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ORIGINAL PAPER

Evaluating mitochondrial DNA in patients with breast cancer and benign breast disease

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Abstract
Purpose To evaluate the role of mtDNA in breast cancer.
Methods We carried out an investigation into the mtDNA major control region or D-loop region and an essential and the largest mtDNA protein-coding gene, NADH dehydrogenase subunit 5 (ND5), together with a mitochondrial haplogroup analysis in 64 patients with breast cancer (BC) and 54 patients with benign breast disease (BBD) as controls.
Results Mutations in D-loop region were found in 10/64 or 15.6% of patients with BC and 14/54 or 25.9% of patients with BBD, while mutations in ND5 were detected in 6/64 or 9.4% of patients with BC and 5/54 or 9.3% of patients with BBD. In addition, in patients with BBD, mtDNA mutations were more likely to rise in D-loop region and the mutations were more likely to be heteroplasmic. However, in patients with BC, those with metastatic feature were less likely to carry mutations in D-loop region. Finally, we found haplogroup M has an increased risk of breast cancer compared with haplogroup N.
Conclusion mtDNA mutation may play a role in early stage of tumorigenesis, and mitochondrial haplogroup can also modulate breast cancer occurrence.

Keywords Mitochondrial DNA mutation · Mitochondrial haplogroup · D-loop region · Heteroplasmy · Breast cancer

Introduction

Human mtDNA is a 16.5-kb circular double-stranded molecule. It encodes 13 polypeptides for the oxidative phosphorylation system, including 7 subunits for NADH dehydrogenase (complex I), one subunit for ubiquinol: cytochrome c oxidoreductase (complex III), 3 subunits for cytochrome c oxidase (complex IV) and 2 subunits for ATP synthase (complex V). It also contains genes for 2 rRNAs and 22 tRNAs for translation in mitochondria (Attardi and Schatz 1988). Except for a 1-kb non-coding displacement loop (D-loop) region which contains the sites for mtDNA replication and transcription initiation, the mammalian mtDNA is almost saturated with coding genes (Attardi 1985). Over last two decades, mtDNA mutations have been associated with many human diseases including neurodegenerative diseases, metabolic diseases, and various types of cancer (Lu et al. 2009; Wallace 2005).

Breast cancer is the commonest cancer type among females, accounting for 16% of all cancer cases in women in Wenzhou area (Zheng 2007). Genetic background, environmental exposures, and gene–environment interactions all contribute to development of breast cancer (McPherson et al. 2000). Among the genes associated with

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predispositions for breast cancer are BRCA1/2, PTEN, and Tp53, and they account for 5–10% of all breast cancer cases (Schwartz et al. 2008). Mitochondrial dysfunction has been characterized in several breast cancer cell lines (Ma et al. 2010; Smolkova et al. 2010), and at the same time, mtDNA alterations have also been detected in many patients with breast cancer (Tan et al. 2002; Tseng et al. 2006).

To evaluate the role of mtDNA mutations in breast cancer development, we carried out an investigation into mtDNA of 64 patients with breast cancer (BC) and 54 patients with benign breast disease (BBD) in Wenzhou area. In particular, we sequenced and analyzed the mtDNA D-loop, the highly variable main control region for mtDNA replication and transcription (Brown et al. 1986), and the complex I subunit 5 (ND5) gene, an essential and the largest protein-coding gene in the mitochondrial genome in these patients.

Materials and methods

Sample collection

A total of 64 female patients with BC (average age: 50.70 ± 10.89; age range: 25–77) and 54 patients with BBD (average age: 42.72 ± 10.00; age range: 15–60), who received treatment in the First Affiliated Hospital of Wenzhou Medical College from April 2007 to June 2008, were recruited. The samples subjected to mtDNA analysis included the lesion tissue, its corresponding nearby normal tissue, and blood from the same patient. For haplogroup analysis, 113 peripheral blood samples from age-matched healthy donors were also collected.

All patients were clinically diagnosed. The tumor and adjacent non-tumor tissues were determined by a senior pathologist. The peripheral blood was obtained before any surgery and chemotherapy, radiotherapy or pharmacotherapy. Informed consent from all patients in this study was obtained under protocols approved by the Wenzhou Medical College Ethic Committee.

DNA extraction and amplification and sequencing

The tumor and adjacent non-tumor tissues were isolated by a needle under a microscope. DNA was extracted from paired frozen tissues and the peripheral blood with phenol–chloroform methods (Ding et al. 2010). The mtDNA templates for sequencing were amplified by PCR. The sequences information of the 5 pairs of primers for amplifying and sequencing the mtDNA D-loop region (position 16,024–576) and the ND5 gene (position 12,337–14,148) are as follows (Rieder et al. 1998): 1F: TCA TTG GAC AAG TAG CAT CC, 1R: GAG TGG TTA ATA

Table 1 The mtDNA mutations found in patients with breast cancer (BC)

Case no.	Site	Sample			rCRS	Heter./Homo.	A.A.change	Mutant type	Location
		t	a	b					
BC51	183	G	G	A	A	Homo.	N/A	Transition	D-loop
BC81	16304	T/C	T/C	C	T	Heter.	N/A	Transition	D-loop
	13920	A/C	C/A	C	C	Heter.	No	Transversion	ND5
BC87	16092	C	T	C	T	Homo.	N/A	Transition	D-loop
	16278	C	T	C	C	Homo.	N/A	Transition	D-loop
BC98	13104	G	G	A	A	Homo.	No	Transition	ND5
BC104	12406	G/A	A	A	G	Heter.	I toV	Transition	ND5
BC106	16092	C/T	C/T	C	T	Heter.	N/A	Transition	D-loop
	16362	G/A	G/A	G	T	Heter.	N/A	Transversion	D-loop
BC136	309	C8-C9	C8-C9	C9-C10	C7	Heter.	N/A	Ins C	D-loop
BC139	16129	A/G	A/G	G	G	Heter.	N/A	Transition	D-loop
BC143	16249	T	T	A/T	T	Heter.	N/A	Transition	D-loop
BC145	16391	T/C	T/C	C	G	Heter.	N/A	Transversion	D-loop
	14020	C	N/A	T	T	Homo.	No	Transition	ND5
BC153	217	T/C	T/C	C	T	Heter.	N/A	Transition	D-loop
BC154	14050	T	T	C	T	Homo.	P to S	Transition	ND5
	13629	G	G	A	A	Homo.	No	Transition	ND5
BC155	14050	C/T	C	T	T	Heter.	S to P	Transition	ND5
BC162	309	C7-C8	C7-C8	C9-C10	C7	Heter.	N/A	Ins C	D-loop

t Lesion tissue, *a* adjacent tissue, *b* blood, *rCRS* revised Cambridge reference sequence, *Heter.* heteroplasmic mutation, *Homo.* homoplasmic mutation, *A.A.change.* Amino acid change

Table 2 The mtDNA mutations found in patients with benign breast disease (BBD)

Case no.	Site	Sample			rCRS	Heter./Homo.	A.A.change	Mutant type	Region
		t	a	b					
BBD22	309	C8-C9	N/A	C9-C10	C7	Heter.	N/A	Ins C	D-loop
BBD62	499	G	N/A	A	G	Homo.	N/A	Transition	D-loop
	530	C	N/A	C/T	C	Heter.	N/A	Transition	D-loop
BBD63	16136	T	N/A	C	T	Homo.	N/A	Transversion	D-loop
BBD65	16391	G/A	N/A	G	G	Heter.	N/A	Transition	D-loop
	170	C	N/A	C/A	C	Heter.	N/A	Transversion	D-loop
BBD72	309	C8-C9	N/A	C7	C7	Heter.	N/A	Ins C	D-loop
	16168	C/A	N/A	C	C	Heter.	N/A	Transversion	D-loop
BBD73	94	G	N/A	A	G	Homo.	N/A	Transition	D-loop
	150	C	N/A	T	C	Homo.	N/A	Transition	D-loop
BBD90	309	C8-C9	N/A	C9-C10	C7	Heter.	N/A	Ins C	D-loop
BBD21	309	C8-C9	N/A	C7	C7	Heter.	N/A	Ins C	D-loop
	13928	C	N/A	G	G	Homo.	S to T	Transversion	ND5
BBD22	150	C	N/A	T/C	C	Heter.	N/A	Transition	D-loop
	12477	T	N/A	C	T	Homo.	No	Transition	ND5
BBD24	316	G/A	N/A	G	G	Heter.	N/A	Transition	D-loop
BBD26	489	C	N/A	T	T	Homo.	N/A	Transition	D-loop
	12705	T	N/A	C	C	Homo.	No	Transition	ND5
BBD34	16093	C/T	N/A	C	T	Heter.	N/A	Transition	D-loop
BBD46	12705	T	N/A	C	C	Homo.	No	Transition	ND5
BBD60	309	C8-C9	N/A	C9-C10	C7	Heter.	N/A	Ins C	D-loop
BBD65	309	C7-C8	N/A	C8-C9	C7	Heter.	N/A	Ins C	D-loop
BBD68	13589	G	N/A	G	A	Homo.	No	Transversion	ND5
	13926	T	N/A	C	T	Homo.	No	Transition	ND5

t Lesion tissue, *a* adjacent tissue, *b* blood, adjacent tissues are not available with this group of patients, *rCRS* revised Cambridge reference sequence, *Heter.* heteroplasmic mutation, *Homo.* homoplasmic mutation, *A.A.change.* amino acid change

GGG TGA TAG, 2F: CAC CAT TCT CCG TGA AAT CA, 2R: AGG CTA AGC GTT TTG AGC TG, 3F: TAT CAC TCT CCT ACT TAC AG, 3R: AGA AGG TTA TAA TTC CTA CG, 4F: AAA CAA CCC AGC TCT CCC TAA, 4R: TCG ATG ATG TGG TCT TTG GA, 5F: ACA TCT GTA CCC ACG CCT TC, 5R: AGA GGG GTC AGG GTT CAT TC.

All PCR products were purified with Takara DNA purification Kit Ver.2.0 (Takara, Japan). The PCR condition was as follows, pre-denatured at 95°C for 5 min, then 94°C for 30 s, 59°C for 45 s, and 72°C for 1 min for 30 cycles, and the final extension at 72°C for 10 min.

mtDNA haplogroup analysis

To assign a mtDNA haplogroup to each sample, all sequences were compared with the revised Cambridge Reference Sequence (rCRS) (Andrews et al. 1999) and aligned using the CodonCode Aligner 3.0.1 (CodonCode Corporation, USA) (Rieder et al. 1998) software program.

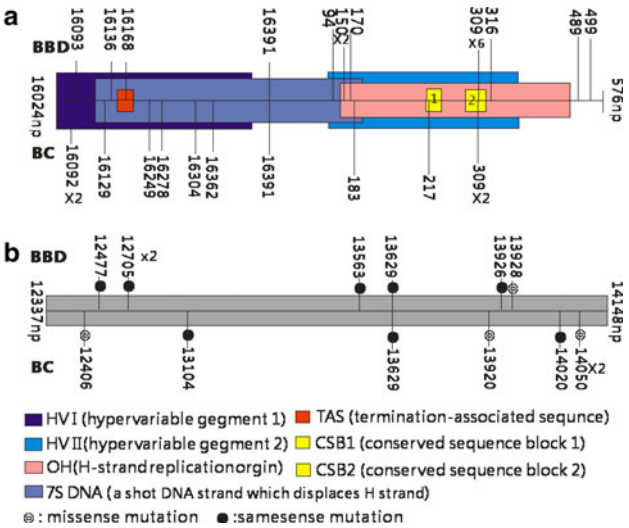

Fig. 1 The distribution and properties of mutations in D-loop (**a**) and ND5 gene (**b**) in patients with BC and in patients with BBD. In both **a** and **b**, the *upper part* exhibits mutations in BBD group, and the *lower part* indicates mutations in BC group. ×*n*: *n* indicates number of samples exhibited such mutations

Based on the most recent refined East Asian mitochondrial haplogroup tree (Kivisild et al. 2002; Kong et al. 2006; Yao et al. 2002), the initial assignment was performed with the sequencing information from the D-loop, ND3, and ND4L regions. When necessary, additional information was obtained by restriction fragment length polymorphism (RFLP) analysis at sites: 663 (*Hae*III), 3394 (*Hae*III), 4833 (*Hha*I), 5178 (*Alu*I), and 9824 (*Hin*fI). With some samples, the 9-bp deletion at the COII-tRNA^{lys} junction was also detected to further identification.

Statistical analysis

A software SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Pearson chi-square test or Fisher's exact test were used to reveal the statistical differences between groups. A P value less than 0.05 was considered to be statistically significant.

Results/discussion

Mutation detection in D-loop region and ND5 gene in patients with BC and in patients with BBD

The sequence information of mtDNA D-loop and the complex I subunit 5 (ND5) gene of 64 patients with BC and 54 patients with BBD was summarized in Tables 1 and 2. In BC group, 10 out of 64 or 15.6% patients carried mutations in D-loop and 6 out of 64 or 9.4% patients carried mutations in ND5 gene. Among them, 2 patients had two D-loop mutations; 2 had one D-loop, and one ND5 mutation; 1 exhibited two ND5 mutations; 6 had one D-loop mutation and 3 had one ND5 mutation. In BBD group, 14 out of 54 or 25.9% patients carried D-loop mutations, and 5 out of 54 or 9.3% patients carried ND5 mutations. Among them, 4 had two mutations in D-loop; 3 had one mutation in D-loop and one mutation in ND5 gene; one had three mutations in ND5; 7 had one mutation in D-loop and one with one mutation in ND5.

Among the D-loop mutations detected in these patients, heteroplasmy at D310 C repeats was the most common one, and it was found in 2 patients with BC and in 6 patients with BBD. And a T to C transition at position 150 was also found in 2 patients with BBD. For mutations found in ND5 gene, a missense mutation at position 14,050 was detected in 2 patients with BC, and a C to T silent mutation at position 12,705 was found in 2 patients with BBD.

Previously, using a temporal temperature gradient gel electrophoresis, Tan et al. (2002) scanned the whole mitochondrial genomes of 19 breast cancer tumor samples in United States. They found 12 out of 19 or 63.2% of them had mutation in D-loop, and, as in our patient, mutation at

Table 3 mtDNA mutations in D-loop and ND5 regions and their homo/heteroplasmy in BC and BBD groups

	BC		BBD		ND5		D-loop	
	D-loop	ND5	D-loop	ND5	BC	BBD	BC	BBD
Patients with mutations detected	10	6	14	5	6	5	10	14
Patients without mutations detected	54	58	40	49	58	49	54	40
P-value	0.424		0.041		1.000		0.178	
Number of hetero-mutations	9	3	13	0	3	0	9	13
Number of homo-mutations	3	4	5	7	4	7	3	5
P-value	0.326		0.002		0.192		1.000	

P-values were calculated with Fisher's exact test

Table 4 The effect of haplogroup in BC and BBD groups and the effects on mtDNA mutations

Region	Haplogroup	BC	P_1-value	BBD	P_2-value	NC	P_3-value
N/A	M	43		24		59	0.037
	N	21		30		54	
D-loop mutation detected	M	13.95%	0.717	16.67%	0.218	–	
	N	19.05%		33.33%		–	
ND5 mutation detected	M	6.98%	0.385	12.5%	0.646	–	
	N	14.29%		6.67%		–	

P-values were calculated with Fisher's exact test

P_1, the contribution of haplogroup M and N to BC group in D-loop or ND5 gene; P_2, the contribution of haplogroup M and N to BBD group in D-loop or ND5 gene; P_3, the distribution of haplogroup M and N among BC/BBD/NC groups

the D310 C repeats was the most common one. In another study conducted in Taiwan with 60 patients with breast cancer (Tseng et al. 2006), 18 or 30% were found to have mutation in D-loop region and 12 or 20% at the D310 C stretch. Besides the difference in population, stringent verification for each mutation detected as suggested in our recent paper (Fang et al. 2009) could be a reason for the relative low frequency of mutation found in our study.

Analysis of mtDNA mutations found in patients with BC and in patients with BBD

The distribution and properties of mtDNA mutations found in this study were illustrated in Fig. 1. Except for the D310 C stretch, there seemed no obvious hot spots for mutations in either D-loop or ND5 gene. Several mutations were found at the conserved sequence blocks in the mtDNA replication origin region, indicting a potential effect on mtDNA copy number control. Majority of the ND 5 mutations detected in this study were silent mutations. However, in patients with BC, among 6 ND5 mutations, 3 were missense mutations. Although the functional consequences of these ND5 mutations have not been determined, it is possible that a compromised complex I function could be involved as complex I deficiency has been implicated in several studies in cancer cells recently (Lu et al. 2009; Park et al. 2009).

To determine whether there is a difference in mtDNA mutation accumulation in non-coding D-loop and coding ND5 gene in BC or BBD cases, we compared the mutation frequencies in these two areas in patients with BC and in patients with BBD. As shown in Table 3, in patients with BC, we did not see any differences in mutation occurrence between D-loop and ND5 gene. Moreover, we did not detect any differences in mutations occurrences between patients with BC and patients with BBD in both D-loop and ND5 gene, either. However, in patients with BBD, mtDNA mutations were more likely to rise in non-coding D-loop region ($P = 0.041$).

Heteroplasmy is a unique feature for mtDNA genetics (Pang et al. 2008; Schon 2000). In particular, in patients carrying sporadic pathogenic mtDNA mutations, the mtDNA is more likely to be heteroplasmic. We thus examined the mtDNA heteroplasmy/homoplasmy status for the mutations detected in the patients with BC and in patients with BBD. Among 19 mtDNA mutations found in patients with BC, 12 were heteroplasmic mutations and 7 were homoplasmic mutations. Among 25 mutations detected in patients with BBD, 13 were heteroplasmic and 12 were homoplasmic. As shown in Table 3, in patients with BBD, compared with mutations found in ND5 gene, the D-loop mutations were more likely to be heteroplasmic ($P = 0.002$).

Analysis of mtDNA haplogroup

As a quality control measure for the sequencing analysis, we also determined the haplogroup of each sample to avoid sample mix-up or contamination (Fang et al. 2009; Kong et al. 2008). Surprisingly, as shown in Table 4, we found

Table 5 mtDNA mutations and other clinical data in BC group

Parameter	D-loop			ND5		
	Mutation detection		*P*-value	Mutation detection		*P*-value
	+	−		+	−	
Age						
<50	2	26	0.165	2	26	0.688
≥50	8	28		4	32	
Metastasis						
+	0	18	0.047	1	17	0.660
−	9	34		5	38	
Unknown	1	2		0	3	
ER						
−	4	21	1.000	3	22	0.678
+	6	31		3	34	
Unknown	0	2		0	2	
PR						
−	5	21	0.729	3	23	0.689
+	5	31		3	33	
Unknown	0	2		0	2	
P53						
−	3	21	1.000	2	22	1.000
+	4	20		2	22	
Unknown	3	13		2	14	
BCl-2						
−	3	16	1.000	2	17	1.000
+	6	29		4	31	
Unknown	1	9		0	10	
Her-2						
−	4	22	1.000	1	26	0.367
+	5	28		4	29	
Unknown	1	4		0	4	
Tumor size (diameter)						
<1 cm	4	16	0.713	2	18	1.000
≥1 cm	6	37		4	39	
Unknown	0	1		0	1	
History of other tumors						
+	1	7	1.000	0	8	1.000
−	9	47		6	50	
Family history of BC						
+	0	2	0.340	0	2	1.000
−	10	52		6	56	

P-values were calculated with Fisher's exact test

Unknown, Cases no corresponding test was conducted

Table 6 mtDNA mutations and other clinical data in BBD group

Parameter	D-loop			ND5		
	Mutation detection		*P*-value	Mutation detection		*P*-value
	+	−		+	−	
Age						
<50 years	10	31	0.722	4	37	1.000
≥50 years	4	9		1	12	
Tumor size (diameter)						
<1 cm	9	21	0.540	3	27	1.000
≥1 cm	5	19		2	22	
History of other tumors						
+	6	11	0.516	2	15	0.645
−	9	28		3	34	

P-values were calculated with Fisher's exact test

that there was a significant difference among patients with BC, patients with BBD, and health controls in the distributions of mega-haplogroup M and N, namely, haplogroup M had a higher correlation with the breast cancer occurrence.

We then checked the mutation frequency in D-loop and ND5 gene in M and N haplogroups patients. As shown in Table 4, however, we did not see any significant differences in the mutation occurrences between M and N in D-loop or ND5 of patients with BC or of patients with BBD.

mtDNA mutations and clinical features

In order to determine whether mtDNA mutation accumulation has any implication in BC or BBD development, we examined the relationship between the mtDNA mutation status and some clinical presentations in both patients with BC (Table 5) and patients with BBD (Table 6). We compared age, metastasis status, ER and PR expression, mutation detection in Tp53, Bcl 2, and Her 2, tumor size, whether the patient or her family had history with other types of tumor or BC, between BC patients with and without mtDNA mutations detected in D-loop or ND5 gene. The only significant correlation we found was BC patients without mutations in D-loop region were more like to be metastatic (Table 5).

In the patients with BBD, we did not detect any significant differences between patients with and without mtDNA mutations detected in D-loop or ND5 gene in tumor size and history of other tumors (Table 6).

Overall, our results indicated that mtDNA mutations could play a role in pathogenesis of BC and BBD. Since BC patients with metastatic feature were less likely to carry mutations in D-loop region, mtDNA mutations may appear in early stage of cancer development. In addition, mitochondrial polymorphism as defined by haplogroups could also modulate breast cancer occurrence.

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Conflict of interest statement We declare that we have no conflict of interest.

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