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Ki67 IN PRIMARY BREAST CANCER AND AN ATTEMPT AT SURROGATE MOLECULAR CLASSIFICATION USING IMMUNOHISTOCHEMISTRY

Histopathology

Dr. Monika Singh Senior Resident, BJ Medical College, Pune.
Dr. Savita Sanjay Patil Associate Professor, BJ Medical College, Pune.
Dr. Ishita Sharma* Senior Resident, BJ Medical College, Pune. *Corresponding Author

ABSTRACT

Breast cancer is the second most common malignancy worldwide and exhibits considerable biological heterogeneity. Molecular classification using gene-expression profiling, as described by Perou et al., identifies distinct subtypes; however, its use is limited in many settings. Immunohistochemistry (IHC) offers a practical surrogate approach for subtyping, with Ki-67 serving as an important marker of tumour proliferation. This study evaluates the association of Ki-67 with key clinicopathological parameters in primary breast carcinoma and explores its role in IHC-based molecular classification. Conducted as a descriptive cross-sectional study in a tertiary care centre, it included patients with histologically confirmed primary breast cancer. The mean age of participants was 51.25 years. A significant inverse association was observed between Ki-67 and ER status, with mean indices of 18.75% in ER-positive and 43.47% in ER-negative tumours. All triple-negative breast cancers demonstrated high Ki-67 expression, with an average index of 54.89%. Luminal A and Luminal B subtypes were more common compared with other published series. Overall, Ki-67 showed significant correlations with tumour grade, perinodal spread and hormone receptor negativity. The findings support the utility of Ki-67 as a valuable biomarker and highlight its importance in distinguishing Luminal B tumours using IHC-based surrogate molecular classification.

KEYWORDS

Breast cancer; Ki-67; Immunohistochemistry; Molecular subtypes; Luminal A; Luminal B; HER2; Triple-negative breast cancer; Proliferation index; Surrogate classification.

INTRODUCTION:

Breast cancer is second most common malignancy worldwide after lung cancer. It is the most common cancer in women, accounting for 20% of all cancers in women and the leading cause of cancer related death among women [1,2].

Breast cancer is a heterogeneous disease with both intra-tumoral (within each individual tumor) and inter-tumoral (among different patients) heterogeneity [3]. Traditional morphology alone is insufficient for prognostication. Tumors belonging to the same histological type can have different clinical course. Additional information on such tumors can also be obtained by further ancillary testing including immunohistochemistry and molecular techniques.

Before the subtyping of breast cancers came, patients would undergo surgery as the first treatment followed by radiation or chemotherapy or both [4].

Perou et al. showed that breast cancers could be classified into fundamentally different subtypes on the basis of molecular analysis with gene expression profiling [5]. Each subtype behaves in a different manner. Four main groups were identified (Luminal A, Luminal B, HER2 enriched and Basal-like). Gene-expression profiling is limited by cost, time, and technical requirements. Cheang et al. [6] proposed immunohistochemical surrogate classification which was further studied by various researchers. Immunohistochemistry has now become an integral part of a complete and comprehensive histopathology report of breast carcinoma. In terms of prognosis and prediction of response to therapy, in addition to histological grade, tumor subtype and lymph node status, the assessment of triple markers – ER, PR, and HER2/neu, and proliferative activity based on Ki- 67 score have become essential requirements for the oncologist. Ki-67 on IHC is a widely used indicator of tumour proliferation. The existing guidelines of the American Society of Clinical Oncology do not include Ki-67 in the list of required routine biological markers. There is no standard operating procedure or generally accepted cut off for Ki67. And, hence, Ki67 staining is not implemented in standard routine pathology and even when used, there is lack of uniformity of reporting.

This study is to identify the utility of Ki-67 in the immuno histochemical subtyping of breast cancer along with ER, PR, HER2. Also, the expression and correlation of these IHC markers with histopathological findings in primary breast carcinoma. In the end we attempt to use IHC as a surrogate for classification of PBC into molecular subtypes.

MATERIALS AND METHODS

This is a descriptive cross-sectional study that was conducted in a tertiary health care center. Study duration was from January 2019 to June 2020. Study was conducted after getting ethical approval from the Ethical Committee. Study population was patients having histologically confirmed primary breast carcinoma. We studied a total of 55 cases of primary breast carcinoma.

We included, histopathologically confirmed case of primary breast carcinoma either biopsy, lumpectomy or mastectomy specimens. Secondary breast carcinoma and cases of primary breast carcinoma who had received neoadjuvant were excluded.

Clinical details such as age, sex, menstrual status, obstetric history was obtained from histopathological requisition form. Grossing was done and representative sections were given.

Formalin fixed paraffin embedded sections were taken and stained with Hematoxylin and Eosin (H&E) and evaluated by two independent pathologists.

Histopathological study of H&E sections was done to assess histological subtype, axillary nodal status, lymphovascular invasion.

Histological grading of tumor was done according to modified Bloom–Richardson grading [7] and staging according to pTNM classification designated by American Joint Committee on Cancer [8].

Immunohistochemical examination was done on representative tumor paraffin blocks. The immunostained slides were examined for nuclear staining in the case of ER, PR, and Ki-67, and membrane staining in the case of HER2/neu and recorded in CAP biomarker template[9].

The IHC panel (ER, PR, HER2, Ki67) was obtained from Biogenex. IHC was performed according to the protocol prescribed by Biogenex life sciences manual. The panel of antibody included were against ER, PR, HER2 and Ki67. The antibodies were provided as ready-to-use.

Known positive controls were used with each batch of IHC.

For hormone receptors, the proportion of positive staining tumor cells (expressed in percentage) and the average intensity of staining were evaluated based on Allred score method [10].

HER2/neu staining will be scored from 0 to 3+ as mentioned in CAP protocol.

Ki-67 was given as percentage of positively stained cells among the total number of malignant cells.

A cut off of 20% was considered in this study to categorize into High and Low Ki67. The relationship between various parameters such as age, menopausal status, tumor size, tumor extent, histologic type, histologic grade, lymph node status, and the expression of ER, PR, HER2/neu, and Ki-67 index was studied. And an attempt to make a surrogate molecular classification according to the IHC findings was made.

Statistical Analysis:
Data was collected and entered in a systematic format in Microsoft excel 2013. All the parameters in the data were analyzed for mean, frequency, percentage. The statistical analysis for correlation among these parameters was determined using Chi-square test. We used Statistical Package for Social Sciences (SPSS) 22.0; IBM Analytics, New York, U.S.A. All p values < 0.05 was taken as statistically significant.

RESULTS AND OBSERVATIONS:

Mean age of the study participants in our study was 51.25±14.75 years. 15(27.3%) patients were < 40 years; 40(72.7%) patients were > 40 years. In our study, the average Ki67 index in younger age group was 35.13% while in older age was 21.88%.

Among the total 55 cases; 33(60.0%) cases had tumor size>2 and <5 cm; 18(32.72%) had tumor size >5 cm and only 4(7.28%) had tumor size <2 cm. IDC, NOS was the most common histological subtype identified, 45(80.0%). Other histological types are 6(10.9%) cases of Invasive Carcinoma with Ductal and Lobular Features- Mixed Carcinoma, 2(3.64%) cases of ILC, 1(1.82%) case of Intra-cystic Papillary Carcinoma and 1(1.82%) case of Mucinous Carcinoma. In IDC, NOS, subtypes identified were 1 case of IDC, NOS with Medullary Features, 1(1.82%) cases of IDC and 1(1.82%) case of IDC, NOS with Osteoclastic Giant Cells. Grade I, II, III were 27(49.90%) cases, 22(40%) and 6(10.91%) respectively.

There were 40(72.73%) ER positive cases and 15(27.27%) ER negative cases.

There were 21 cases (38.18%) had high PR; 9(16.36%) had low PR and 25(45.45%) had negative PR.

There were 46 cases (83.64%) had negative HER2; 9(16.36%) had overexpression of HER2.

Out of our 55 cases, 32(58.2%) cases had high Ki67 compared to only 23(41.8%) cases having low Ki67.

Relationship of various parameters with Ki67 is as mentioned in Table 1.

Table 16.361: Relationship between Ki67 and various parameters:

Parameter	Ki 67 low		Ki67 high		Ki67 total		P value
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
Age group							0.545
Age <40	5	33.3%	10	45%	15	27.27	
Age >40	18	66.7%	22	55%	40	72.73	
Tumor size							0.532
<2	2	50%	2	50%	4	7.3%	
>2 and <5	15	46.9%	17	53.1%	32	58.2%	
>5	6	31.6%	13	68.4%	19	34.6%	
Tumor Grade							0.001
Grade I	14	51.9%	13	48.1%	27	49.1%	
Grade II	9	40.9%	13	59.1%	22	40%	
Grade III	0	0	6	100%	6	10.9%	
Necrosis							0.015
Absent	20	52.6%	18	47.4%	38	69.1%	
Present	3	7.6%	14	82.4%	17	30.9%	
Lympho-vascular Invasion							0.39
Absent	11	36.7%	19	63.3%	30	54.6%	
Present	12	48%	13	52%	25	45.4%	
Perineural Invasion							0.19
Absent	19	38.8%	30	61.2%	49	89.1%	
Present	4	66.7%	2	33.3%	6	10.1%	
Pathological stage- primary tumor (pT)							0.33
pT1	3	60%	2	40	5	9.1%	
pT2	14	50%	14	50	28	50.9%	
pT3	4	28.6%	10	71.4	14	25.5%	
pT4b	2	25%	6	75	8	14.6%	
Pathological stage- nodal spread							0.64
pN0	7	43.8%	9	56.3%	16	29.1%	
pN1	8	53.3%	7	46.7%	15	27.3%	
pN2	6	35.3%	11	64.7%	17	30.9%	
pN3	2	28.6%	5	71.4%	7	12.7%	
Perinodal Spread							0.01
Absent	9	47.4%	10	52.6%	19	34.6%	
Present	5	25%	15	75%	20	41.8%	
Not applicable	9	56.2%	7	43.8%	16	23.6%	
ER status							0.0001
ER Negative	0	0.0%	15	100%	15	27.3%	
ER Positive	23	57.5%	17	42.5%	40	72.7%	
PR status							0.002
PR Negative	4	16%	21	84%	25	45.4%	
PR Positive	19	63.3%	11	36.7%	30	54.6%	
HER2 status							0.015
Negative	23	50%	23	50%	46	83.6%	
Positive	0	0.0%	9	100%	9	16.4%	
Triple negative	0	0%	9	100%	9	100%	0.002

We evaluated the cases and categorized them into intrinsic subtypes- Luminal A, Luminal B, HER2 and TNBC. [11]

Luminal A was characterized as ER positive, PR high (Allred score 5 to 8)

Lumina B, HER2- was characterized as ER positive, PR low (Allred score 0 to 4) & any Ki67 or ER positive, any PR & Ki67 high and with negative HER2.

Luminal B, HER2+ was defined as Luminal B as defined above but with overexpression of HER2.

HER2 positive defined as ER and PR negative and HER2 positive

Triple negative breast cancer (TNBC) defined as negative ER, negative PR and negative HER2 expression.

In the IDC, NOS subtype, 33.3% of cases belong to Luminal A, 33.3% belonged to Luminal B, 13.3% belonged to HER2 enriched and 20% had triple negative tumors.

In the invasive carcinoma with ductal and lobular features, 16.7% belonged to Luminal A and remaining 83.3% belonged to Luminal B subtype. Among other subtypes, all cases of ILC belonged to Luminal A while all cases of intra-cystic papillary carcinoma and mucinous carcinoma belonged to Luminal B.

DISCUSSION

Breast cancer is the most common cancer in women in the world and is second leading cause of cancer related deaths. This study evaluated 55 cases of primary breast cancer investigating relationship between Ki67 index, clinic-pathological findings and ER, PR and HER2 expression.

ER/PR/Her2

In our study, ER+/PR+/HER2- cases were 50.9% while ER+/PR-/HER2- cases were 16.4%. In 3.6% cases we found ER+/PR+/HER2+ expression while in 1.8% cases the expression was ER+/PR-/HER2+. There were a total of 9 cases (16.36%) were negative for all three IHC markers- ER, PR and HER2 and all three markers were positive in 2 cases (3.64%).

Kaur et al [12], they found 36% of ER positivity, 36% with PR positivity while Soni et al [13], triple negative was found in 35% of cases and triple positivity in 4% of cases. In various studies by Maffuz-Aziz et al [14], Soni et al [13], they found that 19.6%, 21.8% of cases, respectively had overexpression of HER2 by IHC analyses.

Ki67 Index

High proliferation correlates with poorer prognosis. This proliferation status in any breast cancer can be done using various methods- mitotic index, detection of cells undergoing DNA synthesis using thymidine uptake assay, using flow cytometry to detect number of cells in S phase of cell cycle and detection of antigens associated with proliferation. [15] Mitotic count has been used in breast cancer for this since a long time and is also included in Nottingham grading of breast cancer. The clinical use of Ki67 is still under debate in use as well as to determine the standard cut off value.

In our study we used 20% cut off of Ki67 throughout. [16]

In 2013 St Gallen argued for 20% cut off compared to their previously recommended 14% cut off criteria for categorizing Ki67 as high and low. [17] Also, Park et al [16], compared 14% and 20% cut off value and found 20% cut off value to have more significance. Escala-Cornejo et al [18] studied the correlation surrogate IHC subtypes with PAM50 gene and concluded that at 20% cut off value, Ki67 provides a better surrogate classification by IHC and higher sensitivity for classifying luminal subtypes.

The average Ki67 in our study was 25.5%. There were 32 (58.18%) cases of high Ki67 at >20% cut off in our study. Hashmi et al [19] found average Ki67 was 56.0%. In the 398 cases studied by Liu et al [20], they found mean Ki67 of 62.6% cases of high Ki67 expression which was classified as >20%.

We found statistically significant correlation between Ki67 and grade of tumor, with mean Ki67 highest in grade III. The mean Ki67 in our study was 17.19%, 27.36% and 56% in grade I, II and III tumors respectively. Shokouh et al [21], found that Ki67 increased with tumor grade which is similar to our study. In another study by Mohammed AA [22], he found that there was a significant correlation between Ki67 and tumor grade. Hashmi et al [19], found mean score of 40.3, 56.58 and 71.2 in grade I, II and III tumors.

No statistically significant correlation between Ki67 index and age, tumor size, pT stage of the tumor, although, a greater number of patients in the younger age group had higher Ki67 value. Mohammed AA [22], also found no statistical association between Ki67 and age of patient.

Although in our study we found no significant correlation with lymph node status and Ki67 index, a notable correlation was seen with perinodal spread. In various studies by Mohammed A [22], they found no statistical association between Ki67 and the number of positive lymph nodes. Kaur et al [12], found no direct correlation of Ki67 with lymph node status.

A strong inverse relationship was identified between Ki-67 and hormone receptor status. ER-negative and PR-negative tumours demonstrated significantly higher Ki-67 indices, consistent with the known biology of more proliferative, hormone-receptor–negative breast cancers. These findings align with those reported by Park et al., who also noted substantially higher Ki-67 in ER- and PR-negative tumours. In our study, out of 9 HER2 positive cases, all of them (100%) high Ki67 index. In the HER2 negative cases, 23(50%) had higher Ki67 index and 23(50%) had lower Ki67 index. There was statistically significant correlation with HER2 overexpression and Ki 67 index.

In the 9 cases with triple negative status, all of them had high Ki67 expression. The mean Ki67 in HER2 positive cases was 31.44%. Park et al [16], found mean Ki67 in HER2 positive was 42.23 compared to 26.15 in HER2 negative.

Ki67 and TNBC

In our study, all triple negative breast cancers (TNBC) had high Ki67 index. In triple negative tumors, the average Ki67 index was 54.89% in our study.

MOLECULAR SUBTYPES USING IHC

We in our study employed the criteria using 2013 update of St Gallen. [17] We defined Luminal A as ER+, high PR and Low Ki67 index; Luminal B as ER+, low PR with any Ki67 or high Ki67 with any PR, HER2 +, HER2 enriched as ER-, PR- and HER2+ and TNBC as negative for ER, PR and HER2.

The distribution of luminal A and Luminal B was more in present study compared to other studies. This difference can be attributed to more ER and PR positive cases in present study along with more cases belonging to the older age group and small sample size.

Various other studies by Park et al [16], Maffuz-Aziz et al [14], categorized breast cancers as Luminal A, Luminal B, HER2 enriched and TNBC based on IHC, but we couldn't compare our findings with theirs as they categorized Luminal B based on Ki67 expression or PR expression alone.

Table 14: Distribution Of Molecular Subtypes Using IHC In Various Studies

Study	*Luminal A*	*Luminal B*	*HER2 enriched*	*TNBC*
Liu et al(20)	10%	46.5%	20.6%	22.9%
Serrano-Gomez et al(23)	26.3%	37.2%	8.6%	20.6%
Present study	32.7%	40%	10.9%	16.4%

Limitations Of The Study-

In our study, sample size was small (n=55).

In our study, the histopathological requisition form that we received did not mention cold ischemia time.

In our study, as the data was collected from histopathological requisition form, many parameters like menstrual status, number of children, duration of lactation and other such parameters couldn't be assessed due to lack of the information in the forms.

We assessed intrinsic subtypes of breast cancer using IHC and not gene expression profile. But with our study we highlight the fact that in situations where molecular profiling is not possible, IHC can be a useful surrogate for molecular subtyping of breast cancer into Luminal A, Luminal B, HER2 enriched and TNBC.

CONCLUSION

In conclusion, Ki67 is a valuable biomarker in breast cancer and can be used in treatment and follow up. Higher Ki67 has a statistically significant correlation with tumor grade and presence of perinodal spread. Also, higher Ki67 is found in PR negative cases. Ki67 is especially useful in determination of of Luminal B subtype using IHC. Further studies are needed to standardise Ki-67 assessment.

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