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Research Article

Higher Mitochondrial DNA Content in Peripheral Blood of Stage III Breast Cancer Patients

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Abstract

The aim of this study was to examine the mitochondrial DNA levels at baseline in the peripheral blood of breast cancer patients so as to evaluate its utility in disease management in Indian setup.

Quantitative Polymerase Chain Reaction (qPCR) of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) was carried out in peripheral blood of 126 patients and 29 healthy controls to measure the mitochondrial DNA content (Mitochondrial/ Nuclear DNA ratio).

Patients showed lower levels of mtDNA in peripheral blood in comparison to healthy females. Levels of mtDNA were highest in stages III (69205 ± 58951 pg/μL), than in earlier stages (42864 ± 23734 pg/μL; P = 0.01) and in patients of stage IV (45176 ± 22997 pg/μL; P = 0.03). The mtDNA content was significantly higher in all patients (stages I-IV combined or stages I-III) when compared to healthy controls. When mtDNA content was compared with various clinical and pathological parameters of patients the higher content was found with aggressiveness of the disease. The mtDNA content was highest in patients with stage III, LVI positive, size >4cm and ER/PR/HER2 positive status.

We observed that mtDNA content is significantly altered in the peripheral blood of breast cancer patients at baseline and it increases with the progression of tumor. We did not observe any relation with disease prediction but it seems higher mitochondrial DNA content in peripheral blood of patients might be a compensatory effect for decline in mitochondrial respiratory function. Further studies with large sample size are required to validate its prognostic utility.

Keywords: Mitochondrial DNA; DNA content; Breast cancer

Introduction

Breast cancer is the most common female cancer, with more than 230,000 new cases estimated to be diagnosed in the United States in 2015 alone [1]. It is estimated that 145,000 new cases are diagnosed and 70,000 deaths occur annually in India [2]. In Indian women the average age at diagnosis is 43- 46 years, about ten years lower than the age in the western world [3]. Blood-based biomarkers hold great promise because of their easy access and uniformity [4]. A major advantage of blood-based biomarkers in solid tumors is that they can be used even after the primary tumor has been removed and also for the monitoring of cancer recurrence.

Mitochondria, the cytoplasmic organelles, play an essential role in cellular energy metabolism, generation of free radicals and apoptosis [5]. Mitochondrial dysfunction has been implicated in cancer; the functional deficiency in mitochondria is the trigger for cells to escape apoptosis and thereby promoting neoplastic transformation [6]. Recent evidence suggests that mutation, reduction, or deletion of mtDNA leads to a defective oxidative phosphorylation, increased Reactive Oxygen Species (ROS) production, induction of the glycolytic pathway, over expression of prosurvival proteins, which ultimately results in cancer proliferation and tumorigenesis [7-9]. The changes reported in mitochondrial DNA could either represent the key mechanisms in tumor initiation, promotion, or the secondary effects of tumorigenesis [10].

Each mitochondrion contains many copies of the mtDNA, and the changes in the copy number occur in response to the energy demands of the cell. Studies have reported that mitochondrial DNA can be used as an important diagnostic as well as prognostic biomarker in cancer patients [11-13]. Many studies have reported either increased or reduced mtDNA content in cancer cells [14,15] and in patients with different tumors [16-18]. Here we report that mitochondrial DNA content is increased in breast cancer cases than in healthy controls and among the different stages (I-IV) of breast cancer, it is highest in stage III.

Materials and Methods

Patient selection

Newly diagnosed primary breast cancers attending the outpatient clinic of the department of Medical Oncology at Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi, India during April 2009 to February 2012 were recruited. A written informed consent was obtained from all participants and the Institute's Ethics Committee approved the study.

Samples and clinico-pathological information

Venous blood samples (2ml in EDTA Vacutainer) were collected from cases with American Joint Committee on Cancer (AJCC) stage I

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Table 1: Clinicopathologic characteristics of breast cancer cases.

Characteristic	Frequency (%) (N = 126)
Age (yrs) Mean ± SD	47.7 ±11.58
Duration of lump (months)	
Mean ± SD	8.9 ± 17.22
Median (IQR)	6 (2 - 12)
Localization	
Right	51 (40)
Left	75 (60)
Stage	
I	3 (2)
II	33 (26)
III	36 (29)
IV	54 (43)
Histology Invasive Ductal	126 (100)
Hormone Receptor status	
ER+ & PR+	48 (41)
ER- & PR–	59 (50)
One +, one –	11 (9)
Her2 +	68 (60)
Her2 –	46 (40)
TNBC	23 (20)

to IV of primary breast cancers. In addition, blood samples were also collected from healthy female volunteers. All samples were processed immediately.

DNA isolation and quantitative PCR

DNA was subsequently isolated from whole blood by phenol-chloroform extraction and ethanol precipitation [19]. DNA was isolated from 5 x 106 cells, aliquoted and stored at -80°C. For the quantification of nuclear DNA (40ng) we amplified GAPDH housekeeping gene and for mitochondrial, the mitochondrial DNA encoded ATPase (MTATP) 8 gene was amplified. Gene sequences for primers and probes used for detection of GAPDH and MTATP 8 were: GAPDH (forward): 5'- CCC CAC ACA CAT GCA CTT ACC; (reverse): 5'-CCT AGT CCC AGG GCT TTG ATT; probe 5'-(YAK)-TAG GAA GGA CAG GCA AC (BBQ). Mitochondrial DNA (forward): 5'-AAT ATT AAA CAC AAA CTA CCA CCT ACC; (reverse): 5'- TGG TTC TCA GGG TTT GTT ATA; probe: 5'-(FAM)-CCT CAC CAA AGC CCA TA (BBQ). Quantitative PCR was carried out in duplicates on LightCycler02 (Roche). Each 20µl reaction consisted of LightCycler Taqman Master (Roche), 400nM forward and reverse primers, 200nM probe, and 40ng DNA sample. PCR amplification was carried out according to manufacturer's instruction which was 95°C for 10min, followed by 45 cycles at 95°C for 10s and 60°C for 30s and 72°C for 1s. Each run included 5-fold dilutions of an external standard curve generated from healthy leukocyte DNA (including 31250, 6250, 1250, 250, 50 and 10 pg/µl) and negative control (without template).

Clinical follow-up

Starting from the completion of treatment, patients were followed up every 3 months during the first year, every 6 months during the second year, and then yearly until relapse with clinical, biochemical, and radiological examinations. All the patients received multimodality treatment in the form of surgery (for stages I-III), chemotherapy, radiotherapy and hormonal +/- targeted therapy whenever indicated. Overall Survival (OS) was defined as the period from date of diagnosis to death and Disease Free Survival (DFS) was defined as the period from end of treatment to relapse.

Statistical analysis

Data was expressed as mean ± Standard Deviation (SD). Differences between groups were assessed by using Student's t-test.

Characteristic	Group				P			
	Healthy Control (N = 29)	Breast cancer patients						
		Stage I, II & III (N = 72)	Stage IV (N = 54)	All cases (N = 126)				
	1	2	3	4	1 *vs.* 2	1 *vs.* 3	2 *vs.* 3	1 *vs.* 4
Mitochondrial DNA (pg/µl) Mean ± S.D	63707 ± 48476	56035 ± 46549	45177 ± 22997	51381 ± 38524	0.99	0.50	0.74	0.33
Nuclear DNA (pg/µl) Mean ± S.D	39397 ± 20103	23928 ± 13343	26887 ± 16873	25197 ± 14967	< 0.001	< 0.01	0.85	< 0.001
Mitochondrial DNA Content Mean ± S.D	1.70 ± 1.16	3.49 ± 6.52	4.58 ± 0.26	2.94 ± 5.08	0.03	0.78	0.24	0.03

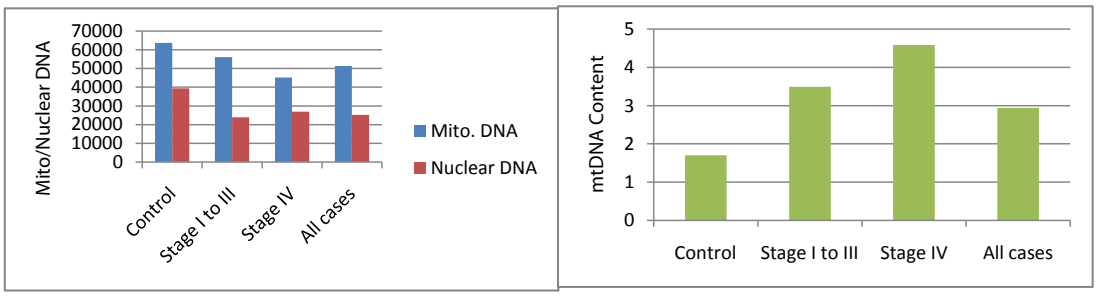

Figure 1: Comparison of Mitochondrial DNA, Nuclear DNA and Mitochondrial DNA content in healthy controls and breast cancer patients.

Variable	Breast cancer patients			P		
	Stage I & II N = 36	Stage III N = 36	Stage IV N = 54			
	1	2	3	1 *vs.* 2	1 *vs.* 3	2 *vs.*3
Mitochondrial DNA (pg/µl) Mean ± S.D	42864 ± 23734	69205 ± 58951	45176 ± 22997	0.01	0.82	0.03
Nuclear DNA (pg/µl) Mean ± S.D	22021 ±12305	25835 ± 14221	26887 ± 16873	0.59	0.34	0.99
Mitochondrial DNA Content Mean ± S.D	2.45 ± 1.58	4.53 ± 9.03	2.21 ± 1.73	0.44	0.99	0.08

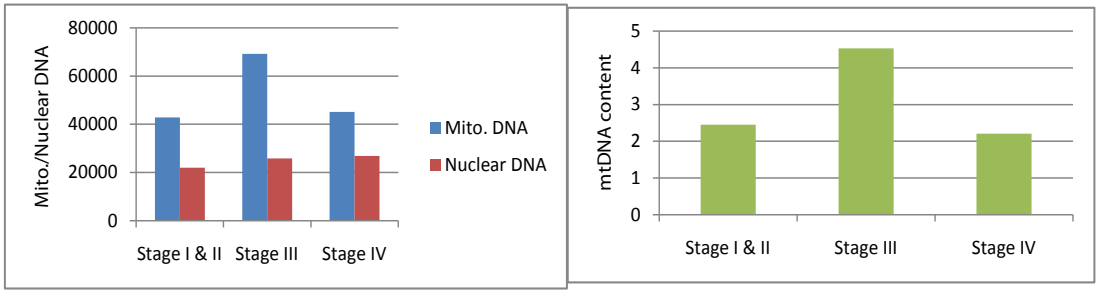

Figure 2: Association of Mitochondrial DNA, Nuclear DNA & Mitochondrial DNA content in different stages of breast cancer patients.

Log transformed values were used for analyses. A P value ≤ 0.05 was considered as significant. OS and DFS were assessed by Kaplan-Meier survival analysis. The association of mitochondrial DNA content with survival was evaluated by Cox proportional hazard regression model. All statistical analysis was done using Stata 12.1.

Results

Patient characteristics

One hundred twenty six patients and 29 healthy female volunteers were enrolled. Mean age of the patients was 47.7 ±11.58 years and that of controls was 42.3 ±12.8 years. Patient characteristics are summarized in Table 1. As can be seen all the cases had ductal carcinoma and about a fifth of them were triple negative.

Mitochondrial DNA levels

Patients showed lower levels of mitochondrial DNA as compared to healthy females though not statistically significant (Figure 1). Among patient groups, levels of mitochondrial DNA were highest in stages III (69205 ± 58951 pg/µL), than in earlier stages (42864 ± 23734 pg/µL) and in patients of stage IV (45176 ± 22997 pg/µL), the difference was statistically significant (Figure 2).

Nuclear DNA levels

The levels of nuclear DNA were significantly higher in healthy controls than in patients (39397 ± 20103 pg/µL *vs.* 25197 ± 14967 pg/µL, P < 0.001, Figure 1) but among the stages (I - IV) levels were similar in all (Figure 2).

Mitochondrial DNA content (mtDNA/nDNA Ratio)

The mtDNA content was significantly higher in all patients (stages I-IV combined or stages I-III) when compared to healthy controls (Figure 1). Among the stages; the ratio was highest in stage III patients and a borderline significance was observed between stage III & IV (Figure 2).

Clinico-pathological parameters

The analysis of the association of various clinico-pathological factors with mitochondrial and nuclear DNA revealed mostly no significant association. The only association was of mitochondrial DNA with the Lymph Node (LN) and Estrogen Receptor (ER) status. The mitochondrial DNA was higher in patients with LN positive disease as compared to LN negative ones (61346.53 ± 52408 *vs.* 42697.50 ± 52408, P = 0.03, Table 2). Patients with ER Negative disease had significantly higher levels of mitochondrial DNA as compared to ER positive patients (53755.56 ± 26803.57 *vs.* 47458 ± 49578.66 P=0.02, Table 2). An increase in mtDNA content was observed in patients with either large tumor size or Lymphoma Vascular Invasion (LVI) positivity (Table 2).

The levels of nuclear or mitochondrial DNA did not reveal any association with the survival analysis of patients (data not shown).

Discussion

One of the important functions of mitochondria is the generation of ATP by oxidative phosphorylation, this process though essential is also associated with increased production of ROS. The higher levels of ROS has been implicated in the oxidative DNA damage, increase in tumorigenicity as well as increase in metastatic ability [20-22] and the changes in mtDNA has long been alleged as the contributors of tumorigenesis [23]. A number of studies have reported either increased or reduced mtDNA content in cancer cells [14,15] or elevated levels in patients with different tumors [24,25]. We observed that mtDNA content (Mitochondrial DNA/ Nuclear DNA) in the peripheral blood of breast cancer patients was elevated in comparison with the peripheral blood from healthy controls. The increase in mitochondrial DNA content and its mass is thought to be the early molecular event in response to the endogenous or exogenous oxidative stress through cell-cycle arrest [26]. Further we observed that among the different stages of breast cancer the mtDNA content in peripheral blood was elevated in stage III patients. In literature, it is reported that the mitochondrial DNA content is reduced in peripheral blood of stage-I breast cancer patients [27] whereas it is significantly elevated in the saliva [24] and the tumor

Table 2: Association of Mitochondrial, Nuclear DNA & Mitochondrial DNA content with clinicopathological features.

Variable	Mitochondrial DNA (pg/µl) Mean ± S.D	Nuclear DNA (pg/µl) Mean ± S.D	Mitochondrial DNA Content Mean ± S.D
Lymph Nodes			
Negative N =24 (33%)	42697.50 ± 26623	21967.08 ± 11020.12	2.40 ± 1.57
Positive N = 49 (67%)	61346. 53 ± 52408	25064.08 ± 14110.74	3.89 ± 7.79
P	0.03	0.44	0. 24
ER			
Negative N=63 (53%)	53755.56 ± 26803.57	28083.81 ± 17105.5	2.57 ± 2.51
Positive N=55 (47%)	47458.18 ± 49578.66	22645.09 ± 12037.47	3.24 ± 7.15
P	0.02	0.11	0.49
PR			
Negative N=66 (56%)	51886.36 ± 26880.14	26555.76 ± 16514.76	2.57 ± 2.46
Positive N=52 (44%)	49467.31 ± 50740.81	24270.76 ± 16514.76	3.28 ± 7.35
P	0.10	0.53	0.39
HER2			
Negative N=46 (40%)	45236.09 ±25823.1	27548.7 ± 18230.34 24094.26 ±	2.1 ± 1.51
Positive N=68 (60%)	54241.76 ± 46634.28	12788.33	3.43 ± 6.07
P	0.28	0.39	0.14
TNBC			
Yes N = 23 (20%)	49926.09 ± 50780.22	29662.17 ± 21160.39	2.16 ± 1.48
No N = 91 (80%)	50780.22 ± 42625.98	24433.19 ± 13826.19	3.07 ± 5.87
P	0.65	0.27	0.67
LVI			
Negative N=33 (51%)	51305.45 ±62601.23	25346.36 ± 15475.97	2.34 ± 1.21
Positive N= 32 (49 %)	60691.88 ± 62601.23	24555 ± 10679.02	4.26 ± 2.34
P	0.80	0.68	0.95
Age			
≤ 48 N = 61 (48%)	50876.07 ± 47010.77	27155.41 ± 17868.86	2.94 ± 6.71
> 48 N = 65 (52%)	51855.69 ± 28744.54	23358.15 ± 11449.64	2.93 ± 2.86
P	0.46	0.29	0.18
Size			
≤ 4cm N=40 (31%)	49817 ± 26635	25598 ± 14124	2.35 ± 1.51
> 4cm N=31 (69%)	64202 ± 63912	22115 ± 12297	4.94 ± 9.68
P	0.57	0.33	0.27
Menopause			
No N = 55 (44%)	49169.82 ± 47132.65	26547.45 ± 18158.45	3.02 ± 7.04
Yes N =71 (56%)	53094.65 ± 30489.61	24150 ± 11963.45	2.87 ± 2.79
P	0.57	0.69	0.28

tissue [28] of advanced stages of head and neck cancer. The increase in the mitochondrial mass and mitochondrial DNA content are thought to be an early molecular event of human cells in response to the oxidative stress exerted through cell-cycle arrest [26]. Thus increase in the mitochondrial DNA content has been looked as a compensatory effect for the decline in mitochondrial respiratory function. This shows a direct proportional relationship between the mitochondrial DNA content and oxidative DNA damage and inverse relationship with respiratory function [28].

Our study shows that the mitochondrial DNA content is significantly altered in the peripheral blood of breast cancer patients in comparison to healthy controls, further our findings provide the first evidence that mtDNA content is elevated in stage III of breast cancer patients. We did not observe any association of mitochondrial DNA content with the survival of patients. Further studies with large sample size are required in this field so as to validate the mitochondrial DNA content in peripheral blood as a potential biomarker for the diagnosis as well as prognosis of breast cancer.

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