Original Article

Immunohistochemical characterization of molecular classification of breast carcinoma and its relation with Ki-67

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ABSTRACT

Background: Breast carcinoma is the leading cause of cancer deaths in women. Molecular classification of breast carcinoma along with Ki-67 index is considered a better predictive factor for prognosis and treatment than routine histopathology. **Aims:** To classify breast carcinoma into the four molecular subtypes defined by immunohistochemical expression of triple markers: Luminal A (estrogen receptor/progesterone receptor-positive [ER/PR+] and human epidermal growth factor receptor 2 HER2/neu), luminal B (ER/PR + and HER2/neu+), triple negative (ER/PR − and HER2/neu−), and HER2 positive (ER/PR−, HER2/neu+), and to correlate the expression of ER, PR, HER2/neu, and classification with Ki-67. **Materials and Methods:** The present study includes sixty breast carcinoma cases studied over a 3-year period. The expression patterns of ER, PR, HER2/neu, and Ki-67 were studied. Clinical features, pathologic features such as size, grade, and lymph node status, and correlation with Ki-67 of the four subtypes were compared. **Results:** Out of sixty cases, most common molecular subtype was triple negative (40.00%) followed by luminal B (23.33%). Most of the tumors showed low proliferative index (low Ki-67); however, triple negative and HER2 positive subtype showed high proliferative index. Most common histological subtype was ductal carcinoma which was mainly triple negative. All medullary carcinoma cases were triple negative. One case of lobular carcinoma and mucinous carcinoma each was HER2 positive and luminal B, respectively. Single case of carcinoma of male breast was luminal B subtype. **Conclusion:** Correlation of molecular classification with age, histological grade, and Ki-67 was statistically significant ($P < 0.05$). ER/PR also correlated with histological grade and Ki-67 ($P < 0.01$). These results emphasize the fact that molecular subtypes correlate with prognosis and aid in targeted therapy.

Key words: Breast carcinoma, estrogen receptor, human epidermal growth factor receptor 2/neu, Ki-67, molecular classification, progesterone receptor

INTRODUCTION

Breast carcinoma is the leading cause of cancer death in women, with more than 1,000,000 cases occuring worldwide annually.[1] With an age-standardized incidence rate of 25.8 cases per 100,000 women per year, it is the most common cancer among Indian women.[2] Gene expression profiling has identified five major patterns of gene expression: Luminal A, luminal B, normal breast-like, triple negative, and human epidermal growth factor receptor 2 (HER2) positive. Usually, the three biomarkers, estrogen receptor (ER), progesterone receptor (PR), and HER-2/neu are routinely assessed and used to approximate the molecular category of breast cancer.[3] Presence of both ER and PR is related to better prognosis and responsiveness to hormonal therapy.[4] A humanized monoclonal antibody, trastuzumab, targeting the HER-2/neu gene product is another example of targeted therapy in breast cancer.[5]

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Ki-67 is an independent factor to predict tumor grade; thus, the use of this proliferation marker in routine approach to patients with breast cancer is recommended.[6] Its prognostic value in breast cancer has not been adequately explored and further studies are needed.

We have classified breast carcinoma based on the expression patterns of ER, PR, HER2/neu, and correlated it with proliferative activity using Ki-67, histological grade, tumor size, lymph node status, and patient age. Thereby, the data would help in planning the prognostic and therapeutic approach in patients with breast cancer.

MATERIALS AND METHODS

A total of sixty patients operated for breast carcinoma between the years 2011 and 2014 were included in the study, of which 46 were modified radical mastectomies, 11 were simple mastectomies, and three lumpectomies. Cases where only a Tru-Cut biopsy had been done and where there was extensive tumor necrosis without sufficient viable tumor cells were excluded from the study. Clinical details such as age, sex, menstrual status, site, and size of tumor were retrieved from the clinical records.

Specimens were subsequently formalin fixed, paraffin embedded, and stained by hematoxylin and eosin for histopathological study to assess histological subtype, axillary nodal status, lymphovascular invasion, and any other significant features [Figure 1]. Histological grading of tumor was done according to modified Bloom–Richardson grading and staging according to TNM classification designated by American Joint Committee on Cancer.

Immunohistochemistry was done on representative tumor paraffin blocks using a standard protocol. 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. 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. HER2/neu staining was scored from 0 to 3+. Ki-67 was scored as percentage of positively stained cells among the total number of malignant cells and divided into three groups - low (≤15%), intermediate (15–30%), and high (>30%).

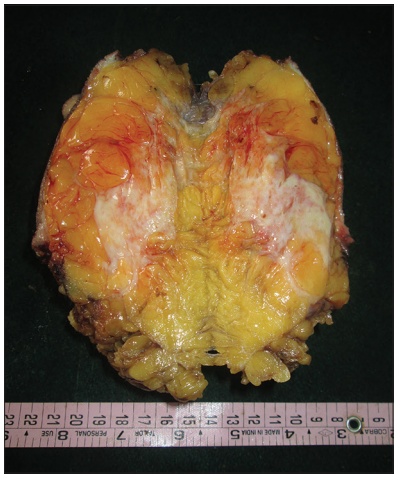

Figure 1: Cut section of mastectomy specimen reveals irregular gray-white mass infiltrating into adjacent tissue

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 were studied and based on their expression classified into luminal A, luminal B, triple negative, and HER2 positive. The statistical analysis for correlation among these parameters was determined using the Pearson Chi-square test. Significance was assumed at $P < 0.05$.

RESULTS

The age of the patients ranged from 22 to 85 years with a mean age of 47.65 years, majority being premenopausal (55.93%). Out of the sixty cases of breast cancer studied, left breast was more commonly involved with tumor size ranging from 1 to 12 cm (mean - 4.52 cm). Majority of the cases were of Stage II (50.00%) and histological Grade I (38.33%). Fifty-four cases (90.00%) were invasive ductal carcinoma (IDC), followed by four cases of medullary carcinoma and one case each of invasive lobular carcinoma and mucinous carcinoma. Lymphovascular invasion was noted in 35 cases (58.33%) and lymph node metastasis in 25 cases (41.67%).

Immunohistochemical expression of ER, PR, and HER2/neu were negative in most of the cases with 55.00%, 58.33%, and 61.67%, respectively. Patients in younger age group (21–30 years) were mostly ER negative, PR negative, and HER2/neu positive indicating a poor prognosis. The two cases that were seen in > 80 years age group were ER and PR positive implying a good response to hormonal therapy. HER2/neu positive cases were more common in premenopausal, whereas negative in postmenopausal women. ER positive, PR positive, and HER2/neu negative tumors were of lower histological grade. This was statistically significant ($P < 0.05$).

Based on molecular classification, most of the cases were of triple negative subtype (40.00%) followed by luminal B, luminal A, and HER2 positive [Chart 1 and Figures 2-5]. Luminal cases were mostly in older age group, and HER2 positive was mainly seen in younger age group of 21–30 years. Majority of luminal A cases were of smaller tumor size (<2 cm) compared to the rest. Luminal A tumors were mainly of low grade (84.62%), whereas other three

molecular subtypes were mostly high-grade tumors. This relation between molecular classification and histological grade was statistically significant ($P < 0.05$). Majority of the cases were positive for lymphovascular invasion and negative for lymph node metastasis irrespective of molecular subtype.

Ki-67 index was low in most cases (31 cases; 56.36%) [Figure 6]. Tumors in the younger age group of 21–30 years showed high Ki-67 index and in the elderly age group of >60 years showed low Ki-67 index. Low Ki-67 tumors were mainly low grade (45.16%), whereas high Ki-67 tumors were mostly high grade. Lymph node metastasis was mostly negative in cases with low proliferative index and positive in high proliferative index cases. ER and PR positive cases were mainly of low Ki-67 index, whereas ER and PR negative tumors were of high Ki-67 index. HER2/neu positive and negative cases were mostly of low Ki-67 index. Correlation of ER/PR status with Ki-67 was

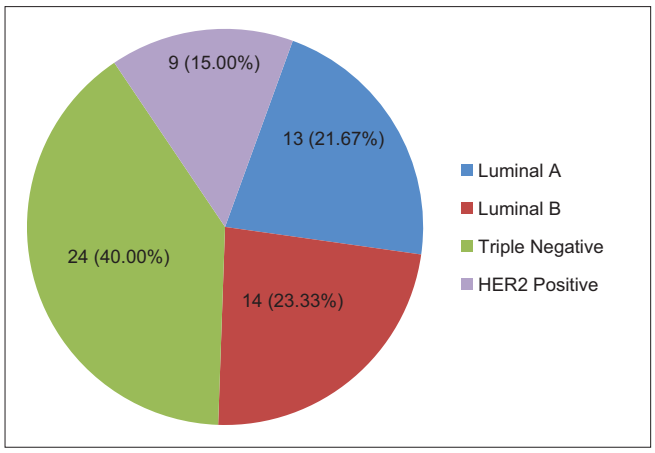

Chart 1: Distribution of breast carcinoma cases according to molecular classification

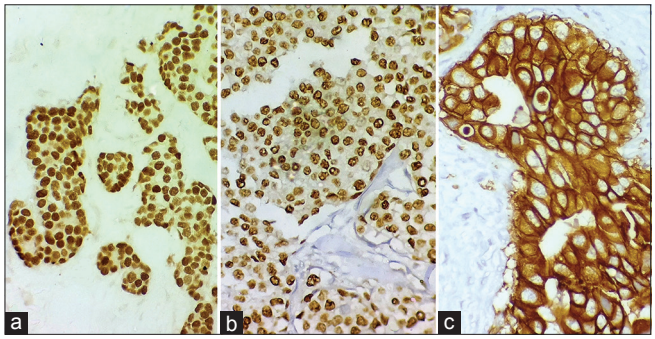

Figure 3: Luminal B subtype. (a) Estrogen receptor positivity, (b) progesterone receptor positivity, and (c) human epidermal growth factor receptor 2/neu positivity (×400)

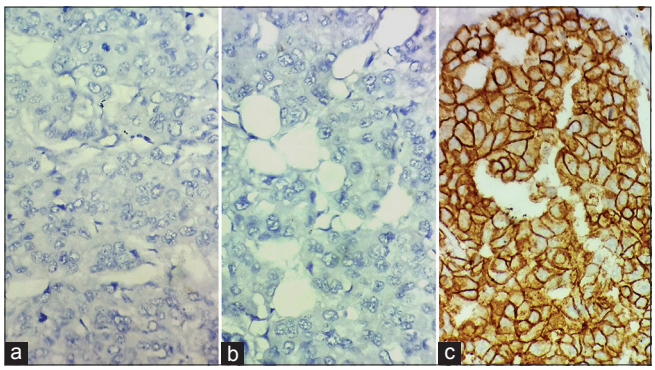

Figure 5: Human epidermal growth factor receptor 2 positive subtype. (a) Estrogen receptor negativity, (b) progesterone receptor negativity, and (c) human epidermal growth factor receptor 2/neu positivity (×400)

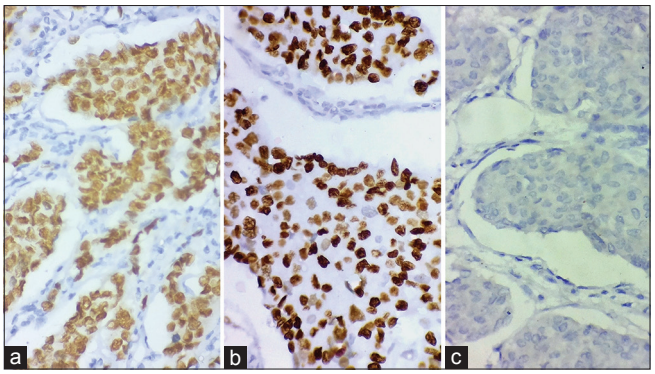

Figure 2: Luminal A subtype. (a) Estrogen receptor positivity, (b) progesterone receptor positivity, and (c) human epidermal growth factor receptor 2/neu negativity (×400)

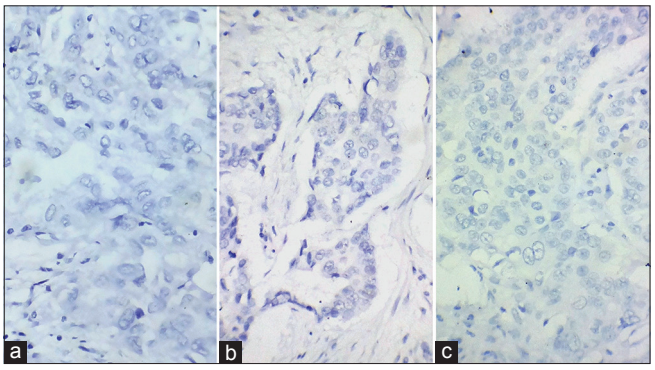

Figure 4: Triple-negative subtype. (a) Estrogen receptor negativity, (b) progesterone receptor negativity, and (c) human epidermal growth factor receptor 2/neu negativity (×400)

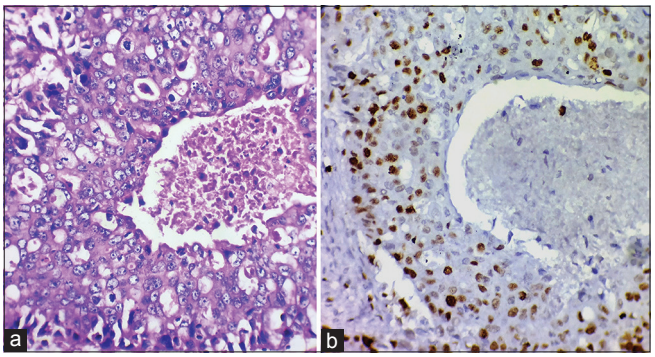

Figure 6: (a) Infiltrating ductal carcinoma with nests of pleomorphic cells having prominent nucleoli, occasional mitosis, and central foci of necrosis (H and E, ×400); (b) Ki-67 in this case showing 75% positivity (×400)

highly significant ($P < 0.01$) [Table 1]. All 12 cases of luminal A subtype were of low Ki-67 index. Most of luminal B cases were also of low proliferative index, whereas triple negative and HER2 positive were of high proliferative index. Correlation of molecular classification with Ki-67 was found to be statistically significant ($P < 0.05$) [Table 2].

Molecular subtype of most cases of younger age group were HER2 positive and triple negative, whereas that of elderly age group were luminal A and luminal B. Ki-67 index showed drastic difference among these age groups with all cases in elderly age group being of low proliferative index, whereas majority of younger age group cases were of high proliferative index. The relation of molecular classification and Ki-67 with age was statistically significant ($P < 0.01$) [Table 3].

Most of the IDC cases were of triple negative subtype (37.04%) and of low proliferative index (57.45%) with a mean Ki-67 score of 26.52%. All four medullary carcinoma cases were ER, PR, HER2/neu negative, and of triple negative subtype. One case of lobular carcinoma and mucinous carcinoma each was HER2 positive and luminal B, respectively. Single case of carcinoma of male breast was seen, which was ER, PR, and HER2/neu positive (luminal B) and of low proliferative index with Ki-67 score of 1%.

DISCUSSION

Use of 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, lymph node status, assessment of triple markers – ER, PR, and HER2/neu, and proliferative activity based on Ki-67 score have become essential requirements for the oncologist. Recent attention has been directed at molecular classification

Table 1: Relationship of Ki-67 with triple markers

Marker	Ki-67			Mean Ki-67 score	Total (*n*=55)	χ^2	*P*
	<15% (*n*=31)	16-30% (*n*=5)	>30% (*n*=19)				
ER							
Positive	19 (79.17)	1 (4.17)	4 (16.67)	13.96	24	9.06	<0.01
Negative	12 (38.71)	4 (12.90)	15 (48.39)	35.52	31		
PR							
Positive	17 (77.27)	1 (4.55)	4 (18.18)	15.18	22	7.723	<0.01
Negative	14 (42.42)	4 (12.12)	15 (45.45)	33.39	33		
HER2/neu							
Positive	10 (47.62)	2 (9.52)	9 (42.86)	28.57	21	1.056	>0.05
Negative	21 (61.76)	3 (8.82)	10 (29.41)	24.59	34		

ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor 2

Table 2: Relationship of Ki-67 with molecular classification

Subtype	Ki-67			Mean Ki-67 score	Total (*n*=55)	χ^2	*P*
	<15% (*n*=31)	16-30% (*n*=5)	>30% (*n*=19)				
Luminal A	12 (100)	0 (0.00)	0 (0.00)	4.58	12	9.29	<0.05
Luminal B	7 (58.33)	1 (8.33)	4 (33.33)	23.33	12		
Triple negative	9 (40.91)	3 (13.64)	10 (45.45)	35.50	22		
HER2 positive	3 (33.33)	1 (11.11)	5 (55.55)	35.56	9		

HER2: Human epidermal growth factor receptor 2

Table 3: Distribution of breast carcinoma cases according to immunohistochemical expression of different age groups

	Younger age ≤30 years (*n*=8) (%)	Middle age 31-60 years (*n*=47) (%)	Elderly age >60 years (*n*=5) (%)	χ^2	*P*
Molecular subtype					
Luminal A	1 (12.50)	11 (23.40)	1 (20.00)	5.73	<0.05
Luminal B	1 (12.50)	10 (21.28)	3 (60.00)		
Triple negative	2 (25.00)	22 (46.81)	0 (0.00)		
HER2 positive	4 (50.00)	4 (8.51)	1 (20.00)		
Ki-67 (%)					
<15	1 (12.50)	25 (59.52)	5 (100.00)	7.12	<0.01
15-30	1 (12.50)	4 (9.52)	0 (0.00)		
>30	6 (75.00)	13 (30.95)	0 (0.00)		
Mean Ki-67	50.00	46.64	4.40		

HER2: Human epidermal growth factor receptor 2

of breast cancer which has prognostic and therapeutic implications.

Leong *et al*.[7] noticed that in Asia, breast cancer incidence peaks among women in forties, whereas in Western countries, it peaks in sixties due to multiple factors such as environmental factors, socioeconomic status, inadequate implementation of screening programs, and lack of appropriate care. In the present study, also most of the cases were in 40–50 years age group with a mean age of 47.65 years. This correlated with a study by Naeem *et al*.,[8] Su *et al*.,[9] and Dang and Mysorekar.[10] Most common histological subtype in this study was infiltrating ductal carcinoma, and lymph node metastasis was absent in most cases. Similar findings were observed in most of the studies.[11-13]

In this study, majority of the cases were ER, PR, and HER2/neu negative. ER and PR were negative in two of the studies by Indian authors,[14,15] whereas in most international studies, hormonal receptors were usually positive.[5,12,13,16,17] This suggests a change in trend in Indian population where patients usually present in advanced stage leading to poor prognosis [Table 4].

In terms of age group, ER/PR positivity was more common among elderly (>60 years) compared to younger women (<30 years) which correlated with a study by Sofi *et al*.[18] Nishimura *et al*.,[16] and Inwald *et al*.[13] also found higher Ki-67 index in women of younger age group than elderly as seen in the present study.

In the present study, most of the cases belonged to triple negative subtype (40.00%) which correlated with a study by Dang and Mysorekar.[10] Luminal A was the most common molecular subtype in all other Indian and international studies.[4,9,11,12,16,19,20] Since the study population of Dang and Mysorekar[10] study included Karnataka region like the present study, regional variation can be attributed to the increased incidence of triple negative cases [Table 5]. Subtypes with poor prognosis such as triple negative and HER2 positive were more common among younger women, whereas those with better prognosis such as the luminal tumors were more common among elderly women. Similar observation was made by Su *et al*.,[9] Nair and Tewari.,[14] and Dang and Mysorekar.[10]

Molecular classification correlated with a study by Onitilo *et al*.[19] Triple negative and HER2 positive tumors were seen in younger age, were of larger size, higher grade, and had more tendency to metastasize to lymph node compared to luminal tumors in both studies.

ER positive, PR positive, and HER2/neu negative tumors were of low proliferative index in most of the studies as in the present study.[4,5,10,13,17] Molecular classification correlated with Ki-67, wherein luminal tumors were of low proliferative index, and triple negative and HER2 positive tumors were of high proliferative index. This was in concordance with studies by Verma *et al*.,[11] Dang and Mysorekar,[10] and Aleskandarany *et al*.[21] Cheang *et al*.[22] suggested that the most appropriate Ki-67 index cutoff point to distinguish

Table 4: Comparative analysis of immunohistochemical expression

Authors	ER (%)	PR (%)	HER2/neu (%)	Ki-67 (mean)
Ivkovic-Kapicl *et al*., 2007 (*n*=20)[6]	Positive (74)	Positive (67)	Negative (81)	-
Kuraparthy *et al*. 2007 (*n*=18)[15]	Negative (61.4)	Negative (66.7)	-	-
Nishimura *et al*., 2010 (*n*=2639)[16]	Positive (74.6)	Positive (61.7)	Negative (78.1)	20
Yang *et al*., 2011 (*n*=34)[17]	Positive (64.2)		Negative (77.6)	24.7
Nair and Tewari 2011 (*n*=50)[14]	Negative (58)	Negative (82)	Negative (62)	-
Verma *et al*., 2012 (*n*=00)[11]	Positive (55)	Negative (45)	Negative (64)	-
Yamamoto-Ibusuki *et al*., 2013 (*n*=235)[12]	Positive (80)	Positive (69)	Negative (87)	24.5
Inwald *et al*., 2013 (*n*=3658)[13]	Positive (85.8)	Positive (77.2)	Negative (81.8)	20.3
Present study 2014 (*n*=60)	Negative (55.00)	Negative (58.33)	Negative (61.67)	23.93

ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor 2

Table 5: Comparative analysis of molecular classification

Authors	Luminal A (%)	Luminal B (%)	Triple negative (%)	HER2 positive (%)
Onitilo *et al*., 2009 (*n*=134)[19]	68.9	10.2	13.4	7.5
Nishimura *et al*., 2010 (*n*=2639)[16]	66.28	9.97	13.49	10.27
Singhai *et al*., 2011 (*n*=90)[5]	56.67	10.00	16.67	16.67
Su *et al*., 2011 (*n*=2791)[9]	48.55	16.73	12.90	21.82
Verma *et al*., 2012 (*n*=00)[11]	47	15	17	21
Dang and Mysorekar 2012 (*n*=72)[10]	27.8	25.0	33.3	13.9
Yamamoto-Ibusuki *et al*., 2013 (*n*=235)[12]	75	5	12	8
Engstrøm *et al*., 2013 (*n*=909)[20]	47.6	35.1	7	6.6
Present study 2014 (*n*=60)	21.67	23.33	40.00	15.00

HER2: Human epidermal growth factor receptor 2

Table 6: Comparative analysis of molecular classification of histological subtypes

Authors	IDC (*n*=54)	ILC (*n*=1)	Medullary (*n*=4)	Mucinous (*n*=1)
Yang *et al*., 2007 (*n*=804)[62]	Luminal A (61.97)	Luminal A (83.12)	-	-
Onitilo *et al*., 2009 (*n*=1134)[47]	Luminal A (62.05)	Luminal A (85.40)	-	-
Su *et al*., 2011 (*n*=2791)[50]	Luminal A (43.3)	Luminal A (66.0)	Triple negative (37.9)	Luminal A (81.2)
Engstrom *et al*., 2013 (*n*=909)[59]	Luminal A (47.01)	Luminal A (54.84)	Triple negative (33.33)	Luminal A (72.09)
Present study 2014 (*n*=60)	Triple negative (37.04)	HER2 positive (100)	Triple negative (100)	Luminal B (100)

ILC: Invasive lobular carcinoma, IDC: Invasive ductal carcinoma, HER2: Human epidermal growth factor receptor 2

luminal B from luminal A tumors was 13.25%. In the present study too, mean Ki-67 score of luminal B tumors was above the cutoff at 23.33%, whereas that of luminal A was 4.58%.

Most common histologic subtype, infiltrating ductal carcinoma was found to be of triple negative molecular subtype in most cases. Although most studies showed luminal A to be most common molecular subtype among IDC cases, triple negative was the second most common.[9,19,20] Among lobular carcinoma cases in majority of studies, luminal A was the most common subtype. However, in the present study, the single case of lobular carcinoma was HER2 positive which was observed in a small percentage of cases in these studies.[9,19,20] Single case is not representative of the whole population leading to this discrepancy. All four cases of medullary carcinoma were of triple negative subtype which correlated with studies by Su *et al*.[9] and Engstrøm *et al*.[20] Mucinous carcinoma case was of luminal type like in study by Su *et al*.[9] and Engstrøm *et al*.[20] [Table 6].

Male breast cancer is an uncommon disease, accounting for approximately 1% of all breast cancer cases and < 1% of all malignancies in men.[23] A case of carcinoma of male breast was noted in the present study which was of luminal B subtype with low Ki-67 index (1%). In a study by Ge *et al*.[23] of 42 male breast cancer cases, most common subtype was luminal A (83%) followed by luminal B (17%). No triple negative or HER2 positive cases were identified in Ge *et al*.[23] study. Wang-Rodriguez *et al*.[24] studied 65 male breast cancer cases and found mean Ki-67 score to be 10.6%, which indicates low proliferative activity like in the present study.

Correlation of molecular classification with age, histological grade, and Ki-67 was statistically significant ($P < 0.05$). ER/PR also correlated with histological grade and Ki-67 ($P < 0.01$). Statistically significant correlation was also found for these parameters in other Indian studies[4,10,11] and few international ones.[6,19] This implies that higher grade and high proliferative index tumors were usually ER/PR negative and of triple negative or HER2/neu molecular subtype.

CONCLUSION

Breast cancer is a global disease with rising incidence in Indian women. As advances are been made in breast cancer diagnosis and treatment, more attention has been directed to markers of increased risk for developing the disease, which are hormone receptors, HER2/neu expression, and proliferative index using Ki-67. Molecular classification based on triple markers which classifies breast carcinoma into luminal A, luminal B, triple negative, and HER2 positive, is considered a better predictive factor for prognosis and treatment than routine histopathology.

In the present study of sixty breast carcinoma cases, most of the cases were of triple negative subtype. Younger patients had a higher frequency of triple negative and HER2 positive tumors, whereas older patients had a higher rate of luminal tumors. Assessment of proliferative activity using Ki-67 revealed low proliferative index in most cases. However, higher Ki-67 index was associated with younger age, higher-grade tumors, lymph node metastasis, negative ER/PR, triple negative, and HER2 positive subtypes. There was a significant statistical correlation between age, molecular classification, ER/PR status, and Ki-67. Correlation of histological grade with molecular classification and hormonal status was also statistically significant.

A greater understanding of the molecular classification of breast carcinoma based on triple markers will help in the development of targeted therapies that will lead to increased efficacy, decreased toxicities, and better selection of patients who will benefit from treatment. Ki-67 being an independent prognostic factor should be evaluated routinely in breast carcinoma cases. It also plays a role in making treatment decisions and to monitor response to treatment.

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Conflicts of interest
There are no conflicts of interest.

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