Cytological Study of Breast Carcinoma Before and After Oncotherapy with Special Reference to Morphometry and Proliferative Activity

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Received: 27 February 2013 / Accepted: 15 May 2013 / Published online: 26 June 2013
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Abstract Our study was done to assess the cytological changes due to oncotherapy in breast carcinoma especially on morphometry and proliferative activity. Cytological aspirates were collected from a total of 32 cases of invasive ductal carcinoma both before and after oncotherapy. Morphometry was done on the stained cytological smears to assess the different morphological parameters of cell dimension by using the ocular morphometer and the software AutoCAD 2007. Staining was done with Ki-67 and proliferating cell nuclear antigen (PCNA) as proliferative markers. Different morphological parameters were compared before and after oncotherapy by unpaired Student's *t* test. Statistically significant differences were found in morphometric parameters, e.g., mean nuclear diameter, mean nuclear area, mean cell diameter, and mean cell area, and in the expression of proliferative markers (Ki-67 and PCNA). Statistical analysis was done by obtaining *p* values. There are statistically significant differences between morphological parameter of breast carcinoma cells before and after oncotherapy.

Keywords Breast carcinoma · Oncotherapy · Morphometry · Proliferative activity

Abbreviations

TDLU	Terminal duct lobular unit
MRI	Magnetic resonance imaging
FNAC	Fine needle aspiration cytology
PCNA	Proliferating cell nuclear antigen
H&E	Hematoxylin and eosin
PAP	Papanicolaou
MGG	May-Grunwald Giemsa
MND	Mean nuclear diameter
MNA	Mean nuclear area
MCD	Mean cell diameter
MCA	Mean cell area
N:C ratio	Nucleocytoplasmic ratio
pCR	Pathological complete remission

Introduction

Breast carcinoma is the most common malignant tumor and one of the leading causes of carcinoma death in women, with more than 1,000,000 cases occurring annually [1]. About 900,000 were diagnosed every year with breast cancer. More than half of these cases are in industrialized countries, about 220,000 in Europe and about 180,000 in North America. Mortality rates from breast cancer have increased during the past 60 years in every country [2]. In India, cancer of the breast is the most common cancer among women in many regions and has overtaken cancer cervix, which was the most frequent cancer a decade ago [3]. In India, the incidence of breast carcinoma in male and

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female is 0.26 and 20.01 per 1 lakh population, respectively, whereas mortality rates are 1.20 and 4.32 per 1 lakh population in male and female, respectively [4]. Most breast cancers usually present as a hard lump which may be associated with indrawing of the nipple. As the disease advances locally, there may be skin involvement leading to *peau d'orange* or frank ulceration and fixation to chest wall cancer *en cuirasse* [5]. Breast carcinoma can be diagnosed by the “triple diagnosis technique,” which involves clinical examination (palpation), mammography, and fine needle aspiration cytology. Patients should also be encouraged for self-breast examination [6]. Beside these, there are core biopsy, open biopsy, MRI, and sentinel lymph node biopsy. The technique fine needle aspiration cytology (FNAC) should generally be preceded by mammographic or ultrasonographic examination to select the most appropriate area to be biopsied. The sensitivity of FNAC is 87 %, specificity close to 100 %, the positive predictive value is 100 %, and the negative predictive value is between 60 and 90 % [7]. Morphometry is the quantitative description of the biological structure. To improve the clinical value of malignancy grading, quantifying nuclear pleomorphism by measuring nuclear features, area, perimeter, and diameter has been suggested [8].

Treatment of breast carcinoma involves different modalities including surgery radiotherapy, chemotherapy, hormonal therapy, and targeted therapy, e.g., herceptin (against Her-2-neu-), lapatinib, and trastuzumab. Neoadjuvant chemotherapy, implying that the patient will receive chemotherapy before the complete surgical removal of the carcinoma, has been recently the standard of care for the treatment of locally advanced breast cancer. One of the major benefits of neoadjuvant chemotherapy is that it can diminish large cancers enabling the surgeon to remove the residual tumor by a more limited operation, such as lumpectomy, instead of mastectomy. The response to neoadjuvant chemotherapy may provide some indication about the potential response of the tumor to further treatment [9]. Radiation can reduce the risk of breast cancer recurrence by about 70 % [10]. Ki-67 is an antigen that appears to correlate with cell proliferation. Tumors with high proliferation fraction have a poor prognosis. Ki-67 is a non-histone protein found on the long arm of chromosome 10 [11]. Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase δ . It seems to be essential for DNA synthesis and is expressed in high concentrations during the cell cycle. Anti-PCNA, also known as cyclin, is a 36-kDa non-histone nuclear protein. It has been demonstrated that PCNA is an auxiliary protein of DNA polymerase- δ and plays a fundamental role in the initiation of cell proliferation. Elevated expression of PCNA appears in the nucleus during late G1 phase immediately before the onset of DNA synthesis, becomes maximal during S phase, and declines again during G2 and M phases.

Therefore, its level correlates directly with the rates of cellular proliferation and DNA synthesis [12].

Our study was done to assess the cytological changes due to oncotherapy, in the case of breast carcinoma, especially on morphometry and to study the relationship between proliferative activity of the tumor (by Ki-67 and PCNA) and effect of oncotherapy.

Materials and Methods

The study was done at the Department of Pathology, with the collected cytological aspirates and tissue specimens of the breast from the patients who visited the Department of Radiotherapy (whenever available), and the cases have been collected over a period of 2 years (June 2010–May 2012). We included those patients who are known, i.e., diagnosed cases of breast carcinoma attending the outdoor of radiotherapy department. We have taken history and have done physical examination and FNAC from the breast lump of these patients before and after receiving oncotherapy. The term “oncotherapy” is used to mean chemotherapy given before mastectomy. Simple random sampling was done. It included few inclusion and exclusion criteria. Those patients who were included in this study were diagnosed with breast carcinoma and who have not received any kind of therapy except the present therapy and whenever available for biopsy. Patients who have received any kind of therapy except the present therapy were excluded from the study. Exclusion criteria also included those patients who could not come for follow-up or discontinue the therapy. The therapy used is given below.

Neoadjuvant chemotherapy protocols for breast carcinoma include the following:

1. FEC protocol:
 - (a) 5-Fluorouracil (injection) 600 mg/m², i.v., day 1
 - (b) Epirubicin (injection) 80 mg/m², i.v., day 1
 - (c) Cyclophosphamide (injection) 600 mg/m², day 1
 Three cycles given at 3 weeks interval
2. PE/TE protocol: This protocol is given in more advanced cases and in cases with more risk factors.
 - (a) Paclitaxel (injection) 175 mg/m², i.v., day 1
 - (b) Epirubicin (injection) 80 mg/m², day 1
 Three cycles at an interval of 3 weeks
 (More advanced cases mean: locally fungated growth/clinically involved axillary or supraclavicular lymph nodes/hepatic/pulmonary/bone metastasis).
 (More risk factors mean: hormonal receptor status ER(–), PR(–), Her 2 neu(+)/premenopausal patients/positive family history.)

Informed consents were taken from the patients. All the patients were thoroughly investigated and examined. The breast was palpated with the palmar surface of the fingers. Four quadrants were palpated systematically and also the axillary tail. If a lump was detected, then the following points are noted: local temperature and tenderness, situation (in which quadrant), number, size and shape, surface, margin, consistency, fluctuation, fixity to skin, fixity to breast tissue, fixity to underlying fascia and muscles, fixity to the chest wall, and palpation of nipple. Then, examination of axillary lymph nodes was done. General examination was done followed by special investigations [13].

First FNAC was done from the breast lump. The cytological aspirates that were obtained were stained by MGG and Papanicolaou (PAP) and morphometry was done. Repeat FNAC was done after the patient has received oncotherapy and the same procedure for slide staining was performed followed by morphometry. For histopathology, biopsy specimens when available were fixed in formalin, processed, and embedded in paraffin wax block. Sections of 3- μm thickness were cut from the paraffin block and stained with H&E. Some unstained slides of cytological aspirates were kept and used for immunocytochemistry for Ki-67 and PCNA. This staining was done on cytological aspirates both before and after oncotherapy. Morphometric analysis was made on PAP-stained slides by the aid of an ocular morphometer attached to $\times 10$ eyepiece of a microscope using a $\times 40$ high power objective. The ocular morphometer was calibrated using a calibration slide or stage morphometer provided with it. One smallest division in stage micrometer is equated with 0.01 mm. One smallest division of ERMA ocular micrometer is equated with 2.5 μm . One hundred random nuclei from the most atypical area of the section were subjected to analysis [14]. The images were also analyzed by using the software AutoCAD 2007. The characteristics studied upon were mean cell diameter (MCD), mean nuclear area (MNA), and mean cell area (MCA) as observed and calculated in different sections.

The nuclear area was calculated by the formula: $A = \text{JLX } a \times b$, where a and b are semi-largest and semi-smallest nuclear diameter. The nucleocytoplasmic ratio (N:C ratio) was obtained by dividing nuclear area by the cell area [14]. Histology slides were examined to validate the diagnosis by FNAC. Staining for Ki-67 and PCNA was done by polymer chain two-step indirect technique [15]. Staining for Ki-67 was done following Cell Marque kit manual using PolyScan polymer HRP/DAB detection system. The antibody stains positive in the nucleus of the cells in cytological smears. At least 1,000 nuclei were counted at $\times 1,000$ magnification and the results were expressed as a ratio of stained to total nuclei counted in percentage (Ki-67 labeling index) [16]. Staining for PCNA index was done by using BioGenex PCNA kit manual [17]. PCNA labeling index was calculated in the

same way as for Ki-67. Finally, a grand chart was prepared tabulating FNAC findings (before and after chemotherapy), histological diagnosis, and Ki-67 and PCNA findings. The results obtained were analyzed. Analysis of data was done by unpaired Student's t test (two-tailed distribution; two-sample equal variance) to calculate the significance of difference in different parameters of breast carcinoma cells before and after oncotherapy. P values below 0.05 were considered as significant.

Results and Discussion

In the 2-year study period, cytological aspirates of breast tissue were collected from 32 cases. Histological samples were collected from six cases. Most of the cases were seen in the age group of 40–50 years. In the age group of 30–40 and 50–60 years, the distribution of patients is equal. The most common mode of presentation of breast carcinoma was seen to be palpable breast lump, which was found in 31 cases out of 32 cases. Nine cases presented with axillary lymphadenopathy. Only one case was seen to be presented with supraclavicular lymph node enlargement. All 32 cases were found to be infiltrating ductal carcinoma. Mean nuclear diameter (MND), MNA, MCD, and MCA have been increased after oncotherapy when compared to these parameters before oncotherapy (Figs. 1 and 2). The MND before oncotherapy was seen to be 11.6 μm , whereas after oncotherapy, it was 14.6.114.3 μm^2 . MNA before oncotherapy changed to 167.32 μm^2 after oncotherapy. Likewise, all the parameters show an increase in value (Table 1).

Unpaired Student's t test (two-tailed distribution; two-sample equal variance) was done thereafter to calculate the significance of difference in different parameters like MND, MNA, MCD, MCA, and N:C ratio between various benign and malignant breast lesions and also with the controls (normal tissue). The results are shown; a p value < 0.05 was considered as significant. The p values of the

Fig. 1 Photomicrograph showing cytology of breast carcinoma (before oncotherapy; PAP stain, $\times 400$)

Fig. 2 Photomicrograph showing cytology of breast carcinoma (after oncotherapy; PAP stain, $\times 400$)

parameters like MND, MNA, MCD, and MCA all were found to be <0.05 , i.e., statistically significant. The mean value of Ki-67 and PCNA labeling was found to be decreased after oncotherapy (Fig. 3). The mean value of Ki-67 index before oncotherapy is 24.38, whereas the mean value of Ki-67 index after oncotherapy was 10.56; so here, we can see that Ki-67 labeling index has decreased much after the oncotherapy. Mean value of PCNA before oncotherapy is 50.40, and the mean value of PCNA labeling index after oncotherapy is 30 (Table 2). The p value of the Ki-67 and PCNA labeling indices shows significant differences in parameters before and after oncotherapy.

A total of 32 patients were included in the study. The study was done with cytological aspirates from those patients before and after oncotherapy. All the cases were seen to be invasive ductal carcinoma. Regarding age, we find that the most common age group for breast carcinoma is 40–50 years. In the study, we found that the most common mode of presentation of breast carcinoma is painless, palpable breast lump. According to Park et al., breast cancer incidence rises rapidly between 35 and 50 years of age [2]. According to Ambreen Muneer et al., the commonest histopathology was infiltrating duct cell carcinoma (60 %), in a study with 214 patients [18]. Chow et al. showed that the mean age of the patients was 56.6 years, which is just higher than our findings. The most common complaint was the presence of a mass which occurred in 635 (90.6 %) patients and 454 (71.5 %) of these patients were painless [19]. Bhattacharya et al. in their study showed that the mean

Fig. 3 Photomicrograph showing Ki-67 staining on cytology smear (before oncotherapy; stained with monoclonal antibody against Ki-67, $\times 400$)

age was 45.7 years in females; most patients presented with a painless lump mainly [20]. Albrektsen et al. in their study showed that ductal tumors, the most common histological type, accounted for 81.4 % of all cases [21].

We performed morphometry in all the cases and controls with an ocular micrometer and a calibrated stage micrometer. Morphometric analysis was performed with PAP-stained smears. In our study, we found a significant difference, i.e., increase in MND, MNA, MCD, and MCA, after oncotherapy (Table 1 and Figs. 1 and 2). In a study with 35 patients, Rasbridge et al. studied the effects of chemotherapy on the tumor morphology and various biological features of breast carcinoma. The characteristic morphological findings that were seen were malignant cells that become enlarged with vacuolated cytoplasm and vesicular nuclei containing prominent nucleoli; occasionally, the nuclei were angular and hyperchromatic. In 15 cases, sufficient material was present in the pretreatment biopsies to compare the grade of the tumors before and after chemotherapy: changes were found in six tumors. Cytotoxic drugs do not induce a consistent pattern of change in the proliferation and apoptotic indices of individual tumors, but there is a tendency to reduce proliferative activity over all the tumors as a group. It was also found that chemotherapy is capable of modifying the expression of the oncoproteins c-erbB-2 and p53 in a minority of cases of breast cancer [22]. According to Grey, after chemotherapy, some nuclei may be very large and there is marked anisonucleosis. The N:C ratio is normal; however, there are some scattered bipolar cells [23]. Radiation therapy

Table 1 Mean of different morphological parameters before and after oncotherapy

Different parameters	Before oncotherapy	After oncotherapy
MND (μm)	11.6	14.6
MNA (μm^2)	114.13	167.32
MCD (μm)	15.48	19.62
MCA (μm^2)	191.18	310.34

Table 2 Mean value of proliferative markers labeling indices before and after oncotherapy

Mean value of labeling indices of proliferative markers	Before oncotherapy	After oncotherapy
Ki-67	24.38	10.56
PCNA	50.40	30

in breast carcinoma may result in bizarre nuclear changes, formation of giant tumor cells, naked nuclei, and abnormal mitotic figures. Extensive tumor necrosis may develop, which may be surrounded by a thick fibrous wall. The most characteristic effect is the presence of atypical epithelial cell in terminal duct lobular unit (TDLU). Chemotherapy can also induce striking morphological changes in tumor cell including a degree of vacuolization to simulate histiocytes [24]. It also results in the atrophy of TDLU with occasional atypia. Radiation therapy-induced cytological atypia may occur in epithelial, endothelial, or stromal cells. The atypical cells display enlarged hyperchromatic nuclei with an increased nucleocytoplasmic ratio. While the nuclei are hyperchromatic, chromatin pattern is blurred (smudge chromatin pattern) [25]. Sneig et al. assess chemotherapy-induced histologic changes in breast cancer to evaluate their relationship with tumor response, treatment, and patient survival. They examined paired pre- and post-chemotherapy tumor specimens from 57 patients with stage II and III breast cancers. Sixty-eight percent of tumors demonstrated moderate to severe cytologic changes in the cytoplasm and nucleus of neoplastic cells. These were characterized by increased overall cell size, cytoplasmic vacuolization, nuclear enlargement, multinucleation, and vesiculation of chromatin. Chemotherapy-induced histologic changes varied within a tumor and between patients. Tumor cytologic changes were significantly associated with histologic ($p=0.03$) as well as clinical ($p=0.02$) tumor response [26]. Some studies showing the effect of oncotherapy on cytological aspirates of other different organs were done. Grogden et al. from the University of Dundee, Scotland showed the effect of radiotherapy on oral mucosa assessed by quantitative exfoliative cytology. A highly significant rise in both nuclear area ($p=0.002$) and cytoplasmic area ($p=0.001$) resulted from exposure to radiation [27].

We find a significant difference in Ki-67 and PCNA labeling indices before and after oncotherapy (Table 2 and Fig. 3).

Tanei et al. showed the prognostic significance of Ki67 index after neoadjuvant chemotherapy in breast cancer. In the 72 non-pathological complete remission (pCR) patients, Ki67 index significantly ($p<0.001$) decreased after NAC, so it was seen that Ki-67 after NAC, but not before NAC, is prognostic in breast cancer patients and might be clinically useful in the prognosis prediction of patients who do not achieve pCR after NAC [28]. DEPES et al. in their study showed that there was a significant decrease in the average index of anti-PCNA-stained cells before (time A) and after (time B) chemotherapy ($p=0.041$). Responder patients displayed a significant decrease in PCNA levels [time A=53.1 and time B=30.7 ($p=0.011$)] [29]. In our study, we find that the mean value of PCNA before oncotherapy was 50.40 and after oncotherapy 30.

There are statistically significant differences between morphological parameter of breast carcinoma cells and also in the expression of proliferative markers before and after oncotherapy

Acknowledgments This study was supported by the departmental fund of the institution supplied by the Government of West Bengal.

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