



## Analysis of mitochondrial DNA copy number variation in blood and tissue samples of metastatic breast cancer patients (A pilot study)

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### ABSTRACT

Changes in mitochondrial DNA (mt-DNA) copy number in blood/tissue have been linked to increased risk of several cancers; however, studies on their association in breast cancer is still lacking. In this pilot study, we investigated mt-DNA copy number variation in peripheral blood and tissue samples from metastatic breast cancer patients and compared their differences. For the study, peripheral blood samples from non-cancer individuals (control) and breast cancer patients, along with resected tissues from adjacent and tumor sites from same breast cancer patients were collected. Total genomic DNA was isolated and changes in mt-DNA copy number were measured by relative quantification using SYBR green based quantitative real time PCR method. Our results indicated a significant reduction in mt-DNA copy number in blood samples of breast cancer patients compared to control. However, a significantly higher mt-DNA copy number was observed in tumor tissue when compared with paired non tumor tissue. There was no significant difference in mt-DNA copy number between blood and adjacent tumor tissue samples of the breast cancer patients. Overall, our study reports for the first time a comparison of mt-DNA copy number in blood and paired tissue together and suggested that mt-DNA copy number is differentially regulated in blood and tumor tissues in breast cancer.

### 1. Introduction

Breast cancer is one of the most commonly diagnosed cancers in females and a leading cause of cancer deaths contributing to 11.6 % death among all cancers worldwide [1]. In recent years, India has seen a tremendous increase in breast cancer incidences, which has surpassed the cervical cancer to reach to the top of the cancer list [2]. Although due to advancement in diagnostic and treatment strategies, the mortality rate has also decreased specially in western countries, however, it has little effect in developing countries like India. In fact, mortality rate is higher in rural area despite of lower incidences of breast cancer compared to urban cities in India, indicating variation in disease susceptibility and clinical outcome [2]. Therefore analysis of risk factors and their role in disease progression are required to develop effective diagnostic and therapeutic approaches.

In recent years, alterations in mitochondrial mechanisms have been

reported in various cancers, and have been linked to carcinogenesis [3]. Specifically, mitochondrial DNA (mt-DNA) alterations such as mt-DNA copy number variations, mt-DNA rearrangements, deletions, and point mutations have been reported in various cancers, and have been investigated for their use as risk factors or early diagnostic markers [4], however role of such alterations in disease progression is still not clear. Mitochondrion contains multiple copies of its DNA (~16.5 kb in size), and due to its close proximity to respiratory chains (RC) complexes and absence of sophisticated DNA repair mechanism, mt-DNA is susceptible to oxidative damage, commonly observed in cancer cells [5]. Therefore alterations in mt-DNA copy number have been reported to be associated with increased cancer risk [6]. However, their positive or inverse relation with cancer incidence is highly variable and depends on various factors including origin of sample and type of cancer. In case of breast cancer, while several studies have reported somatic and germline mutations in mt-DNA, a very few studies have investigated the alterations in

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mt-DNA copy number in patient samples [7–12]. In these studies, copy number variations have been investigated in either peripheral blood samples or paired tissue samples of breast cancer patients. In addition, some of these reports showed contrasting results indicating the complexity of mt-DNA copy number variations in breast cancer [9,12]. Major reason of such discrepancies are the lack of understanding on their tissue specific changes even in same individuals for understanding the effect of mt-DNA copy number on mitochondrial functions [13]. Therefore, in this pilot study, we investigated the variations in mt-DNA copy number in blood and tissue samples. Mt-DNA copy number was measured in peripheral blood samples from healthy and breast cancer patients, and also from paired non-cancerous adjacent and tumor tissue. Results were compared to see the overall changes in mt-DNA copy number in blood and tissue samples of breast cancer patients.

## 2. Materials and methods

### 2.1. Patient samples and DNA isolation

Female breast cancer patient samples; such as peripheral blood and tissue were obtained from Mahaveer Cancer Sansthan, Patna Bihar after approval from Ethics Committee Mahavir Cancer Sansthan to collaborator Dr. Ashok Kumar Ghosh (Ref N. MCS/Admin/2017-18/707). Samples were from those individuals having diagnosed with breast cancer as detailed in Table 1. Pre-surgical blood samples and surgically resected non-cancerous and tumor tissues were collected from same subjects (n = 14). Standard guidelines from American Joint Committee on Cancer (AJCC) were followed for staging of cancer [14]. Briefly, TNM status of tumor was determined considering tumor extent (T), regional lymph nodal spread (N) and distant metastasis (M). It is an indicator of extent of spread of disease and an important prognostic factor for both, cancer free survival and overall survival. Similarly, grade indicates the degree of aggressiveness of the disease and determined by growth of the cells, in which low grade number (grade 1) indicates slow growing cells and less likely to spread, while grade 2 or above reflects fast growing cells and most likely to spread. Lymph Node (LN) status reflects if lymph nodes were involved by tumor, in which N1 reflects that cancer has spread to 1 to 3 axillary lymph nodes, N2 reflects the spread to 4 to 9 axillary lymph nodes and N3 for 10 or more axillary lymph nodes. Tumor diameter is the “T” component of TNM staging depends on tumor size (cm<sup>3</sup>) and helps in deciding the cancer staging. Peripheral blood from female volunteer free from cancer were collected for age matched controls (n = 14). DNA was extracted from 500µl blood samples using a QIAamp Blood Kit (Qiagen, Frederick, MD, USA) according to the manufacturers’ instructions. DNA quantity was measured using Nano-drop spectrophotometer (Thermo scientific, Fitchburg, WI, USA).

**Table-1**

Clinicopathological information of breast cancer patients.

| S. N. | Patient Group | Age (Y)                 | Stage | LN Status | Tumor Grading | Tumor diameter (cm <sup>3</sup> ) |
|-------|---------------|-------------------------|-------|-----------|---------------|-----------------------------------|
| 1     | P-1           | 42                      | 3B    | N1        | 2             | 7                                 |
| 2     | P-2           | 52                      | 3B    | N2        | 2             | 8                                 |
| 3     | P-3           | 55                      | 3A    | N1        | 1             | 4                                 |
| 4     | P-4           | 64                      | 3A    | N1        | 2             | 3                                 |
| 5     | P-5           | 41                      | 3B    | N2        | 2             | 7                                 |
| 6     | P-6           | 45                      | 3B    | N1        | 2             | 8                                 |
| 7     | P-10          | 59                      | 3A    | N2        | 2             | 4                                 |
| 8     | P-11          | 56                      | 3A    | N1        | 2             | 3                                 |
| 9     | P-12          | 44                      | 3C    | N2        | 2             | 12                                |
| 10    | P-15          | 39                      | 3A    | N1        | 2             | 4                                 |
| 11    | P-16          | 40                      | 2B    | N0        | 1             | 3                                 |
| 12    | P-17          | 51                      | 3B    | N1        | 2             | 7                                 |
| 13    | P-19          | 45                      | 3A    | N1        | 2             | 5                                 |
| 14    | P-20          | 41                      | 3A    | N1        | 2             | 4                                 |
|       |               | Mean Age: 48.14 ± 2.133 |       |           |               |                                   |
|       |               | Median Age: 45          |       |           |               |                                   |

### 2.2. Mitochondrial DNA copy number measurement

Relative mt-DNA copy number was measured by quantitative PCR (qPCR) through simultaneous amplification of mt-DNA and nuclear DNA, and calculated after normalizing mt-DNA amplification from nuclear amplification. The mt-DNA specific primers for Human t-RNA leucine 1 gene (forward primer: CACCCAAGAACAGGGTTT GT and reverse primer: TGGCCATGGGTATGTTGTTAA) and nuclear DNA primers for 18s r-RNA gene (forward primer: TAGAGGA-CAAGTGGCGTTC and reverse primer: CGCTGAGCCAGTCAGTGT) were used for quantitative PCR to determine the mt-DNA copy number following previously published protocols [15,16]. The q-PCR reaction was set up according to the manufacturer’s instruction and optimized for more than 95% amplification efficiency (Fast SYBR Green Master Mix, Applied Biosystems) and run on 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative fold changes were calculated by  $2^{-\Delta\Delta C_T}$  ( $\Delta C_T = C_T$  mt-DNA -  $C_T$  n-DNA) method following previously published [16].

### 2.3. Statistical analysis

Statistical analysis was done using Graph Pad Prism software (Version 5.0, San Diego, CA, USA). The continuous variables were compared using Student-t-test between two groups, and more than two groups were compared with ANOVA/Bonferroni’s multiple comparison test when appropriate. The differences between groups with  $P < 0.05$  were considered statistically significant.

## 3. Results

The major aim of this study was to investigate the differences in mt-DNA copy number from blood and tissue compartments of breast cancer patients. Therefore, mt-DNA copy number was determined in 1) blood samples from healthy and breast cancer patients, and 2) paired non-cancerous and tumor tissue from same breast cancer patients who were undergone surgery. The detail of breast cancer patients and age information of control group in this study is summarized in Table 1 and 2 respectively. The mean age of patients and control subjects (n = 14) at the time of study were  $48.14 \pm 2.133$  and  $41.07 \pm 3.489$  respectively and difference between the age of both groups were found non-significant. Almost all the patients in this study were diagnosed with invasive breast cancer of stage 3A-C, and metastatic type of cancer (N1 or more), with an exception of P-16. Out of fourteen, 12 patients had grade 2 or moderately differentiated tumor mass as confirmed through pathological investigations, and overall tumor size varied from 3-12 cm<sup>3</sup> in these patients (Table 1). Total genomic DNA was isolated from peripheral blood samples of control and patient groups and used for

**Table 2**

Age details of the control group.

| S.N. | Control Group | Age (Y)                  |
|------|---------------|--------------------------|
| 1    | C-1           | 31                       |
| 2    | C-3           | 65                       |
| 3    | C-5           | 55                       |
| 4    | C-6           | 50                       |
| 5    | C-7           | 30                       |
| 6    | C-9           | 52                       |
| 7    | C-10          | 32                       |
| 8    | C-12          | 60                       |
| 9    | C-19          | 25                       |
| 10   | C-16          | 39                       |
| 11   | C-18          | 35                       |
| 12   | C-20          | 40                       |
| 13   | C-21          | 36                       |
| 14   | C-14          | 25                       |
|      |               | Mean Age = 41.07 ± 3.489 |
|      |               | Median Age: 37.5         |

analysis of mt-DNA copy number by performing q-RT-PCR through optimized protocol as published earlier [15,16]. The comparison of blood samples showed approximately 34% decrease in mt-DNA copy number in patient groups compared to control group ( $p < 0.0388$ ) (Fig. 1).

To further investigate the tissue specific changes in mt-DNA copy number, we isolated genomic DNA from resected paired non-cancerous and tumor tissues and measured the mt-DNA copy number as discussed previously. Interestingly, we found an opposite pattern from blood investigations and observed a significant 2 fold increase in mt-DNA copy number in tumor tissue than paired non-cancerous adjacent tissue ( $p < 0.0136$ ) (Fig. 2). In order to investigate the changes in mt-DNA copy number across the samples in breast cancer, we compared the fold changes in matched blood, adjacent and tumor tissues of the patients. Relative to blood mt-DNA copy number, a significant 6.36 fold increase was observed in tumor tissue (Fig. 3). However compared to blood, increase in mt-DNA copy number in adjacent tissue was not significant.

Altogether, results on mt-DNA copy number variations indicated that mt-DNA copy number was decreased in blood samples of patients compared to healthy individuals. However, when paired tissue samples were compared, it resulted in an increase in mt-DNA copy number in tumor tissue. Together with paired analysis of mt-DNA copy number in blood and tissue samples of patients, no significant difference was observed in blood and adjacent tissue, while mt-DNA copy number in tumor tissue was significantly elevated.

#### 4. Discussion

Mitochondrial DNA alterations have long been investigated in different cancers to identify their relation to cancer risk; however mt-DNA copy number changes are highly variable and dependent on various factors including contribution of mitochondrial functions in different cancer types [6,17]. In case of breast cancer, only a handful of studies have been reported which have investigated mt-DNA copy number changes, either in blood samples or paired tissue samples with contrasting findings [7–12,18]. For example, some of the studies focusing on blood samples reported a significant increase in mt-DNA copy number [8,10,11]. Conversely, other studies on mt-DNA copy number measurements in paired tissue samples, reported a decrease in mt-DNA copy number [9,12,18–20]. In addition, combined with mt-DNA copy number variations with other cancers, several reports

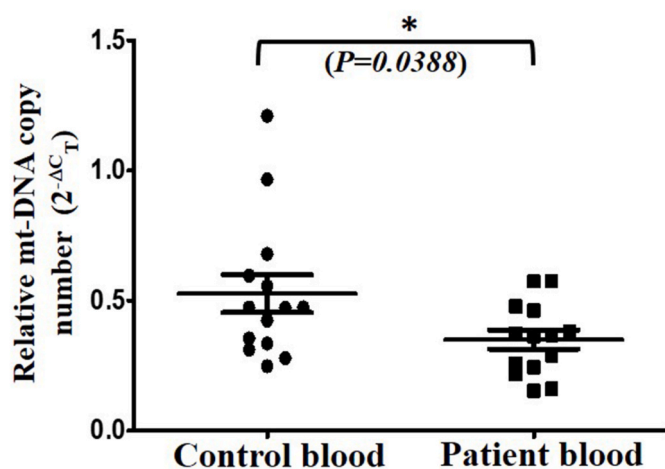


Fig. 1. Mitochondrial DNA copy number variations in peripheral blood samples. Genomic DNA was isolated and used for q-RT-PCR as described in methods. Relative mt-DNA copy number was determined by  $2^{-\Delta C_T}$  method and compared between values (Mean  $\pm$  SD) of patient blood samples from control blood samples. Student's t-test (unpaired, two tailed) was performed to analyze the significance.  $^*(P < 0.05)$ .

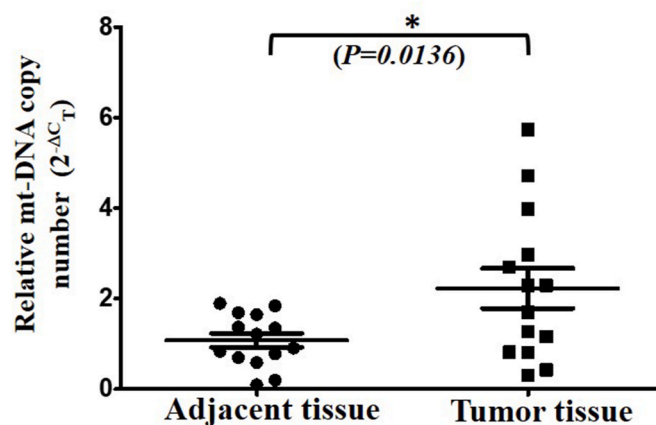


Fig. 2. Variation in mt-DNA copy number in paired breast cancer tissue. Mt-DNA copy number from paired adjacent non tumor and tumor tissue was determined using q-RT-PCR method. The relative fold change ( $2^{-\Delta C_T}$ ) of adjacent and tumor tissue (Mean  $\pm$  SD) is shown. Two tailed paired Student's t-test was used to calculate the significance.  $^*(P < 0.05)$ .

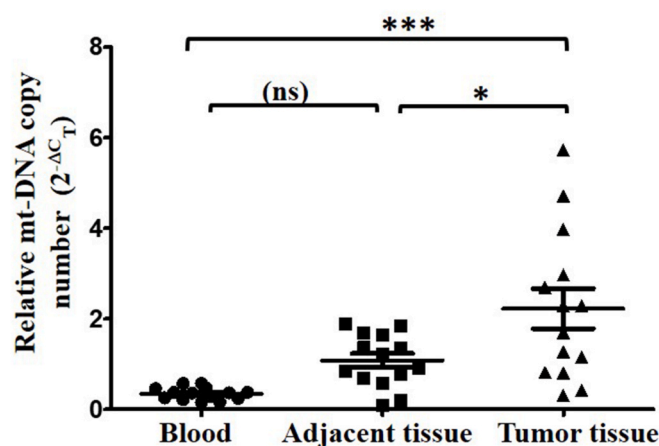


Fig. 3. Comparison of mt-DNA copy number in blood and tissue of breast cancer patients. The relative fold change ( $2^{-\Delta C_T}$ ) of blood, adjacent and tumor tissue (Mean  $\pm$  SD) is shown. One way ANOVA and Bonferroni's Multiple Comparison Test was used to calculate the significance.  $^{***}(P < 0.001)$ ,  $^*(P < 0.05)$ , ns-non significant.

suggested variability in blood vs tissue analysis across different cancers [6,17,21]. For example, in an analysis from The Cancer Genome Atlas (TCGA) data, a decrease in mt-DNA copy number in breast cancer tissue samples has been observed [17]. However in a separate study on meta-analysis, increased mt-DNA copy number was associated with breast cancer combining blood and tissue analysis [6]. There is no clarity on predictive value of mt-DNA copy number in breast cancer, as some indicates low mt-DNA copy number for better prognosis for metastasis [22], while other suggests the contrary [21]. In addition, no data is available on comparison in blood and tissue samples together in breast cancer, which may indicate if such differences co-exist in individual patients, and would allow better understanding of mitochondrial contribution in breast cancer for a given set of conditions. In our study, we selected only metastatic breast cancer cases and measured the mt-DNA copy number in blood and paired tissue samples. Although our study is in line with previous reports on showing inverse relation in blood vs tissue samples, while individual comparisons in blood or tissue samples for mt-DNA copy number resulted in opposite trend from previous reports. In our study, the cancer blood samples were first compared with healthy controls and we found a decrease in the mt-DNA

copy number (Fig. 1). Since previous studies on measurement of mt-DNA copy number in blood samples were reported in patients above 50 years of age [8,10,11,23], this difference could be possibly due to variation in the age group in our study (median age 45). Further, we observed a significant increase in mt-DNA copy number in tumor tissue when compared with its paired non-cancerous tissue (Fig. 2). This is in line with previous reports in which elevated mt-DNA copy number is associated with invasive breast carcinoma along with higher frequency of D-loop region mutations and over expression of several hormone receptors and cancer biomarkers [9]. Since mt-DNA copy number varies with pathological characteristics, the increase in mt-DNA copy number has also been associated with increasing grade (from grade II to III) and very large size of tumor (>50cm<sup>3</sup>) as reported earlier [19]. Similarity, in an early study by Yu et al., although an overall decrease in mt-DNA copy number was observed in paired breast cancer tissue, a significant upregulation was also observed to be associated in patients below 50 years of age (66.7%) and patients with histological grade 2 tumor [12]. In our study, most of the cases had metastatic breast cancer (LN status: N1 or N2, 13/14) and grade 2 (12/14), and mean age below 50 years, corroborating with these reports of higher mt-DNA copy number in paired breast cancer tissue. However, elevation of mt-DNA copy number increase in our case differ from other studies, in which low mt-DNA copy number has been observed in breast cancer tissue samples [12,18,20]. Such discrepancies have also been observed in other cancers and partly account for metabolic flexibility of different cell types in cancer tissue, genetic diversity, tumor purity and functional status of mitochondria [17]. For example, functional analysis of mitochondria in invasive breast cancer patients suggested that epithelial breast cancer cells show upregulation of mitochondrial biogenesis and transcription and may contribute to fueling the invasiveness of breast cancer [24]. However, this upregulation is absent in adjacent stromal cells that have mitochondrial dysfunction and highly dependent on glycolysis supporting Warburg's hypothesis [25]. Similarly, several OXPHOS inhibitors are now in clinical trials for certain cancers, which specifically target upregulated mitochondrial functions indicating an increased dependency on mitochondrial functions in carcinogenesis [26]. Comparison of blood and tissue samples together from breast cancer patients in our study resulted in a consistency increase in mt-DNA copy number in tumor tissue; however, there was no significant difference in the mt-DNA copy number in patients blood to adjacent non-cancerous tissue (Fig. 3). Our study also indicated that changes in mt-DNA copy number from tumor tissues were not necessarily reflected in blood samples of cancer patients, as we found a decrease not increase in the blood samples of patients compared to non-cancer individuals. Since no significant difference was observed in blood and adjacent non-tumor tissue samples of patients; it further suggest that; to obtain a more clear idea on mtDNA copy number variations in cancer, analysis of tissue and blood samples together would be more appropriate choice than analyzing in blood or in paired tissue only. However, with this small sample size and lack of functional studies, it is difficult to conclude whether this increase in mtDNA copy number in tumor tissues indicates functional requirement or compensation for impaired mitochondrial functions.

Therefore, it remains crucial to understand if alterations in mt-DNA copy number has any functional association with mitochondria: whether increased mt-DNA copy number reflects increase compensation for mt-DNA mutations/dysfunction or increased demand for mitochondrial energy/metabolites for growth and invasiveness. Although, from our study, this issue remained unsolved, however based on these preliminary data, future studies are planned in a larger sample size to reconfirm our finding on elevated mt-DNA copy number as well as investigating mitochondrial functions. It will help to understand functional role of mt-DNA copy number variations in breast carcinogenesis and would be useful for breast cancer risk assessment or prognosis.

## Availability of data and materials

The data that support the findings of this study are available from the corresponding author LKS upon reasonable request.

## Authors statement

NKR planned and performed the experiments, compiled and analyzed the data. GP and AKG and were involved in sample collection, histopathological reporting, tabulation of data. RH helped in analyzing and critically proofreading the manuscript. LKS wrote the manuscript. Manuscript was reviewed by all the contributing authors.

## Declaration of competing interest

None.

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