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Original Article

Double-positive in triple-negative? How significant is basal cytokeratin expression in breast cancer?

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ARTICLE INFO

Article history:

Received 8 July 2018

Accepted 30 October 2018

Available online 26 February 2019

Keywords:

Breast carcinoma

Basal phenotype

Triple negative

Immunohistochemistry

Cytokeratins 5/6 and 14

ABSTRACT

Background: Basal-like breast cancer has an unfavorable prognosis. Immunohistochemically, they are predominantly estrogen receptor (ER), progesterone receptor (PR) and CerbB2 receptor (HER2)-negative, show expression of Cytokeratins (CKs) 5/6, CK14, CK 17 and P-cadherin and are associated with germline BRCA1 mutations. Immunohistochemistry (IHC) is an easily available and relatively inexpensive technique that can detect this cancer subtype, and patients can benefit from aggressive management protocols. The aim of this study was to evaluate the expression of CK 5/6 and CK14 in breast cancer and its correlation with age, tumor grade, tumor size, histomorphological pattern, nodal status, ER, PR, HER2/neu, and Ki-67 index.

Methods: Fifty treatment-naïve patients of breast carcinoma who underwent surgery constituted the study group. No core cut biopsy specimens were considered. Histopathological examination along with IHC was performed for CK5/6, CK14, ER, PR, HER2/neu, and Ki-67. Comparison between the expression of CK5/6 and CK14 with age, tumor size, tumor grade, histological subtype, nodal status, ER, PR, HER2/neu, and Ki-67 expression was performed using SPSS 20 version software.

Results: Twenty-six percent of cases showed expression of CK5/6 and CK14. CK5/6 and CK14 expression correlated strongly with ER/PR negativity, young age, and Ki-67 proliferative index greater than 15%. No significant association with HER2/neu negativity was demonstrated. Contrasting results were obtained between CK5/6 and CK14 expression with respect to tumor grade and lymph node status.

Conclusion: IHC can be used to identify patients with basal phenotype breast cancer with good sensitivity and specificity, and such patients can benefit from aggressive management.

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Introduction

Breast cancer is a heterogeneous disease with divergent clinical and prognostic implications. Microarray- and immunohistochemistry (IHC)-based techniques have been used to classify breast cancer into several subgroups based on expression of various genes and receptors that determine the treatment protocol, risk of recurrence, distant metastasis, and period of disease-free survival (Table 1).^{1–8} In addition to conventional hormonal receptor assay, proliferation markers such as Ki-67 are also used as supplementary markers to predict the disease outcome and rationalize treatment protocols. Ki-67 is a nuclear antigen which is expressed in all phases of cell cycle except the G₀ phase. A Ki-67 percentage score of 15% or more is generally considered significant as per the current national and international consensus.^{9–13}

Basal-like is a distinct subtype of breast cancer characterized by expression of genes which are usually found in basal/myoepithelial cells of normal breast. These include high-molecular-weight cytokeratins (CKs) 5/6, 14, 17) and P-cadherin. Morphologically, this subtype is of high grade and often shows medullary-like features and metaplastic differentiation. Immunohistochemically, basal-like subtype is predominantly estrogen receptor (ER), progesterone receptor (PR), and C-erb B2 receptor (HER2) negative and is often associated with germline BRCA1 (breast cancer 1, early onset) mutations. It usually expresses a Ki-67 percentage score of 15% or more, resulting in an aggressive course often with brain and lung metastases.^{1–8,14–23} Therefore, it is important to identify breast cancer patients with a basal phenotype who can benefit from more aggressive chemotherapy and surgery.

However, microarray-based studies cannot be readily applied to formalin-fixed, paraffin-embedded tissues. They are also relatively expensive and require a high level of expertise. IHC in comparison is a more readily available technique and can be performed on formalin-fixed, paraffin-embedded tissues.

This study was undertaken with an aim to evaluate and compare the expression of basal cytokeratins namely CK5/6 and 14 by IHC in carcinoma breast patients and study its correlation with age, tumor grade, tumor size, histomorphological pattern, nodal status, ER, PR, HER2/neu, and Ki-67 expression.

Materials and methods

This descriptive and analytical study was carried out at a tertiary care referral hospital with a malignant disease treatment center. A total of 50 patients of breast carcinoma who underwent modified radical mastectomy (MRM) and breast conservation surgery (BCS) constituted the study group. Specimens that were received retrospectively and prospectively between January 2016 and August 2017 were considered for the study. No core biopsy specimens were included in the study. All patients were treatment-naïve. A pilot study comprising five patients was carried out for understanding the intricacies and limitations of immunostaining procedures. Prior approval for the study was taken from the institutional ethical committee.

Table 1 – Immunophenotype & histomorphology.

Immunophenotype & histomorphology	Luminal A	Luminal B		HER2-enriched	Triple-negative	
		HER2 negative	HER2 positive		Basal-like	Non-classified
ER	++	+	+	–	–	–
PR	++	+	–/low	–	–	–
HER2/neu	–	+	+	+	–	–
CK5/6, CK14	–	–	–	–	+	–
EGFR	–	–	–	–	+	–
Ki-67	<14%	>14%	Any	Any	Any	Any
Morphological subtype	Well-differentiated/tubular/classical Lobular/mucinous	IDS, NOS (less well differentiated)	Grade 2/3 with nodal metastasis and early relapse	IDS, NOS, Pleomorphic Lobular carcinoma	High grade, metaplastic/medullary carcinoma with locoregional/distant metastasis, few secretory and adenoid cystic carcinoma with characteristic molecular changes	
CK, cytokeratin.						

Hematoxylin- and eosin-stained slides were prepared from paraffin blocks of the breast tissue specimen. Representative sections from the tumor were subjected to IHC using monoclonal antibodies to CKs 5/6 and 14, ER, PR, HER2/neu, and Ki-67.

The observations recorded are as follows:

1. Age of the patients.
2. Pathological observations:
 - a. Gross appearance: tumor size
 - b. Microscopic study which included grade of disease as per Nottingham modification of the Bloom–Richardson grading system. Lymph node status was scored as per the TNM staging system.

IHC studies were performed using a Dako Cytomation EnVision + Dual Link System-polymer HRP (horseradish peroxidase) IHC detection system (Dako Cytomation Inc, Denmark). The EnVision+ Dual-Link System-polymer HRP system is based on an HRP-labeled polymer which is conjugated with secondary antibodies. The labeled polymer does not contain avidin or biotin. Hence, non-specific staining resulting from endogenous avidin–biotin activity is significantly reduced. This system is an extremely sensitive method and offers an enhanced signal-generating system for the detection of antigens present in low concentrations. To study the basal phenotype expression, monoclonal antibodies to CK5/6 (CK, rabbit monoclonal antihuman cytokeratin; BioGenex, USA) and CK14 (CK, monoclonal mouse antihuman cytokeratin; BioGenex, USA) were used. Similarly, monoclonal antibodies against ER (monoclonal mouse Antihuman Estrogen receptor; clone 1D5, RTU, Dako Cytomation Inc,

Denmark), PR (Monoclonal mouse Antihuman Progesterone receptor; Clone PgR 636, RTU, Dako Cytomation Inc, Denmark), HER2/neu (polyclonal rabbit antihuman c-erbB-2 oncoprotein; Dako Cytomation Inc, Denmark), and Ki-67 (cell marque Ki-67 (sp6) rabbit monoclonal antibody REF275R18) were used to determine the expression of ER, PR, HER2/neu, and Ki-67, respectively.

For each run, a positive and negative control slide was also prepared. Benign prostatic tissue served as the positive control for CK5/6. For CK14, skin tissue was selected as the positive control.

In case of ER and PR, previously reported confirmed cases of the carcinoma breast that showed strong nuclear staining pattern for ER and PR (percentage score ≥ 4 , intensity score = 3) were taken as the positive control. In addition, the normal, uninvolved breast parenchyma included in the section served as the positive internal control (Fig. 1a&b).

In case of HER2/neu, previously reported confirmed cases of the carcinoma breast that showed strong complete, basolateral or lateral membranous reactivity in $>30\%$ of the tumor cells (score 3+) were taken as positive controls. As negative control for each run, the primary antibody was omitted (Fig. 1c).

The Ki-67 score is defined as the percentage of malignant cells showing nuclear staining with the Ki-67 antibody. Owing to the heterogeneous nature of Ki-67 staining among tumor specimens, scoring is usually carried out at tumor edges and hotspots. The advancing edge of the tumor is generally considered as the most mitotically active part of the tumor. At least three fields were selected at the tumor periphery and examined under a high power objective. A hotspot is an area in a stained slide where Ki-67 staining is particularly

Fig. 1 – Immunohistochemistry images showing strong nuclear positivity for (a) ER; (b) PR in $>67\%$ of tumor cells, Allred score 8 (40 $\times$); (c) membranous positivity for HER2neu, score 3+ (40 $\times$); and (d) nuclear positivity for Ki-67 in tumor cells. ER, estrogen receptor; PR, progesterone receptor.

prevalent. A total of 500 cells were counted in each field. Then, the percentage of Ki-67 positive cells was calculated. Tumors with >15% positive nuclei were classified as having a high expression. Mitotic figures, normal duct, lymphocytes, endothelial, and stromal cells served as an internal control.^{9–13} (Fig. 1d).

A scoring system for reporting the sections stained for CK5/6 and CK14 was formulated based on previous studies by Nielsen et al¹⁴ and Mohammadizadeh F et al.¹⁵ Tumors were classified based on the intensity of staining and percentage of cells showing basal CK expression.

The percentage of cells showing CK5/6 and CK14 expression is as follows: 0, 0%; 1, upto 10%; 2, >10%.

Scoring of staining intensity (cytoplasmic) is given as follows: 0, no staining of the cells; 1, weak staining of the cells; 2, moderate/strong staining of the cells.

These were then multiplied and combined into a final score given as follows: 0 (negative): ≤ 1 , 1 (positive): ≥ 2 [Figs. 2(a & b) and 3(a & b)].

Expression of ER and PR was scored as per the Allred scoring system for ER and PR evaluation.

Scoring of HER2/neu expression was carried out as per the College of American Pathologists protocol.

Statistical analysis

The above observations were analyzed as follows:

1. Expression of basal CKs namely CK5/6 and CK14 and their relationship with conventional prognostic markers such as age, tumor grade, tumor size, histomorphological pattern, nodal status, ER, PR, HER2/neu, and Ki-67 expression.
2. Patient's follow-up, disease-free, and overall survival data were not documented.

Comparison between the expression of basal CKs with age, tumor size, tumor grade, nodal status, ER, PR, HER2/neu, and Ki-67 expression was performed using SPSS 20 version software.

Categorical variables are expressed as 'number of patients' and 'percentage of patients'. CK5/6 positivity and CK14

Fig. 2 – IHC images showing (a) strong diffuse cytoplasmic positivity for CK5/6, IHC score 4 (40 $\times$) and (b) focal positivity for CK5/6 with moderate staining intensity, IHC score 2 (40 $\times$). IHC, immunohistochemistry; CK, cytokeratin.

Fig. 3 – IHC images showing (a) strong diffuse cytoplasmic positivity for CK14, IHC score 4 (40 $\times$) and (b) focal positivity for CK14 with moderate staining intensity, IHC score 2 (40 $\times$). IHC, immunohistochemistry; CK, cytokeratin.

positivity have been compared across the groups using Pearson's Chi Square test for independence of attributes. An alpha level of 5% has been taken, i.e., if any P value is less than 0.05, it has been considered significant.

Results

A total of 50 cases of breast carcinoma on whom MRM/BCS had been performed constituted the study group. The patients ranged in age from 30 to 71 years. Patient demography and association with basal CK expression is depicted in Table 2.

Distribution of histological types, tumor grade, tumor size, and lymph node status along with association with basal CK expression among patients were recorded, and findings are as demonstrated in Table 3.

Immunohistochemical subtyping of the 50 cases under study and statistical correlation of the triple-negative cases with basal CK expression are demonstrated in Table 4.

Expression of ER, PR, and HER2/neu along with association with basal CK (CK5/6 and CK14) expression were noted as depicted in Table 5.

The expression of CK5/6 correlated significantly with a young age of presentation, high tumor grade, and positive node status. In the age group 30–50 years, 47.37% of patients showed CK5/6 expression, whereas only 12.9% of patients in the age group >50 years were positive for CK5/6 expression. Fifty percent of patients with grade III disease showed expression of CK5/6, whereas 0% of patients with grade I disease showed expression of CK5/6. Of node positive cases, 33.33% were positive for CK5/6 whereas 0% of node negative cases were positive for CK5/6.

Similarly, the expression of CK14 correlated significantly with age. In the age group 30–50 years, 36.84% of patients were positive for CK14 expression whereas only 9.68% of patients in the age group >50 years were positive for CK14 expression. However, in contrast to CK5/6 expression, it did not correlate significantly with tumor grade or lymph node status.

In the expression of basal CKs, CK5/6 and CK14 were found to correlate significantly with ER and PR negativity. In the ER negative cases, 60% and 45% were positive for CK5/6 and CK14, respectively, whereas 39.3% and 30.3% of PR negative cases showed positivity for CK5/6 and CK14, respectively. In contrast, only 3.03% of ER positive tumors were positive for CK5/6 and CK14 expression. None of the cases which were PR

Table 2 – Patient demographics and association with CK5/6 and CK14 expression.

Age of patients (in years)	30–50	>50
Total number of patients	19	31
Number and % of patients positive for CK5/6	9 (47.37%)	4 (12.9%)
Association between age and CK5/6	Yes (p<0.05)	No (p> 0.05)
Number and % of patients positive for CK14	7 (36.84%)	3 (9.68%)
Association between age and CK14	Yes (p<0.05)	No (p>0.05)

CK, cytokeratin.

Table 3 – Distribution of patients with statistical association.

Distribution of patients with statistical association	Tumor size			Node status		Histological types				Histological grades		
	T1	T2	T3	Positive	Negative	IDC, NOS	ILC	Med	Muc	I	II	III
Total number of patients	4	35	11	39	11	45	3	1	1	3	31	16
Number and % of patients positive for CK5/6	0	9	4	13	0	12	0	1	0	0	5	8
Association between histomorphology and CK5/6	0%	25.7%	36.6%	33.3%	0%	26.6%	0%	100%	0%	0%	16.1%	50%
Number and % of patients positive for CK14	0	No	No	Yes (p < 0.05)	No	No	No	No	No	No	No	Yes (p < 0.05)
Association between histomorphology and CK14	0%	9	1	10	0	9	0	1	0	0	5	5
	0%	25.7%	9.09%	25.64%	0%	20%	0%	100%	0%	0%	16.1%	31.2%
	No	No	No	No	No	No	No	No	No	No	No	No

CK, cytokeratin; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; Med, medullary carcinoma; Muc, mucinous carcinoma.

Table 4 – Distribution of 50 patients based on immunohistochemical profile.

Non-triple negative					Triple negative
Subtype	Luminal A	Luminal B		HER2-enriched	
		HER2 (-ve)	HER2 (+ve)		
Total no. of patients	17	10	3	4	16
No. of patients positive for CK5/6	0	0	1	0	12
No. of patients positive for CK14	0	0	1	0	09
No. of cases positive for both CK5/6 and CK14	0	0	1	0	09
CK, cytokeratin.					

CK, cytokeratin.

positive showed expression of basal CKs. Another significant finding was the expression of high Ki-67 scores in cases which stained positive for basal CKs. For CK5/6 and CK14, 76.9% and 60% of cases which were positive, respectively, showed a Ki-67 score greater than 15% with a p-value <0.05.

The presence of CK5/6 and CK14 immunoreactivity showed no correlation with tumor size and histologic type. Significantly, no statistically significant association could be demonstrated between the expression of basal CKs and HER2/neu expression.

These findings further reinforce the fact that a cocktail of basal CKs may be more helpful in identification and study of the basal phenotype expression in breast carcinoma cases because of variability and heterogeneity in the staining pattern of individual antibodies.

Discussion

Breast carcinoma is a very diverse disease with variable treatment protocols. By using gene expression profiling, Sørlie et al^{1,2} subclassified breast carcinoma into five different groups, namely, luminal A and B, HER2-positive, basal-like, and so-called normal breast-like tumors. However, gene profiling using microarray-based techniques is an expensive and time-consuming process requiring a high level of expertise and remains unavailable in most centers even today. IHC, in comparison, is a more readily available and relatively inexpensive technique. Several studies have been carried out to investigate the utility of IHC surrogates as a substitute for molecular classification of breast cancer. Based on current recommendations, the immunohistochemical surrogates used for subtyping breast cancer are luminal A and B, HER2-enriched, and triple-negative breast cancer (TNBC). Seminal

studies by Lehmann et al^{8,19,20} have further subtyped TNBC into basal-like (BL 1 and 2), an immunomodulatory type, mesenchymal (M), mesenchymal stem cell-like and luminal androgen receptor subtype. Of these subgroups, BL generated considerable interest among researchers because of their significantly decreased relapse-free survival and poor clinical outcome. Furthermore, there was a strong association with the presence of BRCA1 mutations in patients harboring these 'basal-like' breast carcinomas.^{3–8,19–25}

Despite the existence of numerous similarities between the BL and TNBC, current consensus defines BL as a subtype of breast carcinoma distinct from TNBC. Only 71% of TNBC were found to have a basal phenotype on gene profiling studies whilewhereas only 77% of BLBC exhibited a triple-negative phenotype. In our study, around 75% of the TNBC cases were positive for CK5/6 and CK14, thereby exhibiting significant association with basal immunophenotype (p value < 0.05). However, the rest were found to be negative for expression of basal CKs. This reinforces the fact that although all BLBC are predominantly triple-negative, not all TNBC are basal-type. Molecular profiling, hence, may selectively be applied to identify these non-basal TNBC cases as their prognosis, and treatment regimes may vary considerably from the basal-like cases.^{16,17,19–26} However, basal CK expression is often described as an adverse prognostic factor independent of tumor size, grade, and lymph node status. In seminal studies by Rijn et al²¹ and Perou et al^{1,2}, 16%–18% of the breast cancer cases were positive for CK5/6 or CK 17, and basal CK expression was associated with a poor prognosis.

In the present study, 26% of breast carcinoma cases included in the study showed a basal phenotype. This further reinforces the fact that expression of basal CKs is not an unusual phenomenon in Indian women, and inclusion of basal CKs in current standard IHC protocols for breast cancer cases

Table 5 – Distribution of patients with statistical association.

Distribution of patients with statistical association	ER+ve	ER–ve	PR+ve	PR–ve	HER2/neu			Triple Negative (ER–ve, PR–ve, HER2/neu–ve)
					0/1 +	2 +	3 +	
Total no. of patients	30	20	17	33	43	3	4	16
Patients positive for CK5/6	1	12	0	13	12	1	0	12
Association between CK5/6 and hormone receptor status	No	Yes (p < 0.05)	No	Yes (p < 0.05)	No	No	No	Yes (p < 0.05)
Patients positive for CK14	1	09	0	10	9	1	0	09
Association between CK14 and hormone receptor status	No	Yes (p < 0.05)	No	Yes (p < 0.05)	No	No	No	Yes (<0.05)

CK, cytokeratin; ER, estrogen receptor; PR, progesterone receptor.

may help oncologists to identify patients for more aggressive treatment regimes.

However, no significant association could be documented between CK5/6 and CK14 immunoreactivity and tumor size, histologic type, and HER2/neu expression.

In contrast, the expression of CK5/6 and CK14 was found to correlate significantly with age. In the age group 30–50 years, 47.37% and 36.84% of patients showed CK5/6 and CK14 expression. The fact that basal CK expression correlated with an early age of diagnosis is in agreement with the results brought out by Laakso et al.²², Dent et al.²³, and in more recent studies by Hahnen et al.²⁴ and Zhang et al.²⁵

Similarly, the expression of CK5/6 showed a strong correlation with high tumor grade and positive node status. This is in line with the earlier results published by Rijn et al.²¹, Dent et al.²³, and Boyle.¹⁶ However, few studies by Kim et al.²⁷ and Gangi et al.²⁸ had failed to find any association between basal-like phenotype expression in breast carcinoma patients and adverse outcomes such as decreased relapse free survival, incidence of locoregional, and distant metastasis.

In our study, patient follow-up and response to treatment could not be assessed as being a service hospital; with frequent transfers of nearest of kin, patients often request for a referral and follow-up at other hospitals close to the place of posting of their near and dear ones. So, a larger study which includes these parameters may be needed to corroborate the association between the basal phenotype expression and adverse outcome of disease process.

In contrast to CK5/6 expression, CK14 expression did not correlate significantly with tumor grade or lymph node status. This fact underlines the highly variable and heterogenous staining pattern of these antibodies. This is supported by a study by Rakha et al.²⁹ in which they demonstrated that two basal markers did not exactly identify the same tumors when used alone, and that the associations with overall prognosis were different between tumors defined by CK14, CK5/6, or both CK14 and CK5/6 expression. Consistent with this observation, similar findings with CK5/6 and CK14 and CK5 and CK17 have been reported by Laakso et al.²² and Rijn et al.²¹

The expression of CK5/6 and CK14 correlated significantly with ER and PR negativity of breast carcinoma cases and high Ki-67 proliferative index. This is in line with the results of earlier studies published by Perou et al.¹, Sørli et al.², Fulawka et al.¹¹, Inwald et al.¹³, Nielsen et al.¹⁴, Yue et al.¹⁷ However, no definite correlation could be established between CK5/6 and CK14 expression and breast carcinoma cases which were HER2/neu negative (86% of the cases) and HER2/neu positive cases (14% of cases). This is in line with a previous study by Mohammadizadeh F et al.¹⁵ However, in contrast, Rakha et al.²⁹ and Nielsen et al.¹⁴ in their studies found significant association between basal phenotype expression and HER2/neu negative cases of breast carcinoma. This suggests that a larger study which includes greater number of breast carcinoma cases may be helpful to arrive at a definite conclusion between basal phenotype expression and HER2/neu negative cases.

CK5/6 and 14 have been well accepted as reliable markers for diagnosing breast carcinoma with basal phenotype expression. In the present study, the frequency detected by these markers (26% of the cases) meant that basal phenotype expression is not a rare phenomenon in breast carcinoma patients in Indian

populace. It is well known that patients with breast carcinoma of high grade and ER and PR negativity are characterized by much worse prognosis; so one can probably conclude that analysis of basal phenotype expression of breast carcinoma may be helpful for prognostication and triage of patients with increased risk of recurrence. Moreover, owing to significant association of basal phenotype expression in BRCA 1 mutation associated breast carcinomas, first degree relatives of patients of breast carcinoma with basal phenotype expression may be placed under close observation, and regular checkups for early detection of breast carcinoma as tests for BRCA 1 gene mutation are very expensive and in a developing country such as India, may be well out of reach of a large chunk of the population.

To conclude, basal phenotype expression in breast carcinoma patients is associated with a poor prognosis with increased incidence of relapse and dissemination to the lungs and brain. IHC can be used to identify such cases with good sensitivity and excellent specificity by selecting tumors negative for ER, PR, and HER2/neu but positive for CK5/6 and CK14. However, specific correlation between HER2/neu negativity and basal phenotype expression could not be established through this study. This may be attributed to smaller sample size (50 cases) and also to the fact that only a small percentage of the cases included in this study were found to exhibit HER2/neu positivity. Thus, a larger study and correlation with disease-free survival is needed to corroborate the findings of an adverse outcome associated with expression of basal phenotype as already highlighted by researchers worldwide. The data accrued will continue to provide new insights in some instances or provide tools for improving diagnosis and prognostication in others and may even guide future therapies.

Ethics statement

The study was performed in a manner to conform with the Helsinki Declaration of 1975, as revised in 2000 and 2008 concerning the Human and Animal Rights and the authors followed the policy concerning informed consent.

Conflicts of interest

The authors have none to declare.

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