Morphometric and Histological Study of Breast Lesions with Special Reference to Proliferative Activity and Invasiveness

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Abstract Breast carcinoma is the most common cause of carcinoma death in women. Sometimes, difficulty arises to differentiate between premalignant lesions and carcinoma by routine histopathology. Our study was done to establish the role of morphometry and immunohistochemistry to solve this problem. In this study, total 60 cases of different breast lesions were included and 10 controls were also included to compare the results with the normal findings. They were studied by hematoxylin and eosin-stained sections for morphometry and routine histological study; as well as by proliferative markers such as proliferating cell nuclear antigen and p53. Invasiveness was studied using immunohistochemical staining with 34 β E12 monoclonal antibody. Statistically significant differences were found in morphometric parameters and in expression of proliferative markers between most of them. Morphometry and immunohistochemistry help in the proper diagnosis of different breast lesions that lie in the gray zone on routine histopathology.

Keywords Breast lesions · Morphometry · Markers of proliferative activity and invasiveness

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Abbreviations

H&E	Hematoxylin and Eosin
ME cell	Myoepithelial cell
DCIS	Ductal carcinoma in situ
LCIS	Lobular carcinoma in situ
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
IHC	Immunohistochemistry
UDH	Usual ductal hyperplasia
ADH	Atypical ductal hyperplasia
PCNA	Proliferating cell nuclear antigen
MNA	Mean nuclear area
MNP	Mean nuclear perimeter
MMND	Mean maximum nuclear diameter
Mmnd	Mean minimal nuclear diameter
MND	Mean nuclear diameter
MCA	Mean cell area
MCD	Mean cell diameter
N/C ratio	Nuclear to cytoplasmic ratio
MAb	Monoclonal antibody
PCNA LI (%)	PCNA labeling index
μ	micron
PAP	Peroxidase antiperoxidase
SD	Standard deviation
No.	Number

Introduction

Breast carcinoma is the most common malignant tumor and the leading cause of carcinoma death in women, with more than 10,00,000 cases occurring worldwide annually [1]. In India, cancer of the breast is the most common cancer among women in many regions and has overtaken cervix

cancer, which was the most frequent cancer a decade ago [2]. Benign or malignant lesions do not pose any problem in histopathological diagnosis. But difficulty arises in differentiating between premalignant lesions and carcinoma in situ and minimally invasive carcinoma [3]. There are great differences so far in the prognosis of the lesions concerned. Morphometry and immunohistochemistry (IHC) help to solve this problem.

Morphometry is the quantitative description of the biological structure. To improve the clinical value of malignancy grading, it has been suggested to quantify nuclear pleomorphism by measuring nuclear features such as area, perimeter, and diameter [4].

IHC has an expanding role in distinguishing usual ductal hyperplasia from atypical ductal hyperplasia/low-grade carcinoma in situ, subtyping a lesion as ductal versus lobular or basal versus luminal, helping distinguish true microinvasion from mimics (pseudoinvasion), predicting the likelihood of response to anti-hormonal and other therapeutic agents, improving sentinel node staging, and finally, helping recognize metastatic carcinoma of unknown primary site as originating in the breast [5]. The tumor suppressor gene p53 is one of the most commonly mutated genes in human cancer of all types. Mutation in p53 gene appears to be a strong indicator of poor prognosis independent of other risk factors [6]. Mutation in p53 occurs in 19–57 % of sporadic breast carcinomas [7]. p53 mutations are more frequently reported later in the course of transformation, their presence could reflect a greater malignant potential of the tumor and, thus, an increased probability of metastasis and recurrence after local therapy [8]. Tumor cell proliferation is an important biological variable, which can be regarded as an additional prognostic indicator. The proliferative activity of breast cancers has been evaluated using several methods, among which is the study of the percentage of cells expressing cell cycle-related antigens. 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 [9].

The differential expression of keratins in myoepithelial and epithelial cells of the breast makes immunohistochemical distinction of lesions an attractive possibility. High-molecular-weight keratin, 34 β E12, is a monoclonal antibody that recognizes keratins 1, 5, 10, and 14. Because myoepithelial cells predominantly express keratins 5 and 14 and epithelial cells predominantly express keratins 8 and 18, it is natural to assume that 34 β E12 may be a good marker of myoepithelial cells, but not epithelial cells [5, 10]. So, this marker can be used to detect the presence of invasion.

The objectives of this study –were as follows:

1. To perform routine histopathological study of breast lesions.
2. To assess the morphometric parameters in different breast lesions.
3. To assess the proliferative activity and invasiveness in different breast lesions with the help of IHC.

Materials and Methods

The study was performed over a period of 2 years (June 2009–May 2011). The specimens were taken from the mastectomy and excised breast lumps. Detailed history, clinical findings, and radiological parameters were noted from the patients admitted in the surgical ward. Total 60 cases and 10 controls were included in this study. Controls were taken from the adjacent normal area of the lesions. The specimens were fixed in formalin, processed, and embedded in paraffin wax block. Sections of 3-micron thickness were affixed on egg albumin-coated slides and poly-L-lysine-coated slides. The former were stained with hematoxylin and eosin (H&E) stains and the later group were for use in IHC (p53, 34 β E12 and PCNA index study) [11, 12]. H&E-stained slides were examined thoroughly and a provisional diagnosis of each case was performed. Morphometric analysis was done on H&E-stained histological sections with the aid of computer software AutoCAD 2007. Microphotographs were taken at high power (400 $\times$ magnification) and they were analyzed. Hundred random nuclei from the most atypical area of the sections were subjected to analysis. Morphometric analysis was performed in terms of mean nuclear diameter (MND), mean nuclear area (MNA), mean cell diameter (MCD), mean cell area (MCA), and nuclear to cytoplasmic ratio (N/C ratio). For calculating for PCNA labeling index (PCNA LI%) at least 1000 nuclei were counted under 1000 $\times$ magnification [3]. Regarding the interpretation of the result of p53 immunostaining, a tumor was considered as positive for p53 when it showed nuclear staining in more than 1 % of tumor cells [13]. To assess the presence of invasion, the results of 34 β E12 were interpreted as whether the myoepithelial cell layer is present or not.

Finally, a grand chart was produced tabulating histological diagnosis, morphometric findings, interpretation of p53 staining (whether positive or negative), PCNA LI% and the status of invasion. The results obtained were then analyzed by unpaired Student's *t* test (two-tailed distribution; two-sample equal variance) to calculate the significance of difference in different parameters of various breast lesions and also with that of controls (normal breast epithelium).

P values below 0.05 were considered as significant.

Table 1 Age distribution of the cases ($n=60$)

Histopathological diagnosis (H&E) (60)	Total no. of cases 60	<20 years	20–40 years	41–60 years	61–80 years
Fibroadenosis	15	3	12	0	0
Fibroadenoma	24	9	13	2	0
Tubular adenoma	2	1	1	0	0
Benign phylloides tumor	2	0	1	1	0
IDC (Gr. I)	2	0	1	1	0
IDC (Gr. II)	8	0	4	4	0
IDC (IDC III)	4	0	1	1	2
ILC	3	0	1	2	0

Results

Table 1 shows the distribution of age as well as histopathological diagnosis of the 60 cases. We found 15 cases of fibroadenosis, 24 cases of fibroadenoma, 2 cases of tubular adenoma, 2 cases of benign phylloides tumor, 14 cases of invasive ductal carcinoma (IDC), and 3 cases of invasive lobular carcinoma (ILC). Of the 14 cases of IDC, 2 cases were graded as grade I lesions, 8 cases were graded as grade II lesions, and 4 cases were graded as grade III lesions as per Nottingham's modification of Bloom Richardson's grading system (Table 1). The results of morphometric analysis are depicted in Table 2. We found maximum MND and MNA in cases of IDC grade III (Fig. 2) while that of benign diseases were found to be quite lower (Fig. 1, Table 2). Table 3 shows the PCNA LI (%) of various breast diseases. PCNA LI (%) was found to be highest again in grade III IDC and lowest in fibroadenoma, whereas that of ILC (Fig. 3) was found to be comparable to that of grade I IDC (Table 3). Table 3 also shows the results of immunohistochemical assay with p53 monoclonal antibody in different breast lesions. All the benign lesions except 1 case of fibroadenoma were found to be negative for p53 staining, whereas 7 of the total 14 cases of IDC showed positive nuclear staining

in more than 1 % of the tumor cell nuclei (Fig. 4, Table 3). Finally, the presence of invasiveness was studied using immunohistochemical staining with monoclonal antibody against 34 β E12. We found that all the controls and benign breast lesions have continuous basal or myoepithelial cell layer, whereas all the cases of invasive carcinoma showed discontinuous pattern of staining (Table 3).

Discussion

Table 1 shows that the most common breast lesion was fibroadenoma in less than 40 years age group followed by fibroadenosis. Thus, both of these benign lesions were found to be common below the age of onset of menopause. This finding corroborates with the findings of Manna et al. [3]. They also found fibroadenoma as the most common breast tumor in the age group of 21–40 years. Among the cases of IDC, the most common was grade II lesion. Radwan et al. [4] also found the grade II lesions as the most common type among IDC cases. We found most of the cases of carcinoma in 41–60 years age group.

In our study, MND of various benign lesions of breast, such as fibroadenosis (Fig. 1), fibroadenoma, tubular

Table 2 Results of morphometric study (on H&E stained sections)

Histopathological diagnosis	MND $\pm$ 2 SD (μ)	MNA $\pm$ 2 SD (μ 2)	MCD $\pm$ 2 SD (μ 2)	MCA $\pm$ 2 SD (μ)	N/C ratio $\pm$ 2 SD
Control	5.075 $\pm$ 0.541	20.444 $\pm$ 4.45	12.45 $\pm$ 2.204	120.223 $\pm$ 52.191	0.176 $\pm$ 0.056
Fibroadenosis	5.609 $\pm$ 0.786	25.549 $\pm$ 8.157	11.992 $\pm$ 1.882	116.253 $\pm$ 35.611	0.222 $\pm$ 0.049
Fibroadenoma	4.969 $\pm$ 0.697	18.740 $\pm$ 3.364	11.135 $\pm$ 1.495	98.722 $\pm$ 28.062	0.203 $\pm$ 0.045
Tubular adenoma	6.125 $\pm$ 0.177	29.489 $\pm$ 1.701	12.5 $\pm$ 0.707	122.964 $\pm$ 13.889	0.242 $\pm$ 0.041
Benign phylloides tumor	5.875 $\pm$ 0.177	27.132 $\pm$ 1.632	12.125 $\pm$ 0.177	115.524 $\pm$ 3.367	0.235 $\pm$ 0.007
IDC (Grade I)	10 $\pm$ 0	78.57 $\pm$ 0	16.125 $\pm$ 1.945	210.285 $\pm$ 42.913	0.395 $\pm$ 0.091
IDC (Grade II)	10.219 $\pm$ 0.339	83.685 $\pm$ 7.236	15.314 $\pm$ 0.655	184.937 $\pm$ 15.113	0.453 $\pm$ 0.027
IDC (Grade III)	14.438 $\pm$ 2.125	166.436 $\pm$ 46.065	19.565 $\pm$ 2.696	306.797 $\pm$ 78.144	0.54 $\pm$ 0.054
ILC	8.633 $\pm$ 0.153	58.575 $\pm$ 2.078	10.855 $\pm$ 0.728	92.864 $\pm$ 12.298	0.64 $\pm$ 0.078

Fig. 1 Photomicrograph showing histology of Fibroadenosis (H & E, x400)

adenoma, and benign phylloides tumors were found to be $5.61 \pm 0.79 \mu$, $4.97 \pm 0.7 \mu$, $6.13 \pm 0.18 \mu$, and $5.88 \pm 0.18 \mu$, respectively, and that of malignant cases, such as IDC grade I, IDC grade II, IDC grade III (Fig. 2), and ILC were found to be 10μ , $10.22 \pm 0.34 \mu$, $14.44 \pm 2.13 \mu$, and $8.63 \pm 0.15 \mu$, respectively (Table 2). Radwan et al. [4] found that mean maximal nuclear diameter (MMND) of benign and malignant lesions of breast were in the range of $8.50 \pm 0.77 \mu$ and $(11.10 \pm 1.34) \mu$, respectively [4]. Ikapatt et al. [14] found the MND of IDC grade I, grade II, grade III lesions and that of ILC were in the range of $8.1 \pm 1.5 \mu$, $9.7 \pm 1.3 \mu$, $11.6 \pm 1.7 \mu$, and $8.6 \pm 1.4 \mu$, respectively. So, the values obtained in their studies are corroborative with the values found in our study.

In this study, MNA of fibroadenosis, fibroadenoma, tubular adenoma, benign phylloides tumor, IDC grade I, IDC grade II,

IDC grade III, and ILC were found to be $25.55 \pm 8.16 \mu^2$, $18.74 \pm 3.36 \mu^2$, $29.49 \pm 1.70 \mu^2$, $27.13 \pm 1.63 \mu^2$, $78.57 \mu^2$, $83.69 \pm 7.24 \mu^2$, $166.44 \pm 46.07 \mu^2$, and $58.58 \pm 2.08 \mu^2$, respectively (Table 2). Thus, MNA of IDC cases were found to be gradually increasing with advancement of the histological grade of the lesion and certainly the values of malignant lesions are higher than that of the controls or normal breast epithelium as well as that of the benign lesions. Skjorten et al. [15] and Ruiz et al. [16] also found a gradual increase in the mean nuclear area from baseline value of normal breast epithelium through benign diseases to invasive cancers in their study.

We concluded that both MNA and MND can be used as an important morphometric parameter to differentiate normal epithelial cells and various benign breast lesions from different malignant breast lesions such as IDC and ILC, as well as IDC from ILC, because the difference in their MND values was found to be statistically significant (P value < 0.05).

We further calculated the N/C ratio of various breast lesions and found a gradual increase in the values through normal epithelial cells (controls) to benign lesions and finally to malignant lesions (Table 2). The P values were found to be highly significant in differentiating controls as well as various benign from malignant lesions of breast, as well as IDC from ILC (P value < 0.05).

Finally, we concluded that nuclear parameters were found to be more significant than the cytoplasmic parameters in differentiating various breast lesions because both the nuclear parameters can differentiate benign breast lesions from ILC. But cytoplasmic parameters cannot do so. Furthermore, nuclear parameters can even differentiate between various grades of IDC, except between grade I and grade II lesions of IDC, whereas cytoplasmic parameters failed to differentiate them from each other.

Table 3 SimplePara>Results of immunohistochemical assay with monoclonal antibody against p53, PCNA, and 34 β E12 in different breast lesions ($n=60$). A tumor with positive nuclear staining with

monoclonal antibody against p53 in more than 1 % of the tumor cells interpreted as having a positive expression [13]

Histopathological diagnosis	Total no. of cases ($n=60$) Total no. of controls= 10	PCNA LI (%)		p53 immunostaining		Staining pattern of basal cell layer with monoclonal antibody against 34 β E12
		Range (%)	Mean (%)	No. of positive cases ($n=8$)	No. of negative cases ($n=52$)	
Control	10	5–7	5.6	0 (0 %)	10 (100 %)	Continuous
Fibroadenosis	15	13–25	15	0 (0 %)	15 (100 %)	Continuous
Fibroadenoma	24	3–7	5.583	1 (4.17 %)	23 (95.83 %)	Continuous
Tubular adenoma	2	5–6	5.5	0 (0 %)	2 (100 %)	Continuous
Benign phylloides tumor	2	23–24	23.5	0 (0 %)	2 (100 %)	Continuous
IDC grade I	2	44–45	44.5	0 (0 %)	2 (100 %)	Discontinuous
IDC grade II	8	44–49	47.625	3 (37.5 %)	5 (62.5 %)	Discontinuous
IDC grade III	4	58–64	61	4 (100 %)	0 (0 %)	Discontinuous
ILC	3	42–44	43	0 (0 %)	3 (100 %)	Discontinuous

Fig. 2 Photomicrograph showing histology of Invasive ductal carcinoma (H & E, x400)

We determined the proliferative activity of various breast lesions with the help of monoclonal antibody against PCNA and p53.

Table 3 shows PCNA labeling index (%) of controls and that of various breast lesions. The range of PCNA LI (%) values in our study was 3–64 %. In controls, it was found to be in the range of 5–7 %. In fibroadenoma, it was 3–7 % with a mean of 5.583 %. In fibroadenosis, the range was 13–25 % with a mean of 15 %. PCNA LI (%) of benign phylloides tumors range from 23 % to 24 % with a mean value of 23.5 %. In invasive carcinoma, PCNA LI (%) range from 42 % to 64 % (Table 3, Fig. 3). The value was found to

Fig. 3 Photomicrograph showing histology of invasive lobular carcinoma breast (monoclonal antibody against PCNA, x400)

be lower in ILC when it was compared with that of IDC. Manna et al. found PCNA LI(%) in the range of 5–7 % (mean 6 %) and 12–17 % (14.5 %) for fibroadenoma and fibrocystic disease with features of adenosis, respectively. They also showed that in the invasive areas PCNA LI (%) was 45–51 % with a mean of 49.3 % [3]. Thus, their findings corroborate with the present study. A study by Tanaka et al. [17] showed PCNA LI (%) of breast carcinomas were 42.9 ± 13.3 % [17]. Sarla et al. [18] found significant correlation between PCNA index and histological grades. They found PCNA LI (%) of majority of the grade I and grade II lesions in the range of 11–50 %, whereas that of grade III lesions were found to be >50 % [18]. Our values also fall in the same range. Using unpaired Student's *t* test a significant lower value of PCNA LI (%) was found in controls as well as in the benign lesions as compared with that of malignant ones. Robbins et al. also found a significant difference between PCNA LI (%) of benign and malignant breast lesions [19]. Regarding p53 staining, all the controls and cases of fibroadenosis, tubular adenoma, benign phylloides tumor, IDC (grade I), and ILC were negative for p53 staining, whereas all the cases of IDC (grade III) (Fig. 4), 1 case of fibroadenoma and 37.5 % cases of IDC grade II lesions were found to be positive for p53 immunostaining (Table 3). Hong Suk Song found p53 overexpression in 51.6 % of breast cancer cases [20]. Koutsellini et al. [21] also studied p53 expression in cytologic specimens from benign and malignant breast lesions. They did not find p53 overexpression in any case of cystic disease of breast. They found only one case of p53 positive fibroadenoma out of 12 cases. Out of total 40 cases of carcinoma, they found 21 cases as positive for p53 overexpression [21]. The findings of this study were also similar to our study.

Fig. 4 Photomicrograph showing histology of invasive ductal carcinoma breast (monoclonal antibody against P53, x400)

Finally, the presence of invasiveness was studied using immunohistochemical staining with monoclonal antibody against 34 β E12. We found that all the controls and benign breast lesions have continuous basal or myoepithelial cell layer, whereas all the cases of invasive carcinoma showed discontinuous pattern of staining (Table 3).

So, from this study, we concluded that morphometry and IHC help in the proper diagnosis of different breast lesions that lie in the grey zone on routine histopathology.

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