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Molecular Evaluation of Subtypes of Breast Cancer by Immunohistochemical expression of Estrogen Receptor(ER), Progesterone Receptor(PR), Her2neu,Cytokeratin 5/6 and Ki 67

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Abstract: ***Objective:- In this study, histopathological examination and subsequent IHC staining using a panel of five Immunohistochemistry (IHC) markers i.e Estrogen Receptor(ER), Progesterone Receptor (PR), HER2neu, Cytokeratin5/6 and Ki-67 was done and on the basis of their results subtyping and molecular classification was done into Luminal A, Luminal B, Her2neu and Basal –like subtypes. Materials and methods:-Specimens of 50 patients with breast carcinoma were evaluated histopathologically and immunohistochemically for a panel of five IHC markers i.e. ER, PR, HER2neu, CK5/6 and Ki-67. Results:- We observed that mean age of presentation was 54.86 years. Infiltrating ductal carcinoma (NOS) was the most common histological subtype with majority of the tumors classified as Grade II. We observed 27(54%) cases as Luminal A, 4 (8%) Luminal B, 6 (12%) as HER2neu and 8 (16%) as Basal-like subtype. Remaining 5(10%) cases were Unclassified. Conclusion:- We concluded that most common subtype was Luminal A with low proliferative activity having good prognosis as compared to Luminal B with higher proliferative index and has poor prognosis. Our study provides a basis for the use of IHC for molecular subtyping of breast cancer as an important prognostic factor as well as target for specific adjuvant chemotherapy.***

Keywords: Breast cancer, Molecular classification, ER/PR, Cytokeratin5/6, Ki67

1. Introduction

Breast cancer in women is a major public health problem throughout the world. It accounts for 22% of all female cancers, 26% in affluent countries, which is more than twice the occurrence of cancer in women at any other site.[1] Different studies in Punjab in India documented an increasing incidence of carcinoma breast from 6.9% to 19.11% of all malignancies in females.[2] In India, the average age of breast cancer patients range from 44.2 years to 49.6 years.[3]

Breast cancer is no longer seen as a single disease but rather a multifaceted disease comprised of distinct biological subtypes with diverse spectrum of clinical, pathologic and molecular features. In the recent past histological typing and grading was the best tool for determining the prognostic implications in breast cancer, but ever since the advent of newer immunohistochemical markers, treatment and prognosis is largely dependent on them.

These markers have diagnostic, prognostic and predictive value. IHC panel including antibodies to ER, PR, HER2/neu, CK 5/6 and Ki-67 helps assign tumors into various molecular subtypes.[4] In our study, we have classified breast cancer patients into molecular subtypes of Luminal A, Luminal B, Her2 neu and Basal-like based on IHC evaluation.

2. Methodology

The present study was conducted in the Department of Pathology, Government Medical College, Amritsar, after approval from the institutional thesis and ethics committee. The test population comprised of 50 specimens of breast cancer patients received in the department.

2.1 Tissue Collection

The tissues of the test population submitted as were evaluated by histopathological processing and examination (HPE). The representative tissue block was selected for Immunohistochemical evaluation for ER, PR, HER-2/neu, CK5/6 and Ki67 markers.

2.2 Tissue processing

Gross examination of the specimen was done regarding size, involvement of overlying skin, nipple and areola with ulceration, retraction of nipple, dimpling and any lymph node involvement. Specimens were cut into slices and fixed in 10% formalin for 24 hours. After 24 hours, tissue samples were taken from different areas of the tumor, nipple and areola, overlying skin and from lymph nodes resected from breast tissue or received separately. These tissues were processed and finally embedded in paraffin wax. Sections of 3-4 um thickness were cut and stained with haematoxylin and eosin (H&E). Following HPE reporting representative sections were selected for Immunohistochemistry.

2.3 Immunohistochemistry procedure

Biogenex was used as antigen retrieval system. Biocare reagents were used for IHC staining process. Antigen retrieval was done as per specification of kit. Slides were immersed in citrate buffer and put in microwave oven (antigen retrieval system) for 2 cycles of 10 and 15 minutes at 85°C and 100°C temperature respectively. The slides were then brought to room temperature and immersed in PBS (phosphate buffer saline). Subsequent steps include blocking peroxidise activity, incubation, twice PBS buffer washes, addition of 2 drops of primary monoclonal antibody, incubation and twice buffer washes. Incubation with linked antibody was done followed by 2 washings with PBS. Addition of enzyme conjugate followed by twice PBS washing. One tablet of DAB was dissolved in reagent and incubated. All incubations were done in a moist chamber. Subsequent steps include washing of sections in deionized water, haematoxylin counterstaining, washing under tap water and then dehydration in ascending concentration of alcohol. Clearing was done in xylene and sections mounted with Distrene dibutyl phthalate xylene. Section was viewed under the microscope.

2.4 IHC Markers

ER, PR and Ki-67 positive malignant epithelial cells show nuclear staining, HER-2neu positive malignant epithelial cells show membranous staining and CK-5/6 positive malignant cells show membrane and Cytoplasmic staining. The IHC score was calculated by combining an estimate of the percentage of immunoreactive cells (quantity score) with an estimate of the staining intensity (staining intensity score).[5]

3. Results

Present study of 50 patients show age variation from 35 to 85 years. Tumor size varied from 1.0 to 11.0 cm. Most of the patients (58%) were having tumor size between 2-5 cm. 17 patients (34%) had tumor size > 5 cm. 26 out of 50 cases (52%) showed lymph node metastasis. Our study showed, out of 17 cases with tumor size > 5 cm, 13 cases (76.47%) had secondary deposits in lymph node. Higher risk of nodal metastasis was observed with increased tumor size predicting the prognostic value of tumor size in breast lesions. The most common histological subtype encountered in the present study was Infiltrating Ductal carcinoma (NOS) with 48 cases (96%). This was followed by Medullary Carcinoma and IDC with mucinous change with 1 case each (2%)

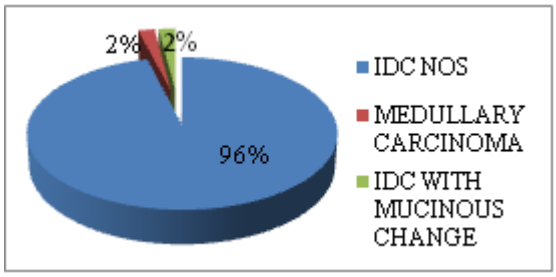


Figure 1: Diagramatic representation of distribution of histological type of cases

Histological grading was done according to modified Bloom-Richardson grading system. Majority of the cases presented with histological grade II (86%) followed by grade I as 4 cases (8%) and grade III only 3 cases(6%) .

Present study showed ER and PR positivity in 31 out of 50 cases, comprising 62 % each and HER2neu positivity in 7 (14%) out of 50 cases.13 cases were triple negative, comprising 26% cases.

Table 1: Showing distribution of cases according to ER, PR and Her2Neu status

IHC Status	*Number Of Cases/50*	*Percentage*
ER/PR+,HER2+	1	2%
ER/PR+,Her2-	30	60%
ER/PR-,Her2+	6	12%
ER/PR-,Her2-	13	26%
Total	50	100%

Out of the 31 cases positive for ER and PR, 27 cases had Ki67<14% and only 4 cases had Ki67>14%. Thus classified as Luminal A and Luminal B respectively.

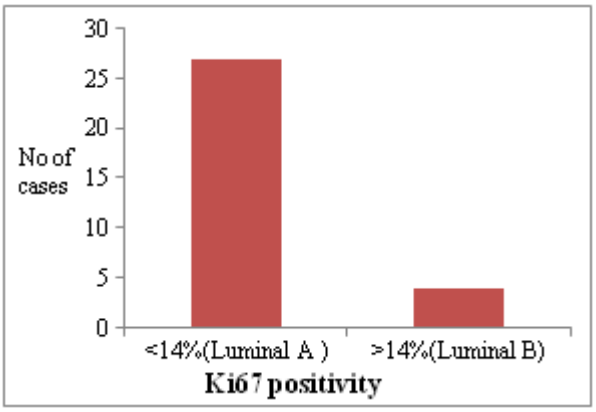


Figure 2: Diagramatic representation of classification of Luminal A and Luminal B

Out of 7 cases positive for Her2 neu, 6 (12%) were ER/PR negative, thus classified as HER2 neu subtype.

In our study 13 cases were obtained as triple negative out of which 8 cases were positive for CK5/6, thus classified as Basal-like subtype and the remaining 5 cases remained unclassified.

Table 2: Basal-like subtype

CK5/6 Positivity in Triple Negative cases	*Molecular subtype*	*Number of Triple negative Cases/50*	*Percentage*
Present	Basal-like	8	16%
Absent	Unclassified	5	10%

Thus in the present study 27(54%) cases were classified as Luminal A, 4 (8%) cases as Luminal B subtype, 6 (12%) cases as HER2neu positive subtype and 8 (16%) cases as Basal-like subtype. Remaining 5(10%) cases were Unclassified

Table 3: Showing distribution of molecular subtypes

IHC Profile	*Luminal A*	*Luminal B*	*HER2/Neu*	*Basal Like*	*Unclassified*
ER/PR	ER/PR+	ER/PR+	ER/PR-	ER/PR-	ER/PR-
HER2 and others	HER2- Ki67(<14%)	HER2- Ki67(>14%)	HER2+	HER2- CK5/6 +	HER2- CK5/6 -
No of cases/50	27	4	6	8	5
Percentage	54%	8%	12%	16%	10%

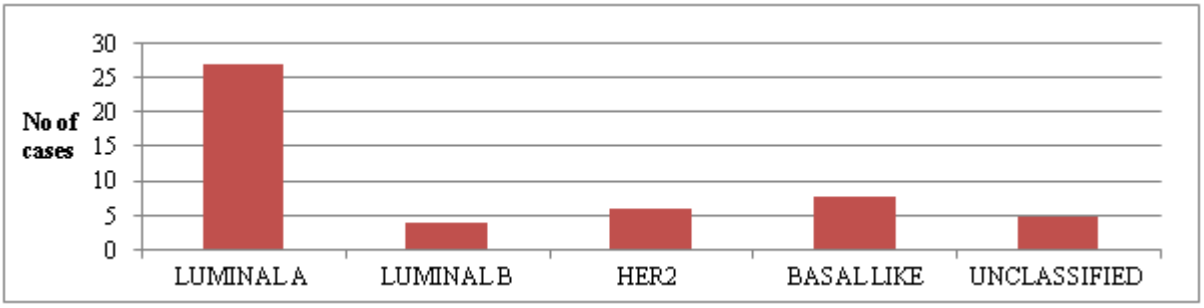


Figure 3: Diagramatic Representation of the distribution of Molecular Subtypes

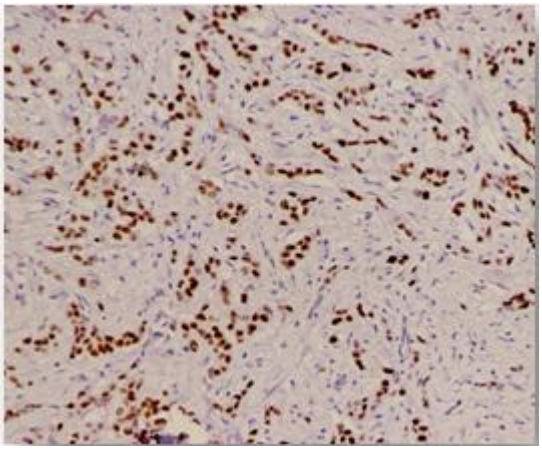
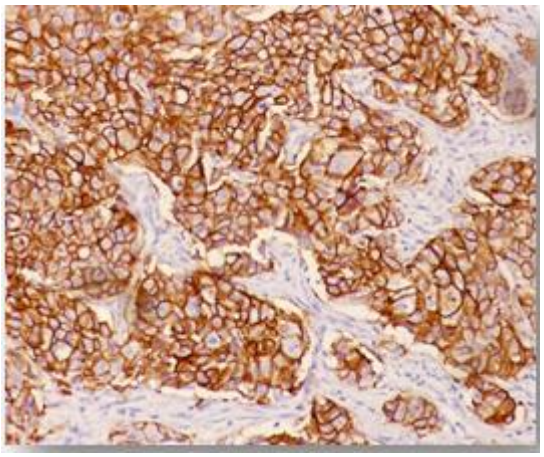
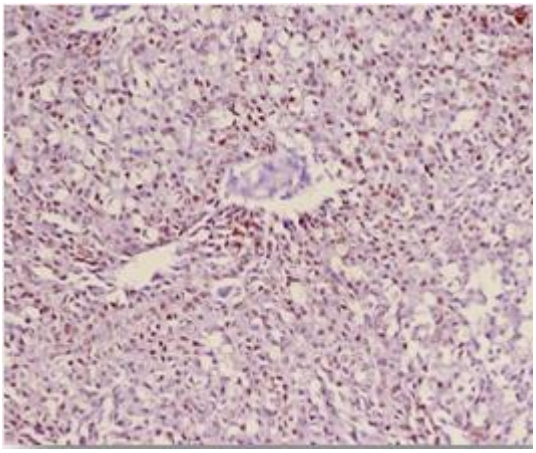


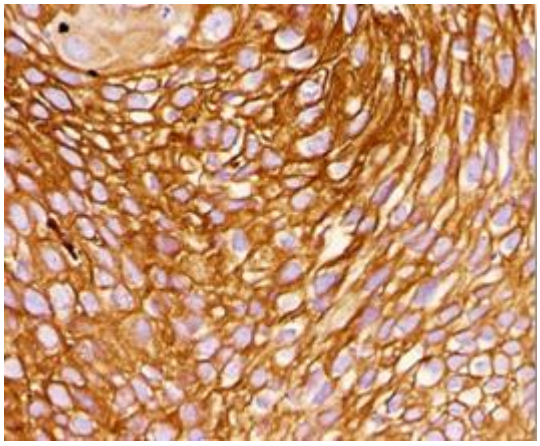
Figure 4: Showing IHC expression for ER, PR, HER2neu , Ki67 and CK5/6



Her2 neu positivity in breast cancer (200X)



Ki67 positivty in breast cancer (200X)



CK5/6 positivity in breast cancer (400X)

4. Discussion

Breast cancer can be divided into intrinsic molecular subtypes which have distinct clinical features, with markedly differing prognosis and clinical outcomes.[6]

The 50 cases diagnosed as carcinoma breast were divided into various age groups. The age varied from 35 to 85 years. Mean age of presentation was 54.86 years. As far as peak age presentation is concerned, our findings were almost

same as that of studies done by Siddiqui et al with mean age of presentation 47.7 +/- 11.8 and 48 years respectively.[7] In a study done by Khokher et al, mean age was 47±12 years.[8]

We observed that tumor size varied from 1.0 to 11.0 cm. Most of the patients (58%) were having tumor size between 2-5 cm. Our study correlated with study done by Patel et al who documented maximum number of cases (64%) with tumor size 3-5 cm.[9]

It was documented 26 (52%) showed lymph node metastasis. Patel et al [9]observed lymph node positivity in 59% of cases. Higher risk of nodal metastasis was observed with increased tumor size predicting the prognostic value of tumor size in breast lesions. In a study done by Carter et al, survival rates were 45.5% for tumor diameter equal to or greater than 5 cm with positive axillary nodes as compared with 96.3% for < 2 cm and with no involved nodes.[10] Tumor diameter and lymph node status were found to act as independent but additive prognostic indicators.

It was observed majority of cases 48(96%) were Infiltrating Ductal Carcinoma (NOS). These correlated with studies by Patel et al[9] (93%), Saxena et al[11] (88.2%) and Hasseini et al [12] (89%) with Infiltrating Ductal Carcinoma (NOS) being the leading histological subtype.

Out of the 31 cases positive for ER and PR, 27 cases had Ki67<14% and only 4 cases had Ki67 > 14%. Thus classified as Luminal A and Luminal B respectively. Out of 7 cases positive for Her2 neu, 6 (12%) were ER/PR negative, thus classified as HER2 neu subtype. In our study 13 cases were obtained as triple negative out of which 8 cases were positive for CK5/6, thus classified as Basal-like subtype and the remaining 5 cases remained unclassified.

Thus in the present study 27(54%) cases were classified as Luminal A, 4 (8%) cases as Luminal B subtype, 6 (12%) cases as HER2neu positive subtype and 8 (16%) cases as Basal-like subtype. Remaining 5(10%) cases were Unclassified.

Our results well correlated with study done by Dawood S et al[13] showing 65.8% as luminal A, 14.3% as luminal B, 4.9% HER2 type, 10.4% were basal-like and 4.6% tumors unclassified.The study by Inwald EC et al[14] observed Luminal A (48.4 %), Luminal B (24.8 %), HER2-like (17.8 %), and Basal-like subtype (9.0 %).

A recent update proposed a panel of IHC surrogates for each subtype of breast carcinoma.[15] These were ER, PR, and HER2, dividing breast carcinoma into luminal, HER2, and triple-negative subtypes. The addition of Ki-67, cytokeratin 5, and epidermal growth factor receptor (EGFR) separates luminal B from luminal A subtypes, and basal-like subtype from triple-negative breast cancer.

A single case of Medullary Carcinoma was observed which was Triple negative and showed positivity for CK5/6, thus classified as Basal-like. It was observed that the women with triple-negative breast cancer were younger, having adverse pathological characteristics as high tumour grade, tumour necrosis and frequent nodal metastases.

We observed that ER/PR positive cases were more common than Her2neu and Basal –like cases in our study population. Thus these patients can be good candidates for targeted chemotherapy

5. Conclusions

It can be concluded that in every case of carcinoma breast hormonal status and subtyping on the basis of Immunohistochemical evaluation is important because these different subtypes have different prognosis and different targeted therapies. Most common subtype was observed to be Luminal A with low proliferative activity thus having good prognosis as compared to Luminal B with higher proliferative index and poor prognosis.

6. Future Scope

Our study provides a basis for the use of IHC for molecular subtyping of breast cancer as an important prognostic factor as well as target for specific adjuvant chemotherapy.

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