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Original Article

Prevalence of molecular subtypes of invasive breast cancer: A retrospective study

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ABSTRACT

Background: Classification of breast cancers into different molecular subtypes using immunohistochemistry as a surrogate tool to DNA microarray technology has been identified. Aim of this study was to find the precise prevalence of molecular subtypes of invasive breast carcinoma using immunohistochemistry and to correlate it with histological and clinical prognostic factors.

Methods: A descriptive study design wherein 56 breast carcinoma cases diagnosed between May 2012 and Apr 2014 were reviewed and molecular subtyped using relevant immunohistochemical markers. Age, histological type, tumor size, tumor grade, lymphovascular emboli (LVE), nodal status and basal marker expressions were analyzed for all cases. Correlations between molecular subtypes and clinico-pathological characteristics were evaluated statistically.

Results: In our study group, average age of patients was 50.5 years and most of patients were diagnosed in stage II (54%). Luminal A subtype was most prevalent 34%, followed by Basal like/Triple negative subtype 25%. Luminal B and Her2/neu subtypes had same prevalence i.e. 18% each and Breast Tissue like/Unclassified subtype/Penta Negative subtype was 5%. All cases demonstrated positivity for the luminal cytokeratins 8/18. Histological grade and ER negative status showed strong correlations with basal markers.

Conclusion: On the basis of hormone receptor, Her2 neu, EGFR and CK 5/6 expressions, breast carcinoma cases were classified into five distinct molecular subtypes which show significant differences in regards to morphological features, prognostic markers and there by predicting possible clinical outcome.

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Introduction

Breast cancer is the most commonly reported cancer world-wide and second leading cause of mortality.¹ Incidence of breast cancer is progressively increasing over last decades,^{2,3} and specially Asian countries have shown marked increase in the incidence.⁴ Breast cancer is a malignancy with varied molecular and clinical characteristics. Breast carcinoma has been sub classified in five subtypes based on gene expression profiling using DNA microarray which is a very expensive technique and cannot be used on formalin-fixed, paraffin-embedded samples. Recently studies have established that similar subtypes can be identified using immunohistochemical specific markers as surrogate tool for DNA microarray.^{5,6} Breast carcinoma subtypes based on immunohistochemical markers are (a) Luminal A – Estrogen Receptor (ER) and/or Progesterone Receptor (PR) positive and Human Epidermal Growth Factor Receptor 2 (Her2) negative, (b) Luminal B – ER and/or PR positive and Her2 positive, (c) Her2neu subgroup – ER and PR negative and Her2 positive, (d) Basal like – ER, PR and Her2 negative, cytokeratin (CK) 5/6 positive and/or Epidermal Growth Factor Receptor (EGFR) positive, and (e) Unclassified/Penta negative (PN) – ER, PR, Her2neu, CK 5/6 and EGFR all negative^{5–7} (Table 1). The molecular subtypes suggest the heterogeneity of breast carcinomas and the possible different cell lineage pathways in breast carcinogenesis. The precise prevalence and clinico-pathological characteristics of these molecular subtypes of invasive breast tumors are not extensively studied in Indian population. The aim of this study was to identify and define the precise prevalence of molecular subtypes of invasive breast carcinoma using immunohistochemistry (IHC) in Indian population and to correlate with the morphological features and prognostic parameters. The morphological features and prognostic parameters i.e. tumor size, tumor type, tumor grade, lymphovascular emboli (LVE) and lymph node status of invasive breast carcinoma of each molecular subtype, were compared.

Materials and methods

A total of 56 histologically confirmed cases of breast carcinomas diagnosed in the Department of Pathology of a tertiary care institution over a period of two years (May 2012–April 2014) were selected for the present study. Cases with adequate material (in the form of adequate representative viable tumor in the sections or the specimen) were studied. All the cases

were processed for paraffin sections for routine hematoxylin and eosin (H&E) staining and IHC using pre-diluted ready to use antibodies (Table 1).

Exclusion criteria

Slides showing quantitatively inadequate material were excluded. Cases which had received chemotherapy prior to surgery, cases with previous lumpectomy followed by mastectomy for residual disease were also excluded in our study.

Light microscopy

H&E stained slides and the paraffin blocks were retrieved from the archives. All the cases were graded and staged according to the 2003 World Health Organization histological classification of tumors of the Breast. The morphological characteristics noted include: tumor size, histological type, tumor grade, mitoses, necrosis, LVE and nodal status.

Immunohistochemistry

The cases of invasive breast carcinoma were classified into molecular subtypes using immunohistochemical markers: ER, PR, Her-2/neu, CK 5/6 and EGFR (Table 2). Negative controls were obtained by omitting the primary antibodies. As per literature description, interpretation for ER, PR, Her-2/neu (score 2 or more taken positive) was done. For CK immunoreactivity in >10% of tumor cells was taken as positive. Statistical analysis and correlations among categorical variables in the univariate analysis were determined using the Pearson chi-square test. Significance was assumed at p-value less than 0.05.

Results

Clinicopathological parameters

Mean age of patients in study population was 50.5 years ranging from 30 to 72 years. 73.2% (41/56) cases presented with non-tender lump breast and rest 26.8% (15/56) presented with other symptoms as positive axillary lymph node, nipple discharge, bone pain etc. The most frequent histological type (89%, 50/56) was infiltrating duct carcinoma, not otherwise specified (IDC, NOS). There were two cases of invasive lobular carcinoma (ILC) and one case each of invasive papillary carcinoma, mucinous carcinoma, medullary carcinoma, and metaplastic carcinoma.

Tumor size

The average tumor size was 3.4 cm ranging from 1.1 cm to 7.8 cm in their largest dimensions. 14.2% tumors (8/56) were <2 cm (T1), 55.4% tumors (31/56) were in the range of 2–5 cm (T2), and 30.4% tumors (17/56) were greater than 5 cm (T3) in the largest dimensions on presentation. 16/17 tumors (94.1%) in TN group (i.e. Basal like plus PN subtypes) were >2 cm in size followed by Her2/neu+ group with 90% (9/10) cases. LUM

Table 1 – Pre-diluted ready-to-use antibodies used in the study.

Antibody	Manufacturer	Clone
CK 8/18	Novocastra	NCL-5D3
CK 5/6	DAKO	D5/16B4
ER	DAKO	1D5
PR	DAKO	PgR636
Her2/neu	DAKO	Polyclonal
EGFR	DAKO	E30

Table 2 – Molecular subtypes of breast cancer based on immunohistochemical characterization.

Molecular subtypes	Immunohistochemical characterization
LUM A	ER+ and/or PR+, HER2–, CK 8/18+
LUM B	ER+ and/or PR+, HER2+, CK 8/18+
HER2/neu	ER–, PR–, HER2+
Basal like	ER–, PR–, HER2– and CK 5/6+ and/or HER1+
Unclassified	ER–, PR–, HER2–, CK 5/6–, HER1–

ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; HER1 = human epidermal growth factor receptor 1; CK = cytokeratin.

A and LUM B groups had 78% (15/19) and 80% (8/10) cases respectively which were >2 cm (Table 3).

Tumor grade

Majority of cases, were of histological grade II (59%, 33/56), followed by grade III (27%, 15/56), and remaining 14% (8/56) cases were grade I tumors. Among grade III tumors the maximum numbers were found in Basal like (9/15) subtype. None of the TN cases i.e. in Basal like and PN group were grade I. IHC subtypes were significantly different by histological grade ($p = 0.001$). The basal-like and unclassified subtypes represented a higher percentage of cases with histological grade III (64.3% & 33%) respectively.

LV emboli and lymph nodes

The highest incidence of LVE was observed in the LUM B group (70%) followed by basal like group (64.3%) and the lowest was seen in the LUM A group (5.3%). 18/56 (32%) cases had no metastases to axillary lymph nodes out of which majority (14/18) of cases were in LUM A subtype. All cases in LUM B, Basal and Unspecified groups were positive for lymph node metastasis. The correlation between no of positive lymph nodes and

molecular subtype was found to be statistically significant ($p = 0.0004$).

ER status

ER expression was positive in 28 (50%) cases (Fig. 1), as one case LUM B subtype showed positivity only for PR which was grade II. 6/28 cases (21.4%) were Grade-I tumors, 18/28 (64.3%) Grade-II tumors and 4/28 (14.3%) were Grade-III tumors. The correlation between ER status and grade was found to be statistically significant ($p < 0.05$).

Her2/neu status

There were (20/56) Her2/neu positive cases (Fig. 2), comprising 7/56, 12/56 and 1/56 tumors of histological grade I, II and III respectively. The correlation between Her2/neu status and grade was found to be statistically significant ($p = 0.0001$).

Classification of cases based on cytokeratin expression

All cases (100%) were positive for the luminal CK 8/18. In addition, 23% (13/56) of these tumors showed positive expression for CK 5/6. The staining intensity of the CK 5/6 was moderate to strong in eleven cases of IDC-NOS and in one case each of metaplastic carcinoma and lobular carcinoma.

Comparison between TN (ER–/PR–/Her2/neu–) cases with respect to basal marker expression

Among the TN group, basal like subtype 64% (9/14) of tumors were of histological Grade-III; whereas only 33% tumors were Grade-III in the TN group without basal marker expression. Only 1/3 case in unclassified/TN non-basal showed LVE while 9/14 cases in basal like had LVE. All cases of the basal like subtype were large in size (>2 cm) while 33% (1/3) cases in unclassified/PN/non-basal group was smaller than 2 cm.

Table 3 – Clinicopathological features of breast tumors according to immunoexpression of hormonal receptors/Her2/CK 5/6/EGFR expression.

		LUM A (19/56)	LUM B (10/56)	Her2 neu (10/56)	Basal (14/56)	Unclassified (3/56)
Tumor size (cm)	<2	4 (21%)	2 (20%)	1 (10%)	–	1 (33.3%)
	2–5	12 (63.2%)	7 (70%)	8 (80%)	3 (21.4%)	1 (33.3%)
	>5	3 (15.8%)	1 (10%)	1 (10%)	11 (78.6%)	1 (33.3%)
Tumor grade	I	1 (5.3%)	5 (50%)	2 (20%)	–	–
	II	14 (73.7%)	5 (50%)	7 (70%)	5 (35.7%)	2 (66.7%)
	III	4 (21%)	–	1 (10%)	9 (64.3%)	1 (33.3%)
Lymph nodes	0	14 (73.7%)	–	4 (40%)	–	–
	<4	2 (10.5%)	3 (30%)	1 (10%)	3 (21.4%)	1 (33.3%)
	4–9	3 (15.8%)	5 (50%)	5 (50%)	9 (64.3%)	2 (66.7%)
LV emboli	≥10	–	3 (30%)	–	1 (14.3%)	–
	Positive	1 (5.3%)	7 (70%)	3 (30%)	9 (64.3%)	1 (33.3%)
	Negative	18 (94.7%)	3 (30%)	7 (70%)	5 (35.7%)	2 (66.7%)
CK 5/6	Positive	0 (0%)	0 (0%)	1 (10%)	12 (85.7%)	0 (0%)
Expression	Negative	19 (100%)	10 (100%)	9 (90%)	2 (14.3%)	3 (100%)
EGFR	Positive	0 (0%)	0 (0%)	1 (10%)	6 (42%)	0 (0%)
Expression	Negative	19 (100%)	10 (100%)	9 (90%)	8 (58%)	3 (100%)

Fig. 1 – Microphotograph showing strong immunopositivity for ER (Score-3).

Fig. 2 – Microphotograph showing strong membrane immunopositivity for Her2neu (Score-3).

Combined classification (ER/Her2/neu, CK 5/6 and/or EGFR (Her1+) expression

On the basis of ER/PR/Her2neu/ EGFR/CK5/6 expression, the cases were categorized into five subgroups: LUM A – 34% (19/56), LUM B – 17.8% (10/56), Her2neu overexpressing – 17.8% (10/56), Basal like – 25% (14/56) and Unspecified – 5.4% (3/56) (Fig. 3). The combined classification was found to be most suited to the luminal A and luminal B cases, as the number of cases with luminal phenotype was the same by both ER/Her2/neu and CK classification systems. Of the 17 TN cases (ER/PR/Her2/neu negative), fourteen cases (82.3%) were also positive for basal markers (CK 5/6 and/or EGFR) while rest three (17.7%) were negative for all five markers (unclassified subtype). One case with CK 5/6 expression was also Her2/neu overexpressing. However, the others (19/20) Her2/neu positive (ER/Her2/neu classification) did not express any basal markers. The basal markers showed positive correlations with higher histological grade and ER negative status. Morphological characteristics in

Fig. 3 – Distribution of cases as per molecular subtypes.

Basal-like carcinomas showed high histological grade, high mitotic activity, larger tumor size and prominent lymphocytic infiltrate. Comparison between TN (ER–/PR–/Her2/neu–) cases with respect to basal marker expression – Among the TN basal group, 64.3% of tumors were of histological grade III and all of them expressed basal markers. Other 35.7% tumors were grade II in the TN group and 40% of them were without basal marker expression. All TN cases were positive for axillary lymph node metastasis, irrespective of basal marker expression but only 9/14 cases showed positive for LV emboli and rest 5/14 (35.7%) cases did not show LV emboli. All of the TN tumors were large in size (>2 cm).

Discussion

Breast cancer is a clinically heterogeneous disease with varied clinical profile. Existing histological classifications may not fully correlate the clinical behavior of this disease. ER, PR, Her2neu, EGFR and basal marker expression status i.e. molecular subtypes of carcinoma breast possibly predict/influence the prognosis and response to hormonal and targeted therapies. DNA microarray technology was originally being employed for molecular sub typing, however, at present IHC is preferred method and successfully applied to routinely processed formalin fixed tissue. In this study, we categorized the breast carcinoma cases into five molecular subgroups on the basis of IHC analysis using ER, PR, Her2neu, EGFR and CK 5/6 markers. Out of total 56 breast carcinoma cases – Luminal A subtype –34%, Luminal B and Her2/neu-18% each while, Basal like (TN basal) and Unclassified/PN (TN non-basal) subtypes were 25% and 5% respectively. In a recent study on 163 breast carcinoma cases by Fernandes et al reported the prevalence in his series⁸ as Basal like 29.1%, Her2/neu 16.4% cases, Luminal A 35% and Luminal B 19.4%. Our results also showed a distribution of breast cancer subtypes similar to what was reported by Fernandes et al. In a study by Sorlie T breast cancer subtype with the ER positive and Her2 negative phenotype that corresponds to the luminal A was the most common, like in our series (34%).⁹ Carolina breast cancer study reported incidence

of Her2neu subgroup only 6.6%,⁵ while in our study Her2-neu subtypes was 18% similar to Fernandes et al 16.4%.⁸ ER was expressed in 50% (28/56) of our cases, slightly lower than the mean percentage reported in the literature (60%–70%). Over expression of the protein and/or amplification of the Her2 gene have been reported in approximately 20–30% of breast cancers, but in our study it was slightly higher i.e. 35.7% (20/56). 17/56 (30.3%) of the cases were of triple negative type, out of which 14/17 (82.4%) were basal like tumors. These results are similar to the results in the available literature.^{5,10} The studies^{11–13} have shown that high tumor aggressiveness and worse prognostic factors have an association with the basal like group. In our study, 64.3% cases of basal like group showed LVE and 100% lymph node positivity was observed in the Basal like, Luminal B and Unclassified subgroups (PN). Clinically, the Basal like subtype also has different metastatic patterns in addition to poor clinical outcome, genetic predisposition (BRCA1/2 mutation) and high prevalence of p53 mutation.¹⁴ Patients from this group may benefit from EGFR (Her1) targeted therapy.¹⁵ In this study, along with the prevalence of different molecular subgroups, we established the correlations between clinico-pathological characteristics. Patients analyzed in present study were younger than in western series; the average age at the time of diagnosis was 50.5 years and patients below 35 years were 7%. However, in the European population only 2.7% were below 35 years of age.¹⁶ In terms of morphological grading, the groups differed significantly in terms of tumor grade ($p = 0.001$). The maximum numbers of cases with grade III morphology were TN (64.3%) and least was in Her2neu group (10%). Correlation was also noted in relation to clinical staging and five subtypes. Only 8.9% patients were diagnosed at stage I, while 41.1% were at stage II, 42.9% at stage III and 7.1% at stage IV. Most of stage I cases were ER+ (67%). In TN group all the cases presented in higher stage i.e. stage II and/or higher. In regards to tumor size, significant difference was found among subtypes ($p = 0.004$). Analysis revealed that overall average tumor size in study group was 3.4 cm and in 85.7% (48/56) of cases tumor measured more than 2 cm. In addition, Basal like tumors had the greatest percentage (100%) of large tumors (>2.0 cm) and had the highest incidence of metastatic deposits. 67.9% (38/56) of patients had positive lymph nodes on presentation in our study. This could be due to late consultation during the progression of the disease, lack of screening mammography program and women's awareness trainings particularly in peripheral area. The predominant histopathological type in this study was invasive ductal cancer (89%), as shown in most of the studies on breast cancer worldwide.

Conclusion

Immunohistochemistry can be easily and widely used to molecular subclassify breast tumors. In this study of patients from a tertiary care hospital of armed forces, these subclasses have shown significant differences in relation to tumor size, histological grade, lymph node positivity and LVE. Identification of Basal like breast cancer, a highly aggressive, biologically and clinically distinct subtype different than its non-basal variant, is important for treatment planning and target

therapy. Hence justify the use of basal markers (CK 5/6 and EGFR) in addition to routinely used estrogen receptor, progesterone receptor, and HER2/neu to subclassify breast cancer.

Conflicts of interest

All authors have none to declare.

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