Original Article

Status of HER-2/neu receptors and Ki-67 in breast cancer of Indian women

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Abstract

Background: Breast cancer is a leading cause of death in women. Receptor status is the most important prognostic and predictive marker for breast cancer. **Aims:** The present study was conducted with an aim to analyze breast cancer of Indian women with discordant receptor status, probably hormone dependent estrogen receptor (ER) positive, progesterone receptor (PgR) negative or ER– negative and PgR+ positive subgroup profile, infiltrating ductal breast cancer (IDC) not otherwise specified. **Materials and Methods:** Specimens from 100 IDC were grouped into three categories according to hormonal status (group 1: ER+ positive and PgR+ positive, group 2: ER+ positive and PgR– negative or ER– negative and PgR+ positive, group 3: ER– negative and PgR– negative) evaluated prognostic parameters. **Statistical Analysis:** Statistically significant difference was found between tumor receptor status distribution and menopausal status ($P = 0.0235$), age of patients ($P < 0.001$), histopathologic grade ($P < 0.001$), vascular invasion ($P = 0.006$), *HER-2/neu* status ($P = 0.004$) and Ki-67 proliferation rate ($P < 0.001$). **Results:** Group 1 tumors were found exclusively in post-menopausal patients with average age 68.9 years, most of which had intermediate grade II, without vascular invasion, with *HER-2/neu* status score predominantly 0 or 1+ and lower Ki-67 proliferation rate. Group 2 tumors were found predominantly in younger post-menopausal patients with average age 57.5 years, with vascular invasion found in 23% of cases. Group 3 tumors mostly had higher histopathologic grade, showed the highest percentage of the Ki-67 positive tumor cells and vascular invasion in 30% of the cases. **Conclusion:** It is concluded that patients with group 2 breast cancer were younger post-menopausal women, with tumors moderately differentiated, HER-2/neu score 0 or 1+ and with lower Ki-67 proliferation rate.

Key words: Breast cancer, HER-2/neu, hormone receptors, Ki-67, menopausal status, proliferative markers

Introduction

Estrogen receptor (ER) is the most important prognostic and predictive marker for breast cancer.[1] Presence of both ER and progesterone receptor (PgR) is related to better prognosis and responsiveness to hormonal therapy.[2] Proper understanding of prognostic features of breast cancer can help in the selection of appropriate treatment for the individual patient. These features are lymph node involvement, tumor size and grade, status of ER and PgR, status of the cancer biomarker HER-2/neu gene expression profile, and patient's age.[3] ER– (negative) and PgR+ (positive) tumors should be regarded as histopathologically equivalent to ER+ and PgR+ tumors. However, the response rate to hormonal therapy for ER– and PgR+ tumors is substantially lower than for ER+ and PgR+ tumors, suggesting real differences between the two hormone receptor profiles.[3-5]

Accordingly, the present study was planned with an aim to reconsider discordant receptor status breast cancers with probably dependent hormonal status ER+ and PgR– or ER– and PgR+ subgroup profile and compare their expression and some established prognostic parameters in breast cancer in Indian women, i.e. tumor size, lymph node metastases, histopathologic and nuclear grade, menopausal status, age of the patients, Ki-67 proliferation index and *HER-2/neu* receptor status.

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Materials and Methods

The present study was approved by the institutional ethical committee. A voluntary, informed, written consent was taken from all the patients. Surgically removed breast cancer tissues were collected from 100 patients in a medical college and tertiary care teaching hospital attached to it, from a city in western India. Expressions of ER, PgR, *HER-2/neu* and Ki-67 were analyzed in specimens of infiltrating duct breast cancer tissue of Indian women during modified radical mastectomy and lumpectomy.

After formalin fixation, paraffin embedding and staining with hematoxylin and eosin, histopathologic features were determined by a histopathologist prior to the immunohistochemical examination. Histopathologic grade was assessed using Bloom and Richardson's method, modified by Elson and Ellis.[6]

Laboratory protocol for immunohistochemistry

Tissue samples were fixed in 10% neutral buffered formalin for 12-24 hours. After processing the tissue samples in auto processor, embedding the tissue with paraffin wax on embedding station, and cutting of paraffin blocks by microtome, 4 μm thickness sections were dried overnight at 37°C. Prior to antibody staining, the slides were pre-treated with microwave irradiation to unmask binding epitopes. After blocking of endogenous peroxide activity with a 3% solution of hydrogen peroxide in methanol for 30 minutes, the slides were immersed in 200 ml of 10 mM citric acid (pH 6.0) for 5 minutes on power (100 W), followed by four cycles of 5 minutes each on power (50 W). After topping up of the buffer with distilled water, this step was repeated. The slides were then left to stand for 10 minutes in buffer at room temperature before being washed thoroughly in tap water.

After three washes in Tris-buffered saline (TBS), the slides were incubated with a 1:25 dilution of mouse anti-ER α monoclonal primary antibody (Clone: 1D5; M7047; DakoCytomation, Glostrup Copenhagen, Denmark), 1:25 dilution of mouse anti-PgR monoclonal primary antibody (Clone: PgR 636; M3569; DakoCytomation, Glostrup Copenhagen, Denmark), 1:25 dilution of mouse anti-HER-2/neu monoclonal primary antibody (Clone: CB11; NCL-L-CB11; Visionbiosystems Asia Pacific, Mount Waverley, VIC 3149 Australia), 1:25 dilution of mouse anti–Ki-67 monoclonal primary antibody (Clone: MIB-1; M7240; DakoCytomation, Glostrup Copenhagen, Denmark) in TBS for 1 hour at room temperature. After three more washes with TBS, added secondary antibody (LINK) (K0355; DakoCytomation, Glostrup Copenhagen, Denmark) that is biotinylated goat antibody to mouse/rabbit immunoglobulin; this LINK secondary antibody was diluted (1:100) in TBS and applied for 1 hour at room temperature. After an additional three washes with TBS, another secondary antibody (Enzyme Labeled) that is Streptavidin–Biotin/Horse Radish Peroxidase (HRP) Complex (K0355; DakoCytomation, Glostrup Copenhagen, Denmark) diluted (1:50) in TBS was added. After an additional three washes, the staining was visualized by adding diaminobenzidine (DAB kit; K3467; DakoCytomation, Glostrup Copenhagen, Denmark) for 5 minutes at room temperature. The slides were washed well in tap water and counterstained with Harris's hematoxylin for 10 seconds to 1 minute and then dehydrated, cleared, and mounted in Distrene Plasticiser Xylene (DPX).

Tumor cells displaying a nuclear staining were considered positive. [Figure 1a and b] ER and PgR status was expressed in the form of H-score,[7] based on a summation of the proportion of tumor cells, showing different degrees of reactivity: negative = 0 (0–50), weak = 1 (51–100), moderate = 2 (101–200), strong = 3 (201–300). This gives a maximum total score of 300 if 100% of cells show strong reactivity. Grouping was done as: group 1 ER+ PgR+, group 2 ER+ PgR- or ER- PgR+ and group 3 ER- PgR-.

HER-2/neu status was assessed by a score that includes the intensity and the percentage of positive tumor cells as 0 denoting negative, 1+, 2+ and 3+ denoting strongly positive [Figure 1c]. Only membrane *HER-2/neu* immunostaining was considered positive.

Ki-67 proliferation index was expressed as a percentage of positive cells on total of 1000 tumor cells counted. Tumor cells displaying a nuclear staining were considered positive [Figure 1d].

Statistical analysis

Estimation of immunohistochemical results was performed using the Pearson χ^2. Analysis of variance (ANOVA) was used in comparison analysis of various histopathologic features between the three groups and Kendall Tau correlation test was used for correlation analyses. Statistical differences with *P* value <0.05 were considered significant. The computing was carried out using the SPSS-16 procedure (SPSS Analytical Software Inc., Chicago, IL, USA).

Results

Age of patients at the time of surgery ranged from 30 to 87 years, with a median age of 63 years. There were 12 pre-menopausal and 78 post-menopausal women in the study population. Sixty-two patients were without lymph node involvement and 28 with lymph node metastasis. Ten cases were not reported.

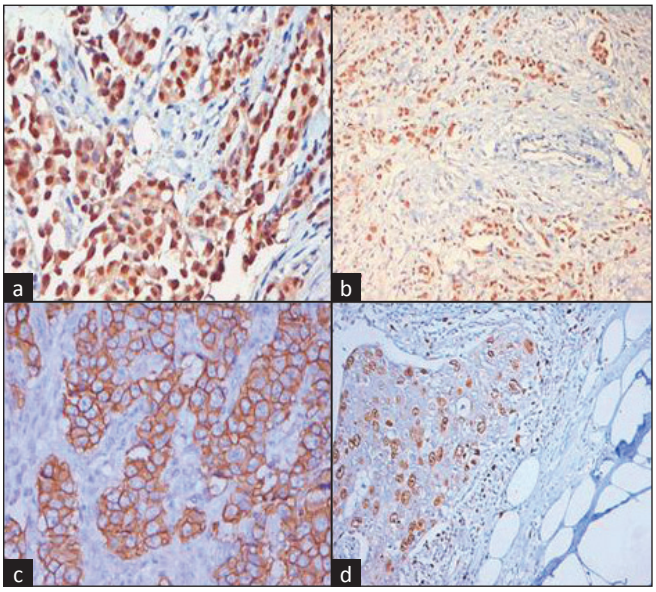

Figure 1: (a) Nuclear positive staining for ER; (b) nuclear positive staining for PgR; (c) membrane positive staining for HER2/neu receptor; (d) nuclear positive staining for proliferation marker Ki-67

A statistically significant difference was found between tumor receptor status distribution and menopause ($P = 0.024$), age of patients ($P < 0.001$), histopathologic grade ($P < 0.001$), vascular invasion ($P = 0.006$), HER-2/neu status ($P = 0.004$) and Ki-67 expression ($P < 0.001$) [Table 1]. Group 1 tumors were found exclusively in post-menopausal women with average age 68.9 years. Most of the tumors had intermediate II grade, showed no vascular invasion, *HER-2/neu* status score was predominantly 0 or 1+ and Ki-67 proliferation rate was lower. Group 2 and 3 tumors were found among both post- and pre-menopausal women with lower average age of 57.5 and 59.7 years, respectively. Vascular invasion was found in 23% of group 2 and 30% of group 3 tumors. While most of the group 3 tumors had higher histopathologic grade. Higher *HER-2/neu* status score of 3+ was found in 40% of group 3 tumors [Figure 1c], with highest Ki-67 expression [Figure 1d]. There was no statistically significant difference between tumor receptor status distribution and tumor size ($P = 0.11$), lymph node status ($P = 0.171$), number of positive lymph nodes ($P = 0.770$), peri-nodal infiltration ($P = 0.430$), findings in peri-tumoral breast tissue ($P = 0.711$), peri-tumoral ($P = 0.431$) and intra-tumoral ($P = 0.660$) lymphatic invasion, lymphocyte infiltration ($P = 0.856$) and type of tumor invasion ($P = 0.955$). Coefficient of contingency found no statistically significant difference in tumor size among group 1, 2 and 3 tumors, although group 3 tumors were bigger and had higher percentage, i.e. 22.4% of positive lymph nodes out of the totally removed axillary lymph nodes, than group 2 (16.8%) and group 1 (17.4%) tumors. Invasion in peri-tumoral and intra-tumoral lymphatic vessels occurred more frequently. Type of tumor growth in 70% of cases was with infiltrating borders.

Discussion

Breast cancer depends on various histopathologic factors including metastatic status of lymph nodes, tumor size, tumor grade, histopathologic grade, *HER-2/neu* status and proliferation markers such as Ki-67. ER and PgR status of these patients could influence these parameters.[8] Growth of breast cancer is often regulated by female sex steroids. Determination of cellular concentrations of ER and PgR in tumor is currently used to predict which patients have good prognosis and may also benefit from anti-hormonal therapy.[9] More than 60% of human breast cancers are ER-positive; no more than two-thirds of these ER-positive tumors respond to endocrine therapy.[10] Some studies have shown that ER-negative breast cancer cell lines do not transcribe ER mRNA due to an extensive methylation of the 5′ promoter of the gene, thus losing ER expression in human breast cancer cells.[11] Measurement of PgR improves predictability of hormone dependency of a tumor, but this relationship remains imperfect. Retrospective clinical studies have demonstrated that only 70% of PgR-positive and 25–30% of PgR-negative tumors respond to hormonal therapy.[12] Still, ER and PgR status at the time of breast cancer surgery is used as a tissue cancer biomarker of both prognosis and hormone dependency to guide adjuvant therapy.[13] ER positivity is strongly associated with age at diagnosis, being more prevalent among post-menopausal women.[14]

In the present study, hormonally dependent patients were exclusively post-menopausal with average age of 68.9 years. It is well known that breast tumors are less well differentiated among younger women. After evaluation of breast cancer in women of 35 years of age or younger, Rosen *et al.* found a high incidence of poorly differentiated tumors (53%) and ER negative cancer.[15,16] In the present study, group 3 patients had average age of 59.7 years, while group 2 patients had average age of 57.5 years. Kollias *et al.* reported similar findings in an evaluation of 2897 women with breast cancer; higher nuclear grade and lympho-vascular invasion observed in women younger than 35 years of age when compared with older women.[17] In the present study, group 3 tumors were predominantly poorly differentiated (60%), while in group 1 tumors, this category was not observed; tumors were moderately differentiated in 63.33% of cases. Group 1 tumors were mostly of grade II (63.33%), and there was no grade III present. Mink *et al.* showed no correlation between steroid ER and PgR expression and grading, but they showed a slight decrease of ER positive cancer with increasing tumor size.[18] In the present study, we observed intra-tumoral lymphatic invasion in similar percentage of all three groups of tumors (10–17%). In the present study, peri-tumoral lymphatic

Table 1: Immunohistochemically determined hormone receptor status in breast cancer in Indian women

Hormonal status	Total patients (*N* = 100)*		
	Group 1 (*n* = 30)	Group 2 (*n* = 30)	Group 3 (*n* = 30)
Menopausal status			
Pre-menopausal	0	7	5
Post-menopausal	30	23	25
Age in years	68.9 ± 8.3	57.5 ± 13.5	59.7 ± 12.1
Tumor size in cm	2.22 ± 1.36	2.3 ± 2.32	3.47 ± 3.54
Histological grade†			
Grade I	11	7	3
Grade II	19	18	9
Grade III	0	5	18
Type of tumor growth			
Infiltration	20	21	20
Expansive	10	9	10
Histopathologic lymph node status‡			
pN0	23	22	17
pN1	1	4	1
pN2	3	2	7
pN3	1	2	4
pN1mi	2	0	1
Intra-tumoral lymphatic vessel invasion			
Negative	27	27	25
Positive	3	3	5
Peri-tumoral lymphatic vessel invasion			
Negative	17	14	12
Positive	13	16	18
HER-2/neu status¶			
0	13	14	10
1	14	10	5
2	3	2	3
3	0	4	12
Ki-67 expression (% of positive cells)	14.64	12.91	28.85

*Ten cases not reported. †I – well differentiated; II – intermediate; III – poorly differentiated. ‡pN – regional lymph nodes; pN0 – no regional lymph node metastasis; pN1 – metastasis in one to three ipsilateral axillary lymph node(s), and/or in internal mammary nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent; pN2 – metastasis in four to nine ipsilateral axillary lymph nodes or in clinically apparent ipsilateral internal mammary lymph node(s) in the absence of axillary lymph node metastasis; pN3 – metastasis in 10 or more ipsilateral axillary lymph nodes, or in infra-clavicular lymph nodes, or in clinically apparent ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes, or in more than three axillary lymph nodes with clinically negative, microscopic metastasis in internal mammary lymph nodes, or in ipsilateral supraclavicular lymph nodes; pN1mi – micro metastasis larger than 0.2 mm, but none larger than 2 mm in greatest dimension. ¶0 – negative, 1 – weakly positive, 2 – moderately positive, 3 – strongly positive

invasion was slightly higher in group 3, but without statistical significance. Lymphocyte infiltration was also similar in all the three groups (χ^2 = 0.3; *P* = 0.856). Vascular invasion was not present in group 1 tumors, while in the other two groups it was present in 23% and 30% cases, respectively. Analyzed tumors mostly showed infiltrating borders in 70% of the cases. Their size did not show statistically significant difference in the analyzed groups (*F* = 2.22; *P* = 0.11). It is known that in patients with small tumors treated with adjuvant hormonal therapy, survival was significantly longer. No difference in the changes of surrounding breast tissue was found in the present study in all three groups. *HER-2/neu* gene amplification or protein overexpression is evident in 20–30% of breast tumors and correlates with poor prognosis.[19,20] Reason for this association remains unclear, although it has been suggested to rest in increased proliferation, vessel formation and/or invasiveness.[21] In the present study, group 3 tumors had *HER-2/neu* score 3+ in 40% of cases, a fact not observed in group 1 and 2 tumors. In this group of tumors, there was also a strong correlation between *HER-2/neu* expression and Ki-67 (*P* = 0.025). Once again, it was observed that the group 3 tumors showed highest Ki-67 proliferation rate (χ = 28.85%, *S* = 21.58). So, poor clinical outcome of these breast cancer patients is expected. Lukashina *et al* showed that higher Ki-67 expression was more frequently associated with positive expression of *HER-2/neu*. Thus, aneuploidy tumors with higher proliferative activity and hyperexpression of *HER-2/neu* are more aggressive ones and larger in size.[22] Use of Herceptin has been effective in 20–25% of HER-2/neu positive breast cancer patients, but Witters showed that pre-menopausal women with *HER-2/neu* overexpression and ER positive breast tumors would probably receive little benefit, and possibly detrimental effects, by treatment with *HER-2/neu* inhibitor alone.[23] Status of axillary lymph nodes is one of most important prognostic

factors in patients with breast cancer. Bader *et al.* showed that approximately 13% of patients with well-differentiated or moderately differentiated tumors, less than or equal to 1 cm in size, without lymph or vascular invasion and a low Ki-67 expression had a low risk of axillary lymph node metastases (4.3%). In the present study, no statistical difference was observed in the number of positive axillary lymph nodes in the three groups that had been investigated (χ^2 = 1.5; *P* = 0.17). It had previously been shown that if positive axillary lymph nodes correlated with HER-2/neu overexpression, prognosis was poor.[20] According to Collett *et al.*, PgR and ER status predicted prognosis in middle age patients (40–60 years) with lymph node positive breast cancer. Analyzing the number of peri-nodal infiltrations of total number of positive lymph nodes, no significant difference was found among the three tumor groups.

It is concluded that discordant receptor breast cancer with group 2 hormonal status ER+ positive and PgR− negative or ER− negative and PgR+ positive was found predominantly in younger post-menopausal women, approximately 10 years younger than women with group 1 tumors, mostly with intermediate II histopathologic grade, *HER-2/neu* status 0 or 1+ and lower Ki-67 proliferation rate. Patients with group 1 tumors should be primarily candidates for hormonal therapy, especially in old age, while more aggressive group 2 and especially group 3 tumors should be treated with proper chemotherapy regimens that should give a possibility of lasting remission.

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