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Research Article

ROLE OF CYTOKERATIN BIOMARKERS IN BREAST CARCINOMA

RITU YADAV[1]*, RAJEEV SEN[2], PREETI CHAUHAN[1]

[1]Department of Genetics, M.D. University, Rohtak - 124 001, Haryana, India. [2]Department of Pathology, Pt. B.D. Sharma University of Health Science, Rohtak, Haryana, India. Email: ritu.genetics@rediffmail.com

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ABSTRACT

Objective: Breast cancer comprises different biological subtypes having varied spectrum of clinical and pathological features with different prognostic and therapeutic implications. This study aimed at the identification of patients on the basis of cancer biomarkers and various clinic-pathological parameters.

Methods: Fresh paraffin embedded tissue block sample of 350 patients of breast carcinoma was collected during 2011-2014, from the Pathology Department, Pt. B.D Sharma University of Heath Sciences Rohtak, Haryana and studied in detail to determine the correlation between hormone receptor status/cytokeratin (CK) expression along with clinic-pathological factors. The immunohistochemical assay of 350 patients of breast cancer was performed. Triple-positive (estrogen receptor [ER]+, progesterone receptor [PR]+, and human epidermal growth factor receptor [Her2]/neu+) and triple-negative (ER−, PR−, and Her2/neu−) breast cancer types were studied to identify the basal and luminal phenotypes on the basis of markers CK5, 14, and CK8/18 expression.

Results: The expression of CK5 and 14 was found to be significantly associated with tumor grade (p=0.001 and p=0.0001), tumor size (p=0.001), respectively. Whereas CK8/18 expression did not reveal any significant association with tumor grade, size, lymph node status, and histological type of breast carcinoma.

Conclusion: In conclusion, the prognostic and therapeutic value of research work would be examined and validated further on large number of samples.

Keywords: Breast carcinoma, Biomarkers, Clinic-pathological factors, Cytokeratin, Hormone receptor.

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INTRODUCTION

Breast carcinoma is the most diverse disease in women after cervical cancer. It is estimated that worldwide 50,800 women died in 2011 alone due to breast carcinoma [1]. Incidence rates vary greatly worldwide from 19.3/100,000 women in Eastern Africa to 89.7/100,000 women in Western Europe. In most of the developing regions, incidence rates are below 40/100,000 [2]. Breast carcinoma is a heterogeneous disease such that disease may have different prognoses and respond to therapy differently despite similarities in histological types, grade, stage, and difference in hormone receptors [3]. Genetic expression profile studies have found that breast carcinoma arises both in basal and luminal cells. These cells can be distinguished by their immunophenotype [4,5].

Previous studies on breast cancer classified the disease into different groups based on immunohistochemistry (IHC) profile of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (Her2/neu) expression, positive (+ve) and/or negative (−ve), which are: ER+PR+HER2+, ER+PR+HER2−, ER-PR-HER2+, ER-PR-HER2−, ER+PR-HER2+, ER+PR-HER2−, ER-PR+HER2+, and ER-PR+HER2−. Recent attention has been directed at triple-negative breast cancer (TNBC) (ER-PR-HER2−) and triple-positive breast cancer (TPBC) (ER+PR+HER2+). TNBC is a burning concept worldwide due to unresponsiveness toward effective clinical therapies in comparison to the other types of breast carcinoma. While TPBC provides the significant prognostic information which imparts guidance of response to targeted and proven therapy; for example, endocrine and trastuzumab therapy for tumors expressing ER/PR and Her2/neu [6]. Many studies have reported that tumors expressing triple-negative and triple-positive expression of ER, PR, and Her2/neu receptors were found in basal- and luminal-like breast carcinoma, respectively. Further, basal and luminal carcinomas were observed inconsistent association with expression of cytokeratin (CK)5, 14, and 8/18, respectively, in most of the studies [7,8].

CK is epithelia-specific intermediate filament proteins, which are expressed in a tissue-specific manner. CK tumor markers can accurately predict disease status before conventional methods and offer a simple, non-invasive, cheap, and reliable tool for more efficient management. CK also plays promising role in vaccination trial and in understanding cellular processes such as apoptosis, mitosis, cell cycle progression, and cell signaling, etc. [7-11]. The aim of this study was to analyze the CK5, 14, and 8/18 markers in breast tissue and to determine the correlation between hormone receptor status (ER, PR, and Her2/neu) along with clinic-pathological factors.

METHODS

Sample collection

Fresh paraffin embedded tissue block sample of 350 patients of breast carcinoma was collected during 2011-2014 and studied in detail. This study was designed for screening of patients by use of specific markers. All samples were taken after institutional ethical committee permissions and personal consent of the patients or guardians (Registration no. CBT-360/4.4.12).

Clinic pathological analysis

Age of diagnosis was categorized as <40, 40-49, 50-59, and ≥60 years. Histological assessment of tumor grade (low, intermediate, and high), tumor size (<2, 2-4.9, ≥5 cm), and lymph node status (positive or negative) were performed. The histological parameters of all cases were reviewed by a pathologist, and the histological grade was determined for each case according to the Bloom and Richardson Grading System [12].

IHC analysis and scoring

IHC analysis was done using a protocol which was based on the principle that detected antigens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological

tissues. IHC staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Tissue sections mounted on glass slides were collected. After deparaffinization in xylene, slides were rehydrated through the grades of alcohol. Endogenous peroxidase activity was blocked using 2% hydrogen peroxidase in methanol. Antigen retrieval was performed with heating the coated sections on glass slides in citrate buffer for 20 minutes. The mouse anti-ER and PR monoclonal antibodies, rabbit anti-ErbB-2/Her-2, rabbit anti-CK5, mouse anti-CK14, and 8/18 monoclonal antibodies (Biogenex, USA, CA) were used as primary antibody. Horseradish peroxidase polymer (Biogenex, USA, CA) was used as secondary antibody. The sections were first stained with diaminobenzidine and then using hematoxylin stain. The ER and PR results were screened manually and interpreted as positive or negative on the basis of scores for proportion as well as intensity.

The expression of ER and PR was scored between 0 and 8 - 0 (negative): No nuclei staining; 1 (borderline): 1% of nuclei staining; 2 (positive): 1-10% of nuclei staining; 3 (positive): 11-33%; 4 (positive): 34-66%; 5, 6, and 7 (positive): 100% of nuclei staining. Expression of HER2/neu scored 0-3 as follows: 0 (negative): No membranous staining identified, 1 (negative): Faint staining involving 10% of positive cells; 2 (positive): Weak but definitive staining of the membrane over at least 10% of positive cells; 3 (positive): Strong positive staining of the complete membrane in more than 20% of cells. Expression of CK5, 14, and 8/18 scored 0-2 as follows: 0 (negative): No cytoplasm and membrane staining identified, 1 (Weak): Faint staining involving up to 10% of positive cells; 2 (positive): Strong positive staining of the cytoplasm and membrane in >10% of positive cells.

Statistical analysis

Statistical analysis was performed using SPSS software package version 22.0 (IBM Corp., Armonk, NY, USA) and Microsoft Excel. Results were expressed in number and percentage. Chi-square test, Pearson correlation and regression were performed to find out relation of biomarker expression with different clinic-pathological factors age, tumor size, grade, and lymph node status. The level of statistical significance was set at $p<0.05$.

RESULTS

In the present study, IHC assay of three 150 patients of breast cancer was performed to determine the correlation between hormone receptor status and CK expression along with clinic-pathological factors. 78% of patients were grouped to determine the hormone receptor status and their relation with clinic-pathological factors.

The IHC evaluation of ER was performed, and ER was found to be positive in 74% patients. The negative expression is counted as no nuclear stain for all of three receptors (ER, PR and Her2/neu) (Fig. 1a). Maximum quick score showed by patients was counted in 2+ve (20.8%) and minimum score was counted in 4+ve and 5+ve (9.9%) (Fig. 1b). Progesterone receptor (PR) positive cases were found to be positive in 75.3% patients. Quick scores of PR were counted from 0 to 7+ve on the basis of nuclear stain intensity same as that of ER (Fig. 1c). Maximum frequency of PR positivity showed by patients was counted in 2+ve (22.3%) and minimum score was counted in 4+ve (8.8%). Expression of Her2/neu was counted from negative to 3+ve shown in Fig. 1d. 75% patients were found to be positive for the expression. Maximum frequency was counted as 2+ve (38.5%), whereas minimum score was counted as 1+ve (27.6%) in quick score. Patients with 3+ve quick score were found to be 33.9% patients.

The majority of patients were observed with triple-positive (43%) as compared to triple-negative phenotype. The frequency of ER-PR-Her2+ was observed second in subtyping (26%) and only a few patients (11%) were observed with other subtypes ER+PR-Her2−, ER-PR+Her2+, and ER-PR+Her2−. TPBC and TNBC types were studied to identify the basal and luminal phenotypes on the basis of markers CK5/14 and CK8/18 expression. The basal cells were stained with the antibodies CK5/6, whereas the luminal epithelial cells that were stained with the antibodies CK8/18 [5].

The IHC expression of CK biomarkers (CK5/14 and 8/18) of 172 patients was reported. The IHC score was used to identify basal- and luminal-like breast carcinoma based on positive expression of the CK (5, 14, and 8/18). The positive expression of CK5, 14, and 8/18 was observed 39%, 52%, and 68%, respectively, in patients with triple-positive receptor status (ER +PR+Her2+). In triple-negative cases (ER-PR-Her2−), positive response of CK5, 14, and 8/18 was recorded 59%, 54%, and 34%, respectively. Maximum positive response of CK5 and 14 was observed in triple-negative cases, whereas the majority of the positive response of CK8/18 was shown by the triple-positive phenotype of breast carcinoma (Fig. 2).

The expression of CK was found to be significantly associated with triple-negative and triple-positive phenotypes of breast cancer (Table 1). In this study, it was found that some patients displayed heterogeneous antibody expression of basal markers CK5 and 14 (15%), i.e., the majority of triple-negative patients showed positive expression of the basal-like marker CK5, but 10% patients did not express CK14 marker. On the other hand, 5% cases showed negative expression of CK5 and positive expression of CK14 markers. CK5 and CK14 were co-expressed in many tumors (85%). In CK8/18+ve case, 34% patients were found to be Luminal A type, and 66% of patients were reported with Luminal B type (Table 2).

In the present study, some patients were found with both of basal/luminal expression of CK markers. The majority of patients were found with biomarker panel CK5+ve, CK14+ve, CK8/18+ve (31%), and minimum (number of patients were found with biomarker panel

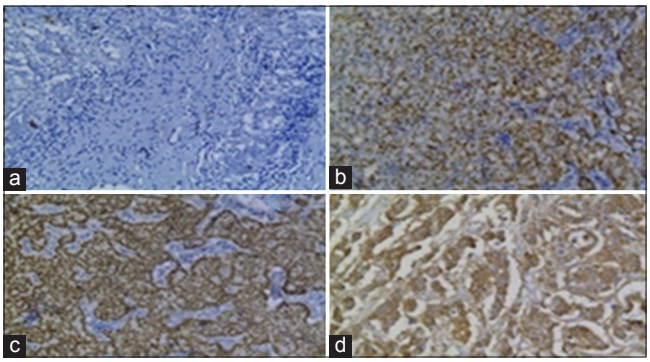

Fig. 1: Microscopic images of the pattern of immunohistochemistry ×100 staining in breast carcinoma. (a) Negative expression of receptors (estrogen receptor [ER], progesterone receptor [PR], and human epidermal growth factor receptor [Her2/neu]) (b) Nuclear stain of ER (c) Nuclear stain of PR (d) Cytoplasmic stains of Her2/neu (n=350)

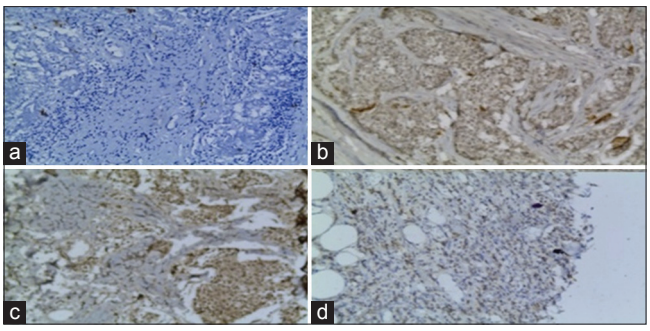

Fig. 2: Cytoplasm membrane stain of cytokeratin (CK)5, 14, and 8/18 (a=−ve, b and c=Cytoplasm membrane stain of CK5, 14 and d=cytoplasm stain of CK8/18) (n=172)

Table 1: Association of cytokeratin markers with triple-positive and triple-negative type of breast cancers

Breast cancer types	CK5+ve	CK5−ve	Significance	CK14+ve	CK14−ve	Significance	CK8/18+ve	CK8/18−ve	Significance
Triple-positive breast cancer type	39	61	*	52	48	NS	68	32	**
Triple-negative breast cancer type	59	41		54	46		34	66	

*Significance level, P<0.05 (χ^2 test); **Significance level, P<0.0001; CK: Cytokeratin. Data are given as percentage (n=172)

CK5−ve, CK14+ve, and CK8/18−ve (10%) (Table 2). The present study has established CK biomarkers 5 and 14 as basal-like and 8/18 as luminal type of breast carcinoma. In addition, a mixed type of breast carcinoma was also observed which may be possible due to the fact that luminal cells may express the genes which were shared with the basal-like and the Her-2 subtypes of breast carcinoma.

The positive expression of CK5 was found more in Grade I (45%) as compared to CK14 and 8/18 which were found more in Grade II (50%, 41%). The expression of CK5 and 14 found to be significantly associated with tumor grade (p=0.001 and p=0.0001), respectively. CK8/18 expression did not reveal any significant association with tumor grade (Table 3). The size of tumors ranged from 0.1 to 12 cm. The maximum patients with positive expression of CK5 and 14 were observed with tumor size >5 cm (47% and 50%, respectively), whereas the expression of CK8/18 was more observed in <2 cm (42%). A significant association was observed between CK5 expression and tumor size (p=0.001). However, no statistically significant association was found among expression of CK14, 8/18, and tumor size (Table 3).

The majority of the patients with <4 positive lymph nodes were found to have negative expressions of CK5, 14, and 8/18 (55%, 59%, and 60%). No statistically significant association was observed among expressions of CK5, 14, 8/18, and lymph node metastasis status (Table 3). Out of the two histological types, i.e., infiltrate ductal carcinoma (IDC) and lobular carcinoma (LC), the majority of the IDC cases were found to have positive expressions of CK5 and 8/18 (90% and 65%) and positive expression of CK14 (73%). However, in lobular histological type, the majority of cases were CK5 and 8/18+ve (40% and 60%), CK14−ve (32%) (Table 3).

Table 2: Classification of breast cancer into various immunohistochemical phenotypes (Basal, Luminal, and mixed types)

Subtype of breast cancer	Percent frequency of patients (%)
Basal-like	
CK5+ve, CK14+ve	85
CK5+ve, CK14−ve	10
CK5−ve, CK14+ve	5
Luminal-like: (CK8/18+ve)	
Luminal A (ER+PR+Her-2/neu−)	34
Luminal B (ER+PR+Her-2/neu+)	66
Mixed type	
CK5+ve, CK14+ve CK8/18+ve	31
CK5+ve, CK14−ve CK8/18+ve	26
CK5−ve, CK14+ve CK8/18+ve	14
CK5+ve, CK14−ve CK8/18−ve	19
CK5−ve, CK14+ve CK8/18−ve	10

n=172; CK, Cytokeratin. Data are given as percentage

DISCUSSION

Breast cancer is a heterogeneous disease and shows various subtypes such as Luminal A (ER+ and/or PR+, Her-2/neu−), Luminal B (ER+ and/or PR+, HER2+), basal-like (ER−, PR−, Her2−, CK5/6+, and/or Her2+), normal-like, and unclassified negative for all the markers [4]. The luminal tumors are suggested to group into two subtypes: Luminal A and Luminal B. The Luminal B tumors have a higher proliferation rate, and they were found to express the genes which were shared with the basal-like and the Her-2 subtypes and are associated with a less favorable outcome [5,13]. Some researchers described that a vast majority of the cases showed positive expression for CK8/18, thus indicating a differentiated glandular phenotype, a finding associated with a good prognosis and longer overall survival, as compared to those with a low or no expression of these markers [14]. CK8 was found to be associated with a better overall survival and as an independent prognostic indicator of the relapse-free survival [14].

There are many reports which combine the triple-negative phenotypes (ER−, PR−, and Her2/neu−) with basal CK (CK5/14) positivity [14,15]. In many studies, CK14 acts as a major partner of CK5 and both of these are associated with the basal phenotype [16,17]. The present results also showed that CK5 and CK14 are co-expressed in some tumors similar to other studies [16-19]. Many studies observed the statistically significant association between tumor grade and expression of CK5/14 but not for CK8/18 similar to the present results [15,16]. However, some studies observed that as the grade increased, the positive expression of CK8/18 also increased. In some studies, basal-like breast cancer was found in 24.6%, 25.7%, 27.2%, and 30.5% of IDC as compare to LC which was found to be similar to the present study [18-21].

A large tumor size was found to be associated with basal-like subtype of breast cancer in many studies similar to the present results [22]. Lymph node involvement is an interesting feature in both basal- and luminal-like subtypes of breast cancer. It has been reported that these cancers favor a hematogenous spread [20-22]. In another study, the rate of lymph node positivity was slightly higher in the basal group compared with the other cancer types [23]. The present study showed lower frequencies of invasion and lymph node metastasis in patients with a basal-like subtype of breast cancer compared with patients having a luminal-like breast cancer. These findings agree with previous reports which have shown that lower lymph node positivity was most described in basal-like subtype of breast cancer [23-25]. The highly metastatic cells were found to be associated with a loss of the CK8/18 expression similar to other studies [26-28].

CONCLUSION

CK5 and 14 as basal-like and 8/18 as luminal type of breast carcinoma may help in early identification and may be a valuable diagnostic tool in detecting breast cancer. In addition, a mixed type of breast carcinoma was also observed which may be possible due to the fact that luminal cells may express the genes which were shared with the basal-like and the Her-2 subtypes of breast carcinoma. These biomarkers do not help only in the classification of breast carcinoma (TNBC and TPBC) but also as a potential selection marker for chemotherapy. Moreover, it may offer the opportunity to develop new biomarkers by providing additional information supplementing currently available clinical and pathological tests and screening procedures. Further keeping in view of the above information validation studies are required on additional biomarkers for basal-like subtype, as basal-like breast cancer account for a high number of breast cancer-related deaths due to limited treatment options. The prognostic and therapeutic value of research work would be examined and validated further on a large number of samples.

Table 3: Association of CK5, CK14, and CK8/18 receptor expression with different clinic-pathological factors

Clinic-pathological factors	CK5+ve	CK5−ve	CK14+ve	CK14−ve	CK8/18+ve	CK8/18−ve
Tumor grade						
I	20	48*	18	46**	34	40
II	35	29	50	36	41	34
III	45	23	32	18	25	26
Tumor size						
<2 cm	23	18*	17	22	42	53
2-4.9 cm	30	58	33	35	34	27
>5 cm	47	24	50	43	24	20
Lymph node status						
<4 positive	43	55	58	59	44	60
>4 positive	57	45	42	41	56	40
Histological type						
IDC	90	60**	68	73	65	40*
Lobular	10	40	32	27	35	60

Significance level, *p<0.05; **p<0.0001 (χ^2 test). CK: Cytokeratin. Data are given as percentage; n=172

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