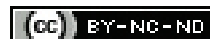


Subtyping of Breast Carcinoma According to ER/PR and HER2/neu Expression: A Cross-sectional Study from Southern Part of Assam, India

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ABSTRACT

Introduction: Globally, breast carcinoma is the most prevalent and lethal form of cancer in women. Breast cancer is no longer considered a single disease but a complex heterogeneous disease with multiple genetic and epigenetic alterations. The prognosis and management of the disease depend on histological stage, type, grade, tumour size, lymph node status, and the status of hormonal receptors like ER, PR, and Her2/neu. Recently, more attention has been given to the molecular classification of breast cancer.

Aim: To analyse and compare the clinicopathological characteristics of invasive breast cancer in the four breast carcinoma subtypes defined by the immunohistochemical expression of Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (Her2/neu).

Materials and Methods: The cross-sectional study was conducted on 64 primary invasive breast carcinoma cases diagnosed on mastectomy specimens between February 1, 2018, and July 30, 2022, in the Department of Pathology at the histopathology section of Silchar Medical College and Hospital, Silchar, Assam, India. Age and tumour characteristics (morphology, grade, stage, and size) and nodal disease status were included in the data for analysis. Immunohistochemical markers were analysed on the sections of these diagnosed cases. IBM Statistical Packages for Social Sciences (SPSS) software was used for data analysis. Qualitative data was presented as frequency and percentage, while quantitative data was presented as mean±{Standard Deviation (SD)}. The Chi-square test was used to determine the statistical significance of

hormonal receptors with the various clinicopathological features. A p-value of <0.05 was considered statistically significant.

Results: In present study of 64 cases of invasive breast carcinoma, the mean age of patients was 51.95±12.72 years. Subtyping was performed based on hormonal receptors. Authors found that the Luminal A variety 33 (51.6%) was the most common hormonal subtype in present study, followed by the basal subtype 24 (37.5%). The Luminal A subtype was found to be predominant among others. The majority of the patients (59.4%) had stage-I tumours, and Ductal type carcinoma was the most common (57.8%). Histologically, most of the tumours were poorly differentiated (28, 43.8%), and most were sized ≤2 cm (41, 64.1%). Lymph nodes were not palpable in most of the patients (43, 67.2%). Subtype comparison with respect to age, stage, histological grade, type, size, and nodal status revealed statistically significant outcomes (p-value of <0.05).

Conclusion: Classification based on Immunohistochemistry (IHC) provides prognostic and therapeutic information that cannot be obtained from either ER/PR or Her2/Neu status alone. The present study provides the incidence of different molecular subtypes in the southern region of Assam, and comparison among them with statistical correlation offers improved and crucial treatment guidance. IHC classification as a clinical tool for ER/PR and Her2/Neu testing is widely accessible, reasonably priced, based on immunophenotype/biologic phenotype categorisation of breast cancer, and is prognostic as well as partly predictive and needs to be practiced invariably.

Keywords: Epidermal growth factor, Estrogen receptor, Grade, Her 2 positive, Hormonal receptors, Molecular subtypes, Observational study, Progesterone receptor, Rabbit monoclonal antibody

INTRODUCTION

Globally, breast carcinoma is the most prevalent and lethal form of cancer in women. Breast cancer is now not a single disease but a complex heterogeneous disease that has multiple genetic and epigenetic alterations [1]. In recent years, due to early detection and effective treatment, there has been a significant decrease in breast cancer deaths and improved outcomes for women with the disease [2,3]. The prognosis and management of the disease depend on the histological stage, type, grade, tumour size, lymph node status, and the status of hormonal receptors like ER, PR, and Her2/neu [4]. Recently, more attention has been given to the molecular classification of breast cancer [5-13]. Among ER, PR, and Her2/neu, ER expression is the most important biomarker as it provides an index of sensitivity for endocrine treatment, which uses steroid hormones as the main growth stimulus. The expression of PR is strongly dependent on ER expression [14].

Her2/Neu, also known as Neu, Cluster of Differentiation (CD) 340, or p185, is a protein encoded by the ERBB2 gene located on the long arm of chromosome 17 (17q12) with tyrosine kinase activity [14]. The Her2/neu gene is located on the 17q12-q21 chromosomal region and acts as an oncogene in several human cancers, encoding a transmembrane growth factor receptor [15].

In present study, breast carcinoma is divided into four groups based on the Immunohistochemistry profile of ER/PR and Her2/Neu expression, and clinicopathological features are compared according to the subtypes. The groups are as follows:

1. ER/PR+, Her2/Neu+ (Luminal B): ER+/PR+, Her2/Neu+; ER-/PR+, Her2/Neu+; ER+/PR-, Her2/Neu+
2. ER/PR+, Her2/Neu- (Luminal A): ER+/PR+, Her2/Neu-; ER-/PR+, Her2/Neu-; ER+/PR-, Her2/Neu-
3. ER/PR-, Her2/Neu+ (HER2-rich): ER-/PR-, Her2/Neu+

4. ER/PR-, Her2/Neu- (Triple negative/basal-like tumours): ER-/PR-, Her2/Neu-

MATERIALS AND METHODS

The present study was a cross-sectional study in which a total of 64 patients diagnosed with primary invasive breast carcinoma in mastectomy specimens at Silchar Medical College, Silchar, Assam, India between January 1, 2018, and June 30, 2022, were included. The present study was approved by the Institutional Ethics Committee of Silchar Medical College with reference no. SMC/4816, dated 21/3/2023.

Inclusion criteria: The study included all individuals with histologically verified mastectomy specimens of invasive breast carcinoma.

Exclusion criteria: The study excluded individuals with inflammatory breast lesions, posttraumatic breast lesions, benign breast conditions, and breast cancer patients who had undergone neoadjuvant chemotherapy.

Study Procedure

Data, including tumour morphology, grade, size, and nodal status, were retrieved from the Pathology Department. Breast carcinoma subtypes were determined based on the expression of ER/PR and Her2/Neu by Immunohistochemistry (IHC), and various clinicopathological parameters were studied. Paraffin blocks containing cancerous tissue were selected from histopathologically confirmed cases of infiltrating ductal carcinoma. Standard IHC staining was performed for ER, PR, and Her2/Neu after slides were prepared from the blocks.

Preparation of slides: Paraffin sections were cut and mounted on saline-coated slides. The slides were heated at 65°C to remove the paraffin and then immersed in xylene. After rehydration of the tissues, the slides were cleaned with distilled water. Subsequently, the slides were washed with Tris buffer and submerged in a 3% peroxide solution for three minutes to remove endogenous peroxidase activity.

Antigen detection and antigen retrieval: Heat retrieval was performed using a decloaking chamber with citrate buffer at 95°C for 40 minutes. The slides were then transferred to Tris-Saline buffer to cool to room temperature. To prevent non specific immunostaining, the tissue sections were treated with 1% mouse serum. Primary antibodies, including rabbit monoclonal antibody QR013 for ER, Rabbit monoclonal antibody QR003 for Her2/Neu, and mouse monoclonal antibody A-2 for PR, were applied to the sections approximately one hour before removal.

Secondary detection of the primary antibody: After 10 minutes of incubation with biotinylated mouse anti-species antibody, sections were washed in Tris buffer. The slides were then treated with a solution of the chromogen 3,3'- Diaminobenzidine (DAB) at a concentration of 1 mg/mL in Tris buffer containing 0.016% fresh H₂O₂. Tap water was used to clean the DAB from the slides.

Counterstaining: Slides were immersed in a solution of haematoxylin diluted 1:1 with distilled water for counterstaining. After counterstaining, the slides were cleaned in distilled water and dehydrated by dipping them in ethanol. Finally, a coverslip was used for viewing and reporting after cleaning in xylene.

Reporting: The reporting was done using the ER/PR score methodology and Allred scoring criteria [16].

Proportion score:

0=No cells are ER positive.

1= \leq 1% of cells are ER positive.

2=2-10% of cells are ER positive.

3=11-33% of cells are ER positive.

4=34-66% of cells are ER positive.

5=67-100% of cells are ER positive.

Intensity score:

0=Negative.

1=Weak.

2=Intermediate.

3=Strong.

Interpretation:

Total score (proportion score+intensity score).

0-2=Negative; 3-8=Positive.

For Her2/Neu scoring, the recommendations of the American Society of Clinical Oncology College of American Pathologists were followed [17].

0=No staining or incomplete faint and barely perceptible staining in $<10\%$ of tumour cells.

1+=Incomplete membrane staining that is faint and barely perceptible and present in $>10\%$ of tumour cells.

2+=Circumferential membrane staining that is incomplete and/or weak/moderate and present in $>10\%$ of invasive tumour cells; or complete and circumferential membrane staining that is intense and present in $\leq 10\%$ of invasive tumour cells.

3+=Circumferential, complete, and intense staining present in $>10\%$ of tumour cells.

For equivocal Her2/Neu positive cases, FISH (Fluorescence In-situ Hybridisation) is typically performed. However, in present study, FISH was not performed. Therefore, Her2/Neu 2+ and Her2/Neu 0 and 1+ results were considered negative. Only IHC results with a 3+ were considered positive.

STATISTICAL ANALYSIS

IBM SPSS software version 21.0 was used for data analysis. Qualitative data was presented as frequency and percentage, while quantitative data was presented as mean (\pm SD). The statistical significance of relationships between axillary lymph node status, patient age, tumour size, tumour grade, and ER/PR and Her2/Neu status in infiltrating ductal carcinoma of the breast was determined. The chi-square test was used to identify significant associations. A p-value of less than 0.05 was considered statistically significant.

RESULTS

A total of 64 patients with invasive breast cancer were included in this study. The mean age of the patients was 51.95 ± 12.72 years. The majority of the patients (59.4%) had stage-I tumours, and the most common type of carcinoma was ductal carcinoma (57.8%). Histologically, most of the tumours were poorly differentiated, and the majority of them were sized ≤ 2 cm. Lymph nodes were not palpable in most of the patients [Table/Fig-1].

Authors found that the Luminal A variety (51.6%, 33/64) was the most common hormonal subtype in present study, followed by the basal subtype (37.5%, 24/64) [Table/Fig-2].

Subject's characteristics	n (%)
Age (years)	51.95 \pm 12.72
Tumour stage	
I	38 (59.4%)
II	20 (31.3%)
III	06 (9.4%)
Cancer type	
Ductal	37 (57.8%)
Lobular	18 (28.1%)
Medullary	03 (4.7%)
Mucinous	03 (4.7%)
Others	03 (4.7%)

Histopathologic grade	
Well differentiated	13 (20.3%)
Moderately differentiated	20 (31.3%)
Poorly differentiated	28 (43.8%)
Missing (data was not recorded in the case sheet which was retrieved from department)	03 (4.7%)
Tumour size	
≤2 cm	41 (64.1%)
2.1 to 5 cm	18 (28.1%)
>5 cm	4 (6.3%)
Missing (data was not recorded in the case sheet which was retrieved from department)	1 (1.6%)
Lymph node status	
Positive	19 (29.7%)
Negative	43 (67.2%)
Not examined	2 (3.1%)

[Table/Fig-1]: Baseline characteristics of sample (N=64).

Subtypes	Number of patients	Percentage
ER/PR+,Her2/Neu-(Luminal A)	33	51.6%
ER/PR+,Her2/Neu+(Luminal B)	5	7.8%
ER/PR-,Her2/Neu+(Her 2 rich)	2	3.1%
ER/PR-,Her2/Neu-(Basal type)	24	37.5%

[Table/Fig-2]: Distributions of tumour subtypes according to ER/PR and Her2/neu status.

[Table/Fig-3] presents the differences in the baseline characteristics among the four subtypes. The difference in tumour subtype across the stage of tumour was found to be statistically significant (p=0.001). The Luminal A subtype was more common in ductal carcinoma, while the Luminal B subtype was predominantly seen in lobular cancer. The basal subtype was more common in ductal carcinoma. The distribution of subtypes across different cancer types was statistically significant (p=0.01).

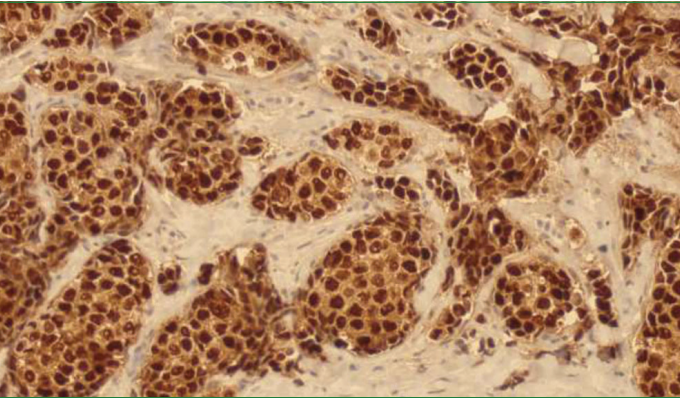
Considering the distribution of tumour subtypes across different histopathologic grades, the Luminal A subtype was more common

Variables	Luminal A (n=33)	Luminal B (n=05)	Her2/Neu rich (n=02)	Basal type (n=24)	p-value
Age (years)	56.79±12.97	51±7.18	45±4.24	46.08±11.19	0.012
Tumour stage					
I	23 (69.7%)	2 (40%)	0	13 (54.2%)	0.001
II	9 (27.3%)	2 (40%)	0	9 (37.5%)	
III	1 (3%)	1 (20%)	2 (100%)	2 (8.3%)	
Cancer type					
Ductal	25 (75.8%)	2 (40%)	1 (50%)	9 (37.5%)	0.01
Lobular	5 (15.2%)	3 (60%)	0	10 (41.7%)	
Medullary	0	0	0	3 (12.5%)	
Mucinous	2 (6.1%)	0	1 (50%)	0	
Histopathologic grade					
Well differentiated	10 (30.3%)	1 (20%)	0	2 (8.3%)	0.045
Moderately differentiated	14 (42.4%)	2 (40%)	0	4 (16.7%)	
Poorly differentiated	7 (21.2%)	2 (40%)	2 (100%)	17 (70.8%)	
Missing	2 (6.1%)	0	0	1 (4.2%)	
Tumour size					
<=2 cm	25 (75.8%)	2 (40%)	0	14 (58.3%)	0.009
2.1-5 cm	6 (18.2%)	1 (20%)	2 (100%)	9 (37.5%)	
>5 cm	2 (6.1%)	1 (20%)	0	1 (4.2%)	
Missing	0	1 (20%)	0	0	

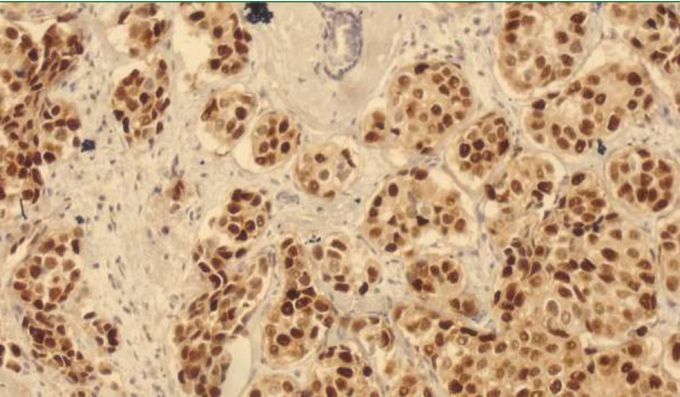
Lymph node status					
Positive	9 (27.3%)	2 (40%)	0	8 (33.3%)	0.013
Negative	23 (69.7%)	3 (60%)	1 (50%)	16 (66.7%)	
Not examined	1 (3%)	0	1 (50%)	0	

[Table/Fig-3]: Distributions of tumour subtype across baseline characteristics.

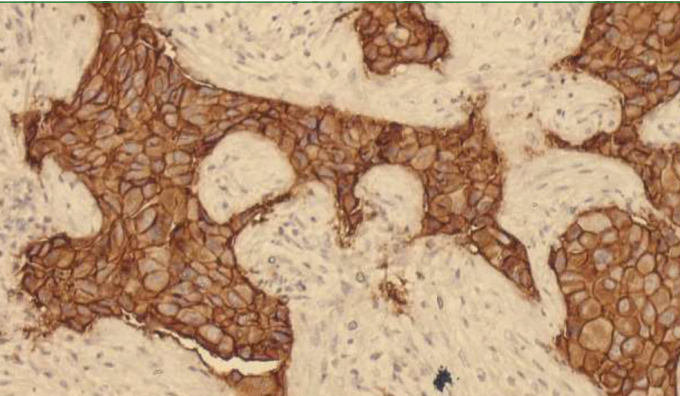
in moderately differentiated carcinoma, while the Luminal B subtype was equally found in moderately differentiated and poorly differentiated carcinoma. The Her2/Neu rich and basal subtype carcinoma were predominantly found among poorly differentiated carcinoma. These distributions were found to be statistically significant (p=0.045). The IHC expression of ER/PR and Her2/Neu was examined [Table/Fig-4-6].



[Table/Fig-4]: IHC expression of ER showing strong nuclear positivity, 200x.



[Table/Fig-5]: IHC expression of PR showing intermediate nuclear positivity, 200x.



[Table/Fig-6]: IHC expression of Her2/Neu with circumferential, complete intense membrane positivity, 200X.

DISCUSSION

The status of hormonal receptors, like ER, PR, and Her2/Neu, is an important factor in the prognosis and management of breast cancer. The present study evaluates the clinicopathological features of breast carcinoma based on the expression of ER/PR and Her2/Neu from a tertiary care centre in Assam.

The mean age of the breast carcinoma patients in this study was 51.95 (±12.5) years. This value was similar to the one reported

by Kanwar A et al., (51.2 years) but slightly higher than the value reported by Vedashree MK and Rajalakshmi V (50.18 years) [18,19]. The most predominant subtype in this study was Luminal A, followed by the basal subtype. This finding is consistent with the results reported by Pandit P et al., [20]. Stage-I had the majority of cases, accounting for 59.3% (38), followed by Stage-II and III with 31.3% (20) and 9.4% (06), respectively. Invasive ductal carcinoma was found in 57.8% (37) of patients, while 28.1% (18) had invasive lobular carcinoma. These findings were similar to those of studies by Pandit A et al., and Elidrissi Errahhali M et al., [20,21].

According to research, 15% to 20% of breast cancers belong to the Her2/Neu high molecular subtype [22]. In present study, 3.1% of patients had the Her2/Neu-rich subtype. However, this number is lower than expected since authors did not include individuals with ambiguous (2+) Her2/Neu receptor status. The inability to treat Her2/Neu equivocal cases using FISH, as recommended by the American Society of Oncology and College of American Pathologists, limits the precise determination of the prevalence of molecular subtypes.

The present analysis shows that 64.1% (41) of the patients had tumours smaller than 2 cm. Similar results were reported by Onitilo AA et al., who found that 71.4% of their cases had tumour size less than 2 cm [23]. This can be attributed to the mammographic screening program and increased cancer awareness in India.

The majority of the tumour in present study were well/moderately differentiated with 51.6% (33) and were lymph node negative. These findings were comparable to other studies [23,24].

The present study reaffirms that breast cancer is a complex disease with various biological subtypes and varied natural histories [25]. Authors findings show statistically significant variations in the clinicopathological characteristics between subtypes.

The classification of breast cancer based on both ER/PR and Her2/Neu status using immunohistochemistry provides prognostic and therapeutic information that cannot be obtained from either status alone. Previous categorisations that divided breast cancer into two groups based solely on the expression of ER were less selective in terms of prognosis, and the additional sub-classification based on the expression of Her2/Neu offers improved and crucial treatment guidance. Moreover, breast cancer has occasionally been dichotomized as triple-negative or another subtype [26].

Authors have classified breast cancer using Immunohistochemistry (IHC) into four global subtypes out of the eight possible subtypes used by other authors. We believe that this classification is effective, straightforward, instructive, clinically beneficial, and fairly discriminatory between the subtypes. The other four groups would be evident if we differentiate between ER+/PR+ and ER+/PR- tumours based on PR expression. Studies with more than four subtypes have generated controversy because PR is regulated by the oestrogen pathway [27].

The most effective technique for molecular classification is to subtype breast cancer using microarrays for gene expression analysis. However, most clinical specimens that have been preserved are not suitable for this type of examination. In addition, these assays were only used in research labs until recently, when commercially available tests such as Oncotype DX and MammaPrint became available. As a result, they were not optimally accessible for routine practice. IHC-based classification systems, such as ER/PR+, Her2/Neu+ with Luminal B, ER/PR+, Her2/Neu- with Luminal A, ER/PR-, Her2/Neu+ Her2/Neu-rich, and ER/PR-, Her2/Neu- with triple-negative/basal-like tumours, are still useful in clinical practice, especially when fresh tissue is not available. They have also been shown to correlate well with intrinsic classification using gene expression microarrays [25,26]. It is important to remember that the Her2/Neu and ER/PR tests do not have perfect reliability. Intralaboratory and interlaboratory variation in ER results is significant due to differences in fixation, antigen retrieval, and staining techniques between laboratories [28-30]. Significant discrepancies in Her2/Neu results obtained from

the same specimen in various laboratories have also been noted [28,31,32]. Continuous efforts need to be made to standardise existing testing and create more reliable and reproducible testing for ER/PR and Her2/neu expression in order for this classification to be more beneficial [28-32].

The majority of patients exhibit low to intermediate histologic grade (51.6%), small tumour size (≤ 2 cm; 64.1%), and negative nodal status (67.2%).

Despite the significant investment and effort directed towards molecular diagnostics, IHC is still relevant, particularly in lower centres. Globally, the predictive value of the assays is limited to recognised targets like the ER/PR protein or the Her2/Neu gene, as new therapeutic target proteins are not being discovered despite the availability of molecular arrays for a decade. Additionally, despite the use of numerous and diverse gene sets in most molecular testing, there is a high degree of concordance in the outcomes predicted for specific patients by these tests, indicating that they are likely tracking a similar set of biological phenotypes that are predominantly influenced by the ER/PR and Her2/Neu gene pathways [33]. Lastly, the argument that molecular technology is superior to IHC testing is purely theoretical and based on the idea that it offers quantification and reproducibility. Some ongoing investigations are based on this speculative notion, which is still unproven.

Limitation(s)

The study has limitations, including its exclusive focus on a single institute and a limited number of cases. The primary drawbacks of IHC methods include restricted technical reproducibility, subjective interpretation, and qualitative results. However, in present study, all IHC procedures and interpretations were carried out in a single laboratory by the same group of pathologists to minimise these concerns. Moreover, FISH was not included for Her2/Neu equivocal cases.

CONCLUSION(S)

The present study is the first to compare the clinicopathological features among the different subtypes in the southern part of Assam. In conclusion, luminal A was found to be the predominant subtype, followed by basal-like, Her2/Neu-rich, and luminal B. The Immunohistochemistry (IHC) classification, used as a clinical tool for ER/PR and Her2/Neu testing, is widely accessible, reasonably priced, based on the immunophenotype/biologic phenotype categorisation of breast cancer, and is both prognostic and partly predictive. Therefore, it should be practiced consistently.

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