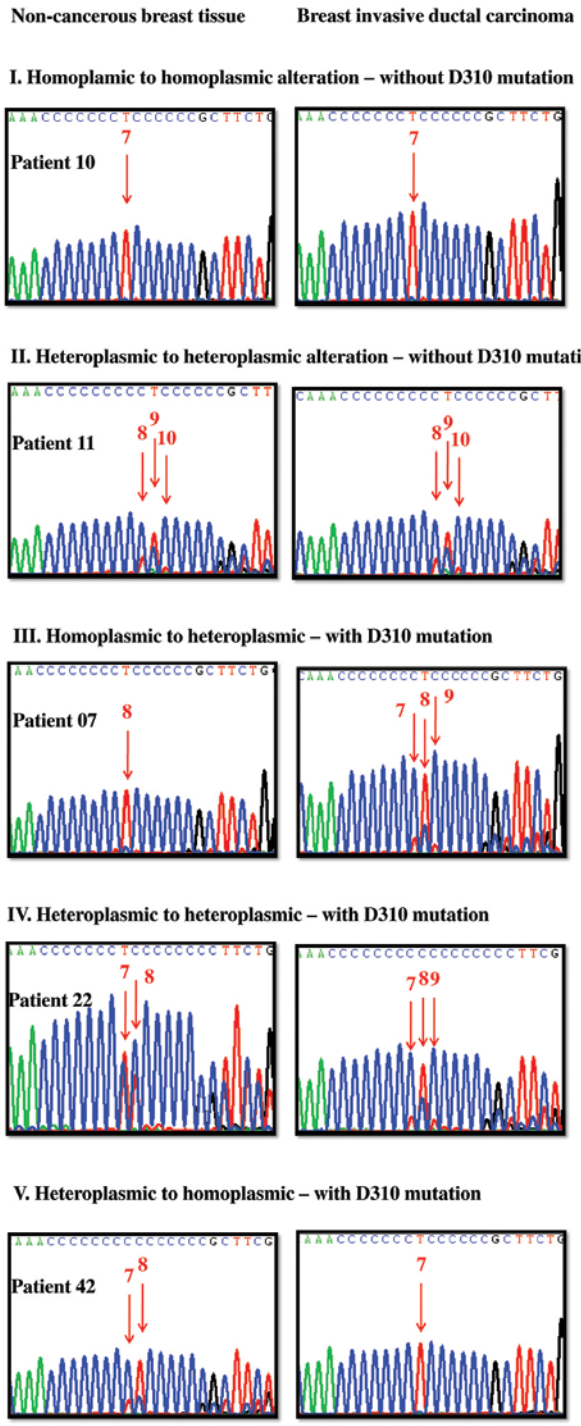

Figure 1. Representative cases to demonstrate the mitochondrial DNA D310 sequence variations and their shifting between non-cancerous breast tissue (left) and paired breast IDC (right). T (thymidine) is shown in red, A (adenine) in green, C (cytidine) in blue and G (guanine) in black during sequencing. The Arabic number above the red arrow denotes the C number before the indicated T peak and the T peak height represents the relative quantity of the D310 variant. Type I, homoplasmic to homoplasmic (first row) without D310 mutation; patient 10 as an example. Her non-cancerous breast tissue harbors one type of D310 variant, the C_7TC_6 and is classified as homoplasmic D310 with C_7TC_6 as the major one. Her BIDC also harbors one type of D310 variant, the C_7TC_6, and is classified as homoplasmic D310 with C_7TC_6 as the major one. As a result, patient 10 was defined as type I homoplasmic to homoplasmic alteration without D310 mutation. Type II, heteroplasmic to heteroplasmic (second row) without D310 mutation; patient 11 as an example. Her non-cancerous breast tissue harbors 3 types of D310 variants, the C_9TC_6, C_8TC_6 and $C_{10}TC_6$ in order, and is classified as heteroplasmic D310 with C_9TC_6 as the major one. Her BIDC also harbors 3 types of D310 variants, the C_9TC_6, C_8TC_6 and $C_{10}TC_6$ in order, and is classified as heteroplasmic D310 with C_9TC_6 as the major one. No significant shifting of the D310 sequence variations was noted. As a result, patient 11 was defined as heteroplasmic to heteroplasmic alteration without D310 mutation. Type III, homoplasmic to heteroplasmic (third row) with D310 mutation; patient 07 as an example. Her non-cancerous breast tissue harbors one type of D310 variants, the C_8TC_6, and is classified as homoplasmic D310 with C_8TC_6 as the major one. Her BIDC harbors 3 types of D310 variants, the C_8TC_6, C_7TC_6 and C_9TC_6 in order, and is classified as heteroplasmic D310 with C_8TC_6 as the major one. Significant shifting of the D310 sequence variations was noted. As a result, patient 07 was defined as homoplasmic to heteroplasmic alteration with D310 mutation. Type IV, heteroplasmic to heteroplasmic (fourth row) with D310 mutation; patient 22 as an example. Her non-cancerous breast tissue harbors 2 types of D310 variants, the C_7TC_6 and C_8TC_6 in order, and is classified as heteroplasmic D310 with C_7TC_6 as the major one. Her BIDC harbors 3 types of D310 variants, the C_8TC_6, C_7TC_6 and C_9TC_6 in order, and is classified as heteroplasmic D310 with C_8TC_6 as the major one. Significant shifting of the D310 sequence variations were noted. As a result, patient 22 was defined as heteroplasmic to heteroplasmic alteration with D310 mutation. Type V, heteroplasmic to homoplasmic (fifth row) with D310 mutation; patient 42 as an example. Her non-cancerous breast tissue harbors 2 types of D310 variants, the C_8TC_6 and C_7TC_6 in order, and is classified as heteroplasmic D310 with C_8TC_6 as the major one. Her breast IDC harbors one type of D310 variant, the C_7TC_6, and is classified as homoplasmic D310 with C_7TC_6 as the major one. Significant shifting of the D310 sequence variations were noted. As a result, patient 42 was defined as heteroplasmic to homoplasmic alteration with D310 mutation.

solution (DAB; + chromogen, DM827; diluted in substrate buffer, SM803; both from Dako). The sections were weakly counterstained with hematoxylin (Merck).

Statistical analyses. All the statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 15.0, software (SPSS, Inc., Chicago, IL, USA). The continuous variables were compared using the Student's t-test/Mann-Whitney U test between two groups or ANOVA/Kruskal-Wallis H test among three or more groups when appropriate. Categorical variables between groups were compared using the Chi-square/Fisher's exact tests or Chi-square test for trend when appropriate. The difference between groups was considered to indicate a statistically significant result when the p-value was <0.05.

Results

mtDNA D310 sequence variations and mtDNA copy numbers of examined tissues and their alterations. The results of the mtDNA D310 sequence variations and mtDNA copy numbers of the non-cancerous breast tissues and paired BIDCs and their alterations are listed in Table II and classified in Fig. 1. For non-cancerous breast tissues, the mean detected D310

Table II. Details of the mitochondrial DNA D310 sequence variations and mitochondrial DNA copy number of the non-cancerous breast tissue and paired breast invasive ductal carcinoma and their alterations in the 51 women.

	Mitochondrial DNA D310 region									Mitochondrial DNA copy no.			
	Non-cancerous breast tissue					Breast invasive ductal carcinoma							
D310 mutation/type of alteration	Pts.	Pattern	Variants	No.	Major	Pattern	Variants	No.	Major	Non-cancerous breast tissue copy no.	Breast invasive ductal carcinoma copy no.	Copy ratio	Change
No (n=20)													
I. Homoplasmic to homoplasmic	02	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.322	0.223	0.692	Decrease
	09	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.313	0.280	0.895	Decrease
	10	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.152	0.214	1.413	Increase
	14	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.173	0.214	1.234	Increase
	18	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.295	0.239	0.809	Decrease
	19	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.276	0.210	0.763	Decrease
	21	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.343	0.281	0.819	Decrease
	23	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.396	0.259	0.654	Decrease
	25	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.303	0.163	0.538	Decrease
	26	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.290	0.235	0.811	Decrease
	27	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.310	0.291	0.939	Decrease
	32	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.230	0.268	1.168	Increase
	33	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.252	0.361	1.435	Increase
	50	Homoplasmy	7	1	7	Homoplasmy	7	1	7	1.250	0.329	0.263	Decrease
	51	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.471	0.229	0.485	Decrease
	52	Homoplasmy	7	1	7	Homoplasmy	7	1	7	0.401	0.233	0.582	Decrease
	04	Homoplasmy	8	1	8	Homoplasmy	8	1	8	0.262	0.324	1.237	Increase
	20	Homoplasmy	8	1	8	Homoplasmy	8	1	8	0.324	0.224	0.692	Decrease
	29	Homoplasmy	8	1	8	Homoplasmy	8	1	8	0.435	0.815	1.872	Increase
	47	Homoplasmy	8	1	8	Homoplasmy	8	1	8	0.232	0.363	1.565	Increase
No (n=2)													
II. Heteroplasmic to heteroplasmic	38	Heteroplasmy	8,7,9	3	8	Heteroplasmy	8,7,9	3	8	0.169	0.320	1.899	Increase
	11	Heteroplasmy	9,8,10	3	9	Heteroplasmy	9,8,10	3	9	0.397	0.281	0.710	Decrease
Yes (n=2)													
III. Homoplasmic to heteroplasmic	05	Homoplasmy	1	1	7	Heteroplasmy	7,10,9	3	7	0.069	0.097	1.421	Increase
	07	Homoplasmy	8	1	8	Heteroplasmy	8,7.9	3	8	0.260	0.311	1.194	Increase

Table II. Continued.

D310 mutation/type of alteration	Mitochondrial DNA D310 region									Mitochondrial DNA copy no.			
	Non-cancerous breast tissue					Breast invasive ductal carcinoma				Non-cancerous breast tissue copy no.	Breast invasive ductal carcinoma copy no.	Copy ratio	Change
	Pts.	Pattern	Variants	No.	Major	Pattern	Variants	No.	Major				
Yes (n=23)													
Heteroplasmic to heteroplasmic	22	Heteroplasmy	7,8	2	7	Heteroplasmy	8,7,9	3	8	0.126	0.262	2.088	Increase
	06	Heteroplasmy	8,7	2	8	Heteroplasmy	8,7,9	3	8	0.170	0.238	1.407	Increase
	08	Heteroplasmy	8,7,9	3	8	Heteroplasmy	7,8	2	7	0.252	0.332	1.320	Increase
	15	Heteroplasmy	8,9	2	8	Heteroplasmy	8,9,7,10	4	8	0.827	0.560	0.676	Decrease
	37	Heteroplasmy	8,9, 7,10	4	8	Heteroplasmy	16,17,15, 18,19,14	6	16	0.131	0.294	2.257	Increase
	39	Heteroplasmy	8,9	2	8	Heteroplasmy	7,8,9,10	4	7	0.193	0.117	0.609	Decrease
	44	Heteroplasmy	8,7	2	8	Heteroplasmy	8,9	2	8	0.326	0.151	0.463	Decrease
	49	Heteroplasmy	8,7	2	8	Heteroplasmy	9,8,10	3	9	0.407	0.452	1.109	Increase
	53	Heteroplasmy	8,7,9	3	8	Heteroplasmy	8,9	2	8	0.263	0.312	1.186	Decrease
	56	Heteroplasmy	8,9,7	3	8	Heteroplasmy	9,8,7,10	4	9	0.290	0.241	0.833	Decrease
	61	Heteroplasmy	8,9	2	8	Heteroplasmy	8,9,10	3	8	0.254	0.228	0.896	Decrease
	62	Heteroplasmy	8,9,7	3	8	Heteroplasmy	8,9	2	8	0.214	0.275	1.285	Increase
	01	Heteroplasmy	9,8	2	9	Heteroplasmy	9,8,10	3	9	0.514	0.430	0.837	Decrease
	03	Heteroplasmy	9,8,10	3	9	Heteroplasmy	9,8,10	3	9	0.192	0.211	1.102	Increase
	13	Heteroplasmy	9,8	2	9	Heteroplasmy	8,9	2	8	0.250	0.351	1.404	Increase
	17	Heteroplasmy	9,8	2	9	Heteroplasmy	9,10,8,11	4	9	0.216	0.116	0.535	Decrease
	28	Heteroplasmy	9,8	2	9	Heteroplasmy	9,10,8	3	9	0.334	0.358	1.075	Increase
	40	Heteroplasmy	9,8,10,7	4	9	Heteroplasmy	8,9	2	8	0.511	0.235	0.460	Decrease
	41	Heteroplasmy	9,8,10	3	9	Heteroplasmy	8,9,10	3	8	0.339	0.383	1.128	Increase
	45	Heteroplasmy	9,8,10	3	9	Heteroplasmy	9,8,10	3	9	0.363	0.485	1.336	Increase
	57	Heteroplasmy	9,8,10	3	9	Heteroplasmy	8,9	2	8	0.437	0.619	1.417	Decrease
	60	Heteroplasmy	9,8,10	3	9	Heteroplasmy	9,8,10	3	9	0.271	0.253	0.932	Decrease
	36	Heteroplasmy	16,17,15, 18,19,14	6	16	Heteroplasmy	16,15,17,14, 18,13,20	7	16	0.245	0.147	0.603	Decrease
Yes (n=4)													
Heteroplasmic to homoplasmic	58	Heteroplasmy	7,9,8	3	7	Homoplasmy	7	1	7	0.489	0.413	0.845	Increase
	42	Heteroplasmy	8,7	2	8	Homoplasmy	7	1	7	0.234	0.406	1.741	Increase
	43	Heteroplasmy	8,7	2	8	Homoplasmy	8	1	8	0.268	0.268	1.000	Decrease
	12	Heteroplasmy	9,8,7	3	9	Homoplasmy	8	1	8	0.124	0.125	1.008	Increase

Pts., patients.

Table III. Distribution of mitochondrial DNA D310 and mitochondrial DNA copy number of the non-cancerous breast tissue and paired breast invasive ductal carcinoma in the 51 BIDC women.

Mitochondrial DNA	Non-cancerous breast tissue	Breast invasive ductal carcinoma
D310 pattern (n, %)		
Homoplasmic	22 (43.1)	24 (47.1)
Heteroplasmic	29 (56.9)	27 (52.9)
No. of D310 variants		
Mean ± SD	2.0±1.1	2.1±1.4
(95% CI of mean)	(1.7-2.3)	(1.8-2.6)
Copy no.		
Mean ± SD	0.317±0.184	0.295±0.130
(95% CI of mean)	(0.269-0.385)	(0.250-0.360)

variants and mean mtDNA copy numbers were 2.0 and 0.317, respectively, and 29 (56.9%) harbored a heteroplasmic D310 pattern. For BIDCs, their mean detected D310 variants and mtDNA copy numbers were 2.1 and 0.295, respectively, and 27 (52.9%) harbored a heteroplasmic D310 pattern (Table III). When compared to the paired non-cancerous breast tissues, 29 (56.9%) BIDCs harbored D310 mutations, and 24 (47.1%) had an increased mtDNA copy number with mtDNA copy ratio >1.000 (Table IV).

Clinicopathological demographic data. The demographic data concerning the clinical, pathological and biochemical analyses of the 51 BIDC women are listed in Table IV. The mean age was 53.6 and the mean tumor diameter was 2.5 cm. After pathological examinations, the mean total dissected axillary lymph nodes was 17.0 with a mean number of positive nodes of 1.8. Concerning the pathological T- and N-status and cancer stage, 24 (47.1%), 21 (41.2%) and 6 (11.8%) belonged to T1, T2 and T3; 30 (58.8%), 13 (25.5), 6 (11.8%) and 2 (3.9%) to N0, N1, N2 and N3; and 17 (33.3%), 26 (51.%) and 8 (15.7%) to stages I-III, respectively. For the histological grade, 4 (7.8%) belonged to grade I, 39 (76.5%) to II and 8 (15.7%) to III, respectively.

Concerning the expression of therapeutic biomarkers, 37 (72.5%), 33 (64.7%) and 14 (27.5%) were positive for ER, PR and HER-2/neu, respectively. For the biomarkers reflecting tumor aggressiveness and proliferation, 14 (27.5%) were positive for p53 expression and 9 (17.6%) had a high Ki-67 expression.

Factors affecting mtDNA copy ratio. The possible factors that affect mtDNA copy ratio are listed in Table V. Advanced T-status (p=0.056), negative-ER expression (p=0.005), negative-PR expression (p=0.007), positive-p53 (p=0.050) and Ki-67 with high expression (p=0.004) were related to a higher mtDNA copy ratio in the BIDCs, respectively.

Factors related to the mtDNA D310 mutation. As shown in Table VI, advanced T-status (p=0.019) and negative-HER-2/neu

Table IV. Demographic data concerning clinical, pathological and mitochondrial DNA features of the 51 BIDC women.

Demographic data	Data
Age (years) mean ± SD (95% CI of mean)	53.6±11.4 (50.5-56.8)
Tumor side, n (%)	
Left	30 (58.8)
Right	21 (41.2)
Tumor location, n (%)	
Outer-upper	28 (54.9)
Outer-lower	9 (17.6)
Inner-upper	9 (17.6)
Inner-lower	3 (5.9)
Sub-areolar	2 (3.9)
Surgical-pathological findings mean ± SD (95% CI of mean)	
Tumor diameter (cm)	2.5±1.6 (2.2-3.0)
Total dissected lymph nodes	17.0±10.0 (14.5-19.0)
Positive-dissected lymph nodes	1.8±4.2 (0.8-3.3)
T-status, n (%)	
T1	24 (47.1)
T2	21(41.2)
T3	6 (11.8)
N-status, n (%)	
N0	30 (58.8)
N1	13 (25.5)
N2	6 (11.8)
N3	2 (3.9)
Cancer stage, n (%)	
I	17 (33.3)
II	26 (51.0)
III	8 (15.7)
Histological grade, n (%)	
I	4 (7.8)
II	39 (76.5)
III	8 (15.7)
Estrogen receptor, n (%)	
Negative	14 (27.5)
Positive	37 (72.5)
Progesterone receptor	
Negative	18 (35.3)
Positive	33 (64.7)
HER-2/neu	
Negative	37 (72.5)
Positive	14 (27.5)
p53	
Negative	37 (72.5)
Positive	14 (27.5)
Ki-67	
Low	42 (82.4)
High	9 (17.6)

Table IV. Continued.

Demographic data	Data
Mitochondrial DNA alteration, n (%)	
D310 mutation	
Yes	29 (56.9)
No	22 (43.1)
Mitochondrial DNA copy ratio	1.052±0.441
mtDNA copy number, n (%)	
Increase (ratio >1.000)	24 (47.1)
Decrease (ratio ≤1.000)	27 (52.9)

expression (p=0.061) were associated with a higher rate of D310 mutations in the human BIDCs.

Discussion

Surgical-pathological T-, N- and M-status and cancer stage of AJCC remain as the gold standard to predict the prognosis of BIDC women. With the advance in cancer research, the oncogenic process and the proliferative aggressiveness of BIDCs were found to be correlated to positive-p53 (tumor suppressor) and high Ki-67 (cell proliferation) expression, respectively. Furthermore, due to the breakthrough of modern molecular biology in the evaluation of specific cellular receptors, hormone ablation or targeted therapy have been advocated for BIDC women harboring positive-ER/PR or positive-HER-2/neu expression to improve their outcomes, in addition to routine adjuvant chemotherapy (15,18,25-29). In this retrospective study, we demonstrated that: i) advanced T-status, negative-ER, negative-PR, positive-p53 and high Ki-67 expression are related to higher mtDNA copy ratios in BIDCs; and ii) advanced T-status and negative-HER-2/neu are related to higher rates of mtDNA D310 mutations in BIDCs. Since mitochondria are the cellular powerhouse for energy production, whether these mtDNA alterations in human BIDCs are related to a metabolic shift warrants further appraisal and discussion. Metabolic shift in human cancer was first described by Dr Warburg 7 decades ago (Warburg effect), and he contended that human cancer tissues exhibited increased glycolysis yet decreased mitochondrial respiration to generate ATP (30-32). It also became the basic theory of positron emission tomography (PET) scan in cancer evaluation.

The changes in mtDNA copy numbers have been analyzed in several human type of cancers. Compared to the paired non-cancerous counterparts, a decrease in mtDNA copy number was reported in lung cancer (33), hepatocellular carcinoma (34) and gastric cancer (35). In lung cancer tissues after neoadjuvant chemotherapy, a progressive decrease in mtDNA copy number was correlated with disease progression (22). These decreases were thought to be decreases in mitochondrial function. On the contrary, an increase in mtDNA copy number was noted in the carcinogenesis of head and neck cancers (36), and the progression of esophageal squamous cell carcinomas (20), particularly in patients who smoked cigarettes. These increases were regarded as a compensation process to overcome the oxidative damages from cigarette smoking and to maintain proper mitochondrial function (19). Concerning BIDCs, a decrease in mtDNA copy number was reported in some research articles (37-39). Nevertheless, we demonstrated a significantly higher mtDNA copy ratio in BIDCs that harbored negative-ER, negative-PR, positive-p53 or high Ki-67 expression. Thus, we focused on the role of ER, PR, p53 and Ki-67 and their relationships to mtDNA in BIDCs.

Table V. Possible factors affecting mitochondrial DNA copy number ratio.

Factors (n, %)	Mitochondrial DNA copy no. ratio	P-value
Age (years)		0.832
≤50 (n=24, 47.1)	1.066±0.502	
>50 (n=27, 52.9)	1.039±0.388	
Pathological findings		
T-status		0.056
T1 (n=24, 47.1)	1.144±0.460	
T2 (n=21, 41.2)	0.878±0.341	
T3 (n=6, 11.8)	1.293±0.520	
N-status		0.131
N0 (n=30, 58.8)	0.969±0.399	
N1 (n=13, 25.5)	1.218±0.470	
N2 (n=6, 11.8)	0.912±0.369	
N3 (n=2, 3.9)	1.637±0.637	
Cancer stage		0.975
I (n=17, 33.3)	1.053±0.412	
II (n=26, 51.0)	1.038±0.452	
III (n=8, 15.7)	1.093±0.518	
Histological grade		0.215
I (n=4, 7.8)	0.834±0.488	
II (n=39, 76.5)	1.042±0.465	
III (n=8, 15.7)	1.206±0.245	
Estrogen receptor		0.005
Negative (n=14, 27.5)	1.291±0.357	
Positive (n=37, 72.5)	0.961±0.440	
Progesterone receptor		0.007
Negative (n=18, 35.3)	1.233±0.372	
Positive (n=33, 64.7)	0.953±0.449	
HER-2/neu		0.301
Negative (n=37, 72.5)	1.012±0.433	
Positive (n=14, 27.5)	1.158±0.459	
p53		0.050
Negative (n=37, 72.5)	0.983±0.426	
Positive (n=14, 27.5)	1.233±0.444	
Ki-67		0.004
Low (n=42, 82.4)	0.977±0.420	
High (n=9, 17.6)	1.399±0.382	

Table VI. Possible factors related to mitochondrial DNA D310 mutation.

Factors (n, %)	Mitochondrial DNA D310 mutation: No n (%)	Mitochondrial DNA D310 mutation: Yes n (%)	P-value
Total	22 (100)	29 (100)	
Age (years)			0.714
≤50 (n=24, 100.0)	11 (45.8)	13 (54.2)	
>50 (n=27, 100.0)	11 (40.7)	16 (59.3)	
Pathological findings			
T-status			0.019
T1 (n=24, 100.0)	9 (37.5)	15 (62.5)	
T2 (n=21, 100.0)	13 (61.9)	8 (38.1)	
T3 (n=6, 100.0)	0 (0.0)	6 (100.0)	
N-status			0.593
N0 (n=30, 100.0)	14 (46.7)	16 (53.3)	
N1 (n=13, 100.0)	5 (38.5)	8 (61.5)	
N2 (n=6, 100.0)	3 (50.0)	3 (50.0)	
N3 (n=2, 100.0)	0 (0.0)	2 (100.0)	
Cancer stage			0.598
I (n=17, 100.0)	6 (35.3)	11 (64.7)	
II (n=26, 100.0)	13 (50.0)	13 (50.0)	
III (n=8, 100.0)	3 (37.5)	5 (62.5)	
Histological grade			0.707
I (n=4, 100.0)	1 (25.0)	3 (75.0)	
II (n=39, 100.0)	17 (43.6)	22 (56.4)	
III (n=8, 100.0)	4 (50.0)	4 (50.0)	
Estrogen receptor			
Negative (n=14, 100.0)	6 (42.9)	8 (57.1)	
Positive (n=37, 100.0)	16 (43.2)	21 (56.8)	
Progesterone receptor			0.889
Negative (n=18, 100.0)	8 (44.4)	10 (55.6)	
Positive (n=33, 100.0)	14 (42.4)	19 (57.6)	
HER-2 /neu			0.061
Negative (n=37, 100.0)	13 (35.1)	24 (64.9)	
Positive (n=14, 100.0)	9 (64.3)	5 (35.7)	
P53			0.214
Negative (n=37, 100.0)	14 (37.8)	23 (62.2)	
Positive (n=14, 100.0)	8 (57.1)	6 (42.9)	
Ki-67			0.163
Low (n=42, 100.0)	20 (47.6)	22 (52.4)	
High (n=9, 100.0)	2 (22.2)	7 (77.8)	

ER and PR both belong to the nuclear receptors, which can interact with the hormone response element (HRE) in nDNA, after the binding of estrogen or progesterone. With regards to ER, it not only can activate estrogen HRE in nDNA to control cell proliferation and apoptosis in several tissues yet also can be imported into the mitochondria to interact with the mtDNA to increase the expression of mtDNA-encoded peptides, and mitochondrial biogenesis due to mtDNA also harbors sequences similar to the HRE of nDNA (HRE-like motif) (40). Furthermore, an *in vitro* study denoted that the interactions among the estrogen, ER and HRE-like motif in mtDNA were crucial to maintain mitochondrial function, inhibit cell apoptosis, and overcome oxidative damage in breast cancer cell line, MCF-7 (41). Concerning PR, similar effects were also observed in benign breast epithelial cells (42). As a result, we proposed that an increase in mtDNA copy numbers in BIDCs that harbor negative-ER or negative-PR expression are thought to be a compensation process to enhance the interaction between ER/PR and HRE motifs on mtDNA to maintain mitochondrial function.

Concerning the associations among p53, mitochondria and mtDNA, pilot studies have shown that functional p53 may participate in the regulation of cellular mitochondrial biogenesis and respiration (43-45), maintenance of mtDNA integrity (46), maintaining mtDNA copy number abundance (46) and the homeostasis of reactive oxygen species (47). In a normal cell, p53 is inactivated by its negative regulator, mdm2, as a result, the protein p53 is usually not detectable (48). Under stress or pathological situations, various pathways lead to the dissociation of the p53 and mdm2 complex, which leads to an activated, accumulated and detectable p53. The activated p53 induces cell cycle arrest to allow either repair and survival of the cell or apoptosis to discard the damaged cells. Although the primary antibody that we used in the present study cannot distinguish wild-type or mutant p53, the positively detected p53 did suggest an elevated stress in BIDCs. It is postulated that the elevated mtDNA copy ratio in BIDCs may indicate an enhanced interaction between p53 and mtDNA toward a possible metabolic shift or survival benefit.

High protein Ki-67 expression denotes a high proliferative status (49). Ki-67 is detectable during all active phases of the cell cycle (G_1, S, G_2 and mitosis) except the resting status (G_0). High Ki-67 expression in BIDCs means a highly proliferation situation of the cancer cells and is associated with a poor outcome (15,16). It is reasonable to hypothesize that the elevated mtDNA copy ratio in BIDCs with high Ki-67 expression is regarded as increased energy expenditure for the rapid growth of cancer cells.

In addition to quantitative change of mtDNA copy number, several studies also evaluated the qualitative mtDNA D310 mutations in human types of cancers, including prostate (50,51), head and neck (52) and lung cancer (53,54), and esophageal squamous cell carcinoma (20), and some showed clinical significance. In breast cancer, a D-loop mutation, including D310 mutation, was identified as a poor prognostic factor (39). Intriguingly, our results showed that negative-HER-2/neu expression was related to a higher rate of D310 mutation in BIDCs. Due to the fact that the mtDNA D310 is located near the binding side of mitochondrial transcriptional factor to control mitochondrial biogenesis (55), whether negative-HER-2/neu expression and D310 mutation are related to the functional alterations in BIDC mitochondria deserve further evaluation. Although the detailed interaction between HER-2/neu and mtDNA remains speculative, a recent novel study revealed that HER-2/neu can translocate into mitochondria to negatively

regulate mitochondrial respiratory functions (56). Thus, it is assumed that an altered mitochondrial function may exist in BIDCs with negative HER-2/neu expression and mtDNA D310 mutation.

In addition to the above mentioned biomarkers of ER, PR, HER-2/neu, p53 and Ki-67, TNM status and cancer stages of AJCC remain the gold standard criteria to predict the prognosis of BIDC women. In our preliminary result, advanced T status was associated with a higher incidence of D310 mutation and an elevated mtDNA copy ratio. It is reasonable to assume that the increase in mtDNA copy number compensated for the mtDNA with D310 mutations when the BIDCs underwent progression, which was proposed in head and neck cancer and esophageal cancer (19,20,36). To verify whether these increases in mtDNA copy number and mtDNA D310 mutations confer enhanced aggressiveness to BIDCs, further *in vitro* study is warrant.

Based on our preliminary results, we conclude that elevated mtDNA copy ratios and D310 mutations, suggesting an altered mitochondrial function, may be relevant biomarkers correlated with pathological T status and ER, PR, HER-2/neu, p53 and Ki-67 expression in BIDCs.

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