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Study of immune histo chemical markers in breast cancer and their correlation with various clinico pathological parameters at tertiary care center in Saurashtra region

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Abstract
Background: Breast cancer poses a huge epidemiological burden in Saurashtra region. Breast cancer can be completely treated if diagnosed at an early stage. Hormone receptors (ER and PR) and human epidermal growth factor receptor-2 (HER-2) are the most widely used biomarkers that are used in stratifying breast cancer treatment and management. Predictive factors evaluate the likelihood of benefit from a specific treatment. ER, PR, and HER2/neu are prognostic as well as predictive factors. The present study was conducted to correlate the expression of ER, PR, and HER2/neu with each other and to various clinopathological parameters as follows: age of the patient, histological grade, tumor size, and lymph node metastasis.
Material and Method: The current study was performed at a tertiary care center in Saurashtra region on Eighty One patients diagnosed with breast malignancy. We analyzed the expression of ER, PR, HER2/neu and Ki67 index by immunohistochemistry (IHC), with each other and to various clinico-pathological parameters.
Result: In the present study the maximum number of patients belonged to the age group 41-59 years (61.7%). The most common histological type was Invasive Ductal carcinoma of No Special Type (IDC NST) with more common involvement of left sided breast .Out of total 81 cases, majority of the cases were classified as Luminal A.
Conclusion: In the present study, majority of the patients were having invasive ductal carcinoma, NST type with various patterns of expression of IHC markers. IHC is an easy and economic method for the prognostic classification of various breast cancer where molecular techniques are unavailable. This is valuable in reducing the overall burden of breast cancer in the Saurashtra region.

Keywords: Breast cancer, various clinico pathological, tertiary care center

Introduction
Breast cancer poses a huge epidemiological burden in Saurashtra region. With increasing awareness, more and more cases are being diagnosed every day. Many histopathological types have been identified, among which Infiltrating Ductal carcinoma is found to me most common [1]. Breast cancer can be completely treated if diagnosed at an early stage. Localized lesions are being treated by surgical intervention, followed by chemotherapy when indicated, radiotherapy and adjuvant hormonal therapy for Estrogen Receptor (ER) and Progesterone Receptor (PR) positive cases [2].
Many prognostic factors has been defined for example tumor size, tumor grade, nuclear grade, axillary lymph node metastasis etc. but biological molecular prognostic factors have received more importance in recent years because a significant number of patients with early-stage breast cancer harbor microscopic metastasis at the time of diagnosis [3]. Hormone receptors (ER and PR) and human epidermal growth factor receptor-2 (HER-2) are the most widely used biomarkers that are used in stratifying breast cancer treatment and management [4]. These markers are used as prognostic and predictive factors in the management of breast cancer. Prognostic factors are those which influences patient's overall outcome such as chances of recurrence after treatment. These factors help in the selection of patients for a specific treatment [5].

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Predictive factors evaluate the likelihood of benefit from a specific treatment. ER, PR, and HER2/neu are prognostic as well as predictive factors [5].

Estrogen receptor
Many of the physiological changes that the breast tissue undergo are mediated by estrogen. Two subtypes of Estrogen receptors have been identified, ERα and ERβ [6]. The role of ERα Receptor has been well identified as prognostic and predictive factor in breast cancer.
The significance of ERβ is not well established [6, 7]. The majority of ER-positive breast cancers contain both ERα and ERβ subtypes; although, some cancers have only ERβ expression. This may lead to distinct clinical behaviors and responses. It is observed that in contrast to ERα, ERβ expression declines during breast carcinogenesis [8].

Progesterone receptor
PR is of two types as follows: PR-A and PR-B. Progesterone acts as a modulator of estrogen function [9]. Studies have shown that ER-positive and PR positive breast cancers have better response to hormonal therapy while ER positive and PR negative breast cancers are less responsive to hormonal treatment. It has been shown that ER and PR expression can change over the natural history of the disease or as consequence of treatment as these are unstable receptors [10].

Human epidermal growth factor receptor-2/neu (c-erbB-2)
This belongs to family of closely related growth factor receptors, including epidermal growth factor receptor or HER1, HER2, HER3, and HER4. HER2/neu amplification or overexpression plays a role in oncogenic transformation and tumor genesis which may lead to increased and uncontrolled cell proliferation, decreased apoptosis, increased cancer cell motility, and angiogenesis resulting in worse prognosis [11].
At present, determining ER, PR, and HER2/neu receptor status in breast cancer have become a common practice, as there is a survival advantage for patients with hormones receptor positive status by treatment with adjuvant hormonal or chemotherapeutic regimens. It is well known that strong ER-positive cases benefit from endocrine therapy alone, in contrast to those with low to moderate ER positivity. PR status is independently associated with disease-free and overall survival. Patients with ER, PR-positive tumors have a better prognosis than patients with ER, PR-negative tumors [12].
The present study was conducted to correlate the expression of ER, PR, and HER2/neu with each other and to various clinopathological parameters as follows: age of the patient, histological grade, tumor size, and lymph node metastasis.

Inclusion criteria
All patients with malignancy of the breast confirmed histopathological were included in the study.

Exclusion criteria
Patients with inflammatory breast lesions, posttraumatic breast lesions and benign breast diseases were excluded from the study.

Materials and Method
The current study was performed at a tertiary care center in Saurashtra region. Eighty One patients diagnosed with breast malignancy were enrolled for the study. Written informed consent was obtained from all patients. We analyzed the expression of ER, PR, HER2/neu and Ki67 index by immunohistochemistry (IHC), with each other and to various clinico-pathological parameters.

Procedure
Paraffin blocks containing cancer tissue were selected from histopathological confirmed cases of infiltrating ductal carcinoma. After preparing slides from blocks, immunohistochemical staining was done for ER, PR, HER2/neu and Ki67 by standard procedure using primary antibodies and secondary antibody Poly-HRP immunostaining kit from DAKO [13].

Preparation of slides
Paraffin sections were cut and mounted on Poly L lysine coated slides. Slides were melted at 65°C and then dipped into xylene to remove the paraffin. After rehydrating tissues by dipping them in graded alcohol solutions, slides were washed with distilled water.

Antigen retrieval and detection of antigens
Heat retrieval was done with Tris EDTA high pH buffer in a pressure cooker for 30 min at 95°C and then brought to room temperature after removing from the pressure cooker and by placing the slides in Tris-Saline buffer (wash buffer) in a moist chamber. After that the sections were covered with freshly prepared 3% hydrogen peroxide solution for 15 minutes and the rinsed with wash buffer. The sections were exposed to the primary antibody for about 1 h, and then primary antibody was washed with the wash buffer.

Secondary detection of the primary antibody
Sections were incubated with biotinylated mouse polymer horseradish Peroxidase (DAKO) for 30 min, and then rinsed in wash buffer. A solution of chromogen, 3, 3'-diaminobenzidine (DAB) at 1 mg/ml in DAB buffer (DAKO) was prepared and added to the slides for 10 minutes. DAB from the slides was washed with tap water.

Counterstaining
A solution of Harris hematoxylin was used by dipping slides for 1.5 minutes. Then, slides were washed in tap water and dehydrated by dipping in graded solutions of alcohol. Washed in xylene and coverslip was applied using DPX for viewing and reporting.

Reporting
Reporting done as per ER/PR scoring system and criteria as per Allred scoring system [14]. The statistical analysis was done using SPSS Version 26 software and the outcomes were compared with those of other similar studies.

Proportion score
0 – No cells are ER +ve.
1 – ≤1% of cells are ER +ve.
2 – 1%–10% of cells are ER +ve.
3 – 11%–33% of cells are ER +ve.
4 – 34%–66% of cells are ER +ve.
5 – 67%–100% of cells are ER +ve.

Intensity score
0 – Negative.
1 – Weak.
2 – Intermediate.
3 – Strong.

Interpretation
Total (proportion score + intensity score).
0–2 = Negative; 3–8 = Positive

Human epidermal growth factor receptor-2/neu scoring system and criteria according to the American Society of Clinical Oncology College of American Pathologists guidelines [15]

0 = no staining or incomplete faint and barely perceptible in < 10% of tumor cells.

1+ = incomplete membrane staining which is faint and barely perceptible and within >10% of tumor cells.

2+ = circumferential membrane staining that is incomplete and/or weak/moderate and within >10% of the invasive tumor cells; or complete and circumferential membrane staining that is intense and within ≤10% of the invasive tumor cells.

3+ = circumferential, complete, and intense staining and within >10% of tumor cells.

FISH is required for equivocal HER2/neu positivity. Hence, HER2/neu 2+ was taken as negative along with her2/neu 0 and 1+. Only 3+ on IHC was taken as positive.

Furthermore, the breast cancer subtypes were classified on the basis of positivity of ER, PR and HER2/Neu along with the Ki67 index into Lunimal A, Luminal B, Her2/Neu overexpression and triple negative breast cancer. This a prognostic classification of breast cancer and can be used in patients who cannot afford to go for molecular and DNA microarray investigations.

Definition of subtypes of breast cancer- St. Gallen classification [25].

Subtypes of breast cancer	Er and Pr	Her-2	Ki67
Luminal A	Er + and/or pr +	Her-2-	Ki67<14%
Luminal B with her-2 negative	Er+ and/or pr+	Her-2-	Ki-67≥14%
Luminal B with her-2 positive	Er + and / or pr +	Her-2 +	Any ki-67
Her-2 enriched	Er-, pr-	Her-2 +	Any ki-67
Basal-like (triple negative)	Er-, pr-	Her-2-	Ck5/6 + and/or egfr +

Results and Observations

Table 1: No of patients in each age group (n=83)

Age group (years)	No. of Patients	Percentage
≤40	18	22.22
41-59	50	61.73
≥60	13	16.05
Total	81	100

In the present study the maximum number of patients belonged to the age group 41-59 years (61.7%). The minimum age of patient was 25 years and maximum age was 74 years with a mean age of 51.3 years.

Table 2: Histological type of breast cancer in each age group (n=81)

Age group (years)	IDC NST	Lobular type	others
≤40	16/18	2/18	00
41-59	46/50	2/50	2/50
≥60	13/13	00	00
Total	75/81(92.59%)	4/81(4.93%)	2/81(2.46%)

The most common histological type was Invasive Ductal carcinoma of No Special Type (IDC NST) which was 92.59% of total cases. Lobular carcinoma was found only in 4 cases (4.93%) out of 81 cases there was one case of papillary carcinoma of breast and one case of malignant phyllodes. All the cases in the with age more than 60 years were diagnosed as IDC NST while the lobular carcinoma belonged to a younger age group, with 2 case from less than 40 years age group and 2 cases from the age group of 41-59 years.

Table 3: Comparison of type of carcinoma with the site of breast lesion

Histological type Site of lesion	IDC NST (75)	Lobular Ca (04)	Others (02)	Total
Left side	39(52%)	1(25%)	2(100%)	42(56%)
Right side	36(48%)	3(75%)	00	39(44%)

In the present study it was found that left sided breast were more common (56%) than the right sided cancers (44%). Out of 75 cases of IDC NST, 39(52%) were left sided and 36(48%) were right sided. 75% of cases of lobular carcinoma of breast had presented with right sided mass.

Table 4: Summary of expression of estrogen receptor, progesterone receptor and Her2Neu with respect to histological type of breast cancer.

Histological type	ER positive	PR Positive	HER2/Neu Positive 3+	Triple negative
IDC NST(75)	41(54.67%)	28(37.34%)	16(21.34%)	21(28%)
Lobular Ca (04)	3(75%)	2(50%)	00	1(25%)
Other (02)	1(50%	1(50%)	00	1(50%)
Total(81)	45(55.56%)	31(38.27%)	16(19.75%)	23(28.31%)

The present study showed that out of 75 cases of IDC NST on histological examination 41 cases (54.67%) were ER positive and 28 cases (37.34%) were positive for PR immune staining. While 3 out of 4 cases (75%) of lobular carcinoma of breast were positive for ER with a strong staining intensity and 2 cases (50%) were positive for PR immune staining. All the Her2/Neu positive cases belonged to IDC NST on histological examination. A total of 23 cases out of 81 cases (28.31%) were found to be Triple negative breast carcinoma.

Furthermore, Ki67 index was studied and found out that mean Ki67 index was 26.59±22.13. this suggested that the overall proliferation rate of the tumor cells in the studied cases was less with minimum being 1% and maximum being 85%. One-way Anova was employed using SPSS V26, and we found that there was significant correlation between ER positivity and Ki67 Index (p=0.034), while PR positivity and Ki67 index were not significantly correlated (p=0.352).

Table 5: Comparison of Luminal subtypes with Age group.

Age group (Years)	Luminal A	Luminal B	Her2Neu Overexpression	Triple negative
≤40	6	6	1	3
41-59	12	7	5	13
≥60	8	6	4	7
Total	28 (34.5%)	20 (24.7%)	10 (12.34%)	23 (28.31%)

Along with ER, PR and Her2Neu, Ki67 staining was also performed in all the cases. The cases that showed the ki67 staining of less than 20% cells was considered low staining index and more the 20% was considered high staining index. Out of total 81 cases, 28 cases (34.5%) were classified as Luminal A, 20 cases (24.7) were classified as Luminal B, 10 cases (12.34%) showed Her2Neu overexpression and the remaining 23 cases (28.31%) were classified as Triple negative breast cancer. It was found that the luminal categorization was not affected significantly by age of presentation.

Table 6: Comparison of Luminal subtypes with Histological subtypes

Histological Type	Luminal A	Luminal B	Her2Neu Enriched	Triple negative
IDC NST(75)	24(32%)	20(27%)	10(13.34%)	21(28%)
Lobular Ca. (04)	3(75%)	00	00	1(25%)
Others (02)	1(50%)	00	00	1(50%)
Total (81)	28(34.5%)	20(24.7%)	10(12.34%)	23(28.31%)

It was also observed that 32% of cases of IDC NST were classified as Luminal A, 27% were classified as Luminal B, 13.34% showed Her2Neu overexpression and 28% were classified as triple negative breast cancer. We found that 75% of lobular carcinoma were classified as Luminal A category which shows that they will respond better to hormonal therapy alone.

Discussion

The present study was conducted on 81 patients with histo pathologically proven cases of breast cancer at a tertiary care center in Saurashtra region in the state of Gujarat in India. Here we have attempted to study and correlate the expression of ER, PR and Her2/neu along with different clinico pathological parameters.

In the present study the mean age of the patients was 51.7 ± 11.01 years with the minimum age of 25 years and the maximum age of 74 years. This is comparable to various similar studies [16, 17, 18]. Many patients with younger age were also seen in the present study. This is a global phenomenon and factors responsible for this demographic change need to be studied further.

In the present study we found that 45 cases (55.56%) were ER positive and 31 cases (38.27%) were PR positive. This was comparable with study conducted in other parts of the country which showed that the number of cases found to be ER and PR positive are less which makes the response to the hormonal therapy questionable [16, 19, 20, 21]. It was found that there was a decrease in ER positivity with increasing age group which is similar finding in study conducted by Azizun-Nisa et al. [22].

In the present study we also found that HER2/Neu positivity (+3) was seen in 16 cases (19.75%) which was lower when compared to some studies [22, 23, 24] while it was higher that

the study conducted by Shukla et al. [16]. It was also seen that 23 cases (28.31%) were found to be triple negative breast cancers (TNBC). This was found to be in concordance with the findings of other similar studies [16, 22, 24].

As we know that Breast carcinoma is a heterogeneous disease with several clinical and histopathological presentations, this is due to several gene expression subtypes and molecular profiles. The different gene expression subtypes and molecular profiles are responsible for giving different predictive and prognostic characteristics for the patients. Microarray technique is used for evaluation of such markers but due to the cost of DNA analysis, the use of immune histochemical analysis of markers, which have been used as surrogate tools for defining subtypes of breast cancer, was generally accepted. According to the 2011 St. Gallen consensus conference, 5 subtypes of breast cancer were defined using the presence of receptors on the surface of the tumour cell, and the measuring values of Ki67 [25, 26, 27]. In the present study prognostic classification has also been done with 28 cases (34.5%) into Luminal A category, 20 cases (24.7%) into Luminal B, 10 cases (12.34%) showing HER2/Neu overexpression and 23 cases (28.31%) were triple negative breast cancer (TNBC). This was in concordance with other studies who also has used this prognostic classification [28, 29].

Conclusion

This study conducted at a tertiary care center of Saurashtra region. Immunohistochemistry has taken a central role in the diagnosis and management of breast cancer. Various patterns of expression of the IHC markers was seen among the histopathological variants and clinical stage of the disease. The study was well in concordance with similar studies conducted in other parts of the country. IHC is an easy and economic method for the prognostic classification of various breast cancer patients especially in Saurashtra region where other molecular techniques for diagnosis like DNA microarray array are unavailable. At our tertiary care center we found that IHC reports has helped clinicians in decision making in terms of treatment plans and hence better management of the patients who belong to economically backward classes of society. This is valuable in reducing the overall burden of breast cancer in the Saurashtra region. Further prospective study in this area is required to better understand the factors affecting the prognosis of the patients in terms of ER, PR and Her2/Neu expression.

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