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Original Research Paper Pathology

Study of CK5/6 expression in invasive breast carcinomas and its utility as an independent prognostic marker.

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ABSTRACT

Background: *Breast carcinoma is currently the commonest invasive cancerin women. Subtyping of the breast carcinoma based on molecular phenotype has highlighted the enormous heterogenicity of this disease. Molecular classification of breast carcinoma commonly performed by immunohistochemistry has great impact on the prognostication and treatment strategy. CK5/6,a high molecular weight cytokeratin, is frequently used to categorize basal-like phenotype of breast carcinoma. It also identifies the tumours with poor prognosis. The aim of the present study was to examine the expression of CK5/6 in randomly selected invasive breast carcinoma cases and to study the correlation of CK5/6 expression and the clinicopathological prognostic markers with statistical analysis.*

Methods and materials: *All the cases of invasive breast carcinoma diagnosed by histopathology were prospectively collected over 2 years. The surgical specimen received were examined grossly and microscopically for histopathological tumour typing. Prognostic indicators like tumour size, presence of tumour necrosis, tumour staging and lymph node status were appropriately recorded.Immunohistochemistry for CK5/6 was performed on all the cases and graded. 0 and 1+ grade was considered as negative staining, 2+ and 3 + were considered as positive staining. The findings among CK5/6 positive cases and CK 5/6 negative cases were compared. The clinicopathological prognostic indicators were correlated statistically with CK5/6 positivity.*

Results: *CK5/6 expression was seen in relatively younger patients with larger tumour size. CK5/6 positivity is associated with tumour necrosis, relative higher tumour grade and higher occurrence of lymphnode metastasis. There was statistically positive association between tumour necrosis, lymphnode metastasis and tumour grading with CK 5/6 positivity.*

KEYWORDS :

Introduction:

Breast cancer is a diverse group of diseases with remarkable variation in the clinical presentation, morphology, molecular characteristics and clinical outcome [1]. It is the most common invasive cancer in women[2]. It is the leading cause of cancer deaths despite the screening and improvement in treatment protocols. Incidence of breast cancer has significantly increased in past years, the phenomenon partlyattributed to changing lifestyle [3]. Worldwide the occurrence of carcinoma of breast vary significantly. In India, as per recent ICMR statistics, breast cancer has surpassed cervical cancer and has been ranked the most common invasive cancer in women[4].

Breast cancer is traditionally classified based on the histological type of the tumour. The decision about the treatment and prognostication is largely based on certain clinicopathological parameters like tumour size, lymph node stage, histological type, tumourgrade, vascular invasion and patients age [1].The advent of gene expression profile (GEP) in breast carcinoma opened a new horizon of molecular classification of breast cancer. This classification offers better prediction of the prognosis and also hasledto individualisedpatient management. Molecular profiling of the breast cancer also throws light into the immense heterogenicity of the disease. Currently four main molecular classes of breast carcinoma are recognised namely, luminal, Her-2 enriched, basal-like and normal like. Amongst these, Basal-like is the most aggressive with poor prognosis and reduced survival[1,2]. It is resistant to existing molecularly-targeted treatment modalities [5].This variant of carcinoma metastasises via lymphaticsalong with a tendency forhaematogenous spread and visceral metastasis [1,6,7]. Though majority of basal-like breast carcinomas are negative for ER, PR and Her2, they are not synonymous with triple negative tumours [1].Cytokeratin(CK) 5/6 being basal markers, essentially identifies majority of the basal like tumours [8]. In addition, the GEP of CK5/6 positive breast carcinoma is said to be like that of BRCA1 mutation positive tumours. This warrants the screening of BRCA1 gene in patients having CK5/6 positive tumours [7,9].Precise identification ofpatients with basal-like phenotype is desirable for appropriate patient management.The aim of thisstudy was to examine theexpression of CK5/6 in randomly selected invasive breast carcinoma cases and to study the correlation of CK5/6 expression and the clinicopathological prognostic markers with statistical analysis

Materials and Method:

This is a prospective study carried out in a rural tertiary care hospital in south India between 2012 to 2014. Patients with his to pathological diagnosis of breast carcinoma were randomly selected for the study. Informed consent was obtained from each patient to use the breasttissues for the study. Also, institutional ethical committee clearance was obtained for the same. The specimen received were of modified radical mastectomy with axillary clearance. Each specimen was grossly examined by the pathologist for the location, size and extend of the tumour, presence of tumour necrosis, and number of axillary lymph nodes excised. Trimming of the specimen was done and representative areas from tumour were selected for making paraffin blocks. Four micron thick sections were taken using microtome and stained with Haematoxylin and Eosin (H&E) stain. The slides were examined by the pathologist for the following.

- Morphology of the tumor and histological type
- Presence of necrosis
- Grading of the tumour using Nottingham modification of The Scarf-Bloom-Richardson System by observing the mitotic activity, nuclear pleomorphism and extent of tumor differentiation.
- All slides of the lymph nodes sections were examined carefully for the presence of metastatic deposits.

IHC procedure

The best paraffin block with highest tumour content was chosen for immunohistochemical(IHC) studies. The Avidin Biotin Complex (ABC) detection system of IHC technique was used. Appropriately selected formalin- fixed paraffin embedded blocks of each casewere cut into sections of 3 - 4 micron thickness, then placed on positively charged slides & left over night to dry at room temperature. Sections were heated in a microwave oven for 30 minutes at 65 ^{0}C and then deparaffinized and rehydrated using xylene and decreasing grades of alcohol respectively. Antigen retrieving was done by placing the slides in a closed jar containing antigen retrieval solution comprising of EDTA buffer at pH 9.0. Endogenous peroxidase activity was blocked by adding peroxidase block to the slide and incubated at room temperature for 10 minutes. Primary antibody- Anti CK5/6 (BIOGENEX)6 ml, ready-To-Use was usedand incubated at 37 ^{0}C for 1 hour. This was followed by application of one to two drops of the biotinylated link (secondary antibody) with dilution of 1:20 and incubated at 37^{0} C for 30 minutes. Following this, Streptavidin – Peroxidase Reagent was applied and sections incubated at 37^{0}C for 30 minutes (dilution of 1:20) The slides were stained using cooled DAB- substrate chromogen solution incubated at room temperature for 20 minutes. followed by counterstainingit by immersing in a bath of Mayer's haematoxylin. After each step the slides were washed using buffer solutionand blotted. Finally, the slides were dehydrated using increasing grades of alcohol and cleared using xylene solution and mounted using DPX

Sections from basal cell carcinoma, well known to be positive for CK5/6 were used as positive control. Negative control was obtained by skipping the application of primary antibody to one slidein each set of immunostaining.

Immunoreactivity was regarded positive when dark brown, homogenous or punctatestaining was seen localized to the cytoplasm or membrane of tumour cells. The staining was graded as follows

0 : No immunostaining
1+ : Weak positive immunostaining (positive cells less than 10 %)
2+ : Positiveimmunostaining (positive cells 10 % to 50%)
3+ : Strongly positiveimmunostaining (positive cells > 50%)

The scores 0 and 1 were counted as negative and scores 2 and 3 were counted as positive cases. The findings of the study were recorded in excel sheet and statistical analysis was done using primer of biostatistics 7.0 pro. The findings among CK5/6 positive and CK 5/6 negative cases were compared. Age of the patient, tumour size, presence of necrosis, lymph node metastatic and tumour grade were correlated statistically with CK5/6 positivity.

Results:

Total of 70 cases were included in the study. All the patients were females between the age group 41 to 68 years with the mean age of 54.5 years. Infiltrating ductal carcinoma not otherwise specified (IDC-NOS) was the predominant histological type (85.71%) (figure1a).Other histopathological types were, 4 cases of medullary carcinoma, two cases each of mucinous carcinoma and papillary carcinoma, one case each of tubule-lobular and lobular carcinoma. CK5/6 was expressed in 28 cases(40%) and negative in 42cases (60%). Age range and mean age of presentation amongst CK 5/6 positive patients were 41-58 years and 51.7 years respectively, while that among the CK 5/6 negative cases were 44-68 years and 54.04 years respectively. The average tumour size was 5.5cm among CK5/6 positive cases and 4.5cm among CK5/6 negative cases. Tumour necrosis was maximum among the CK5/6 positive cases accounting to 57.14% cases whereas tumour necrosis was seen only in 19% of CK5/6 negative cases (figure1b).Axillary lymph node metastasis was in 50 % of CK5/6 positive cases and 19% of negative cases. Amongst CK5/6 positive patients, majority of the tumours were grade 2 (57.2%) followed by grade 3 (42.8%) with no tumours of grade1 category. Whereas grade 1 tumour was most common (38.09%) among the CK5/6 negative patients, followed by grade 2 (33.3%) and grade 3 (28.5%) tumours. Among 28 CK5/6 positive cases 16 had 3+ scoring(Figure 1c). Among 42 CK5/6 negative cases 30 showed absent staining with 0 scoring(figure1d). The findings have been summarised in table 1

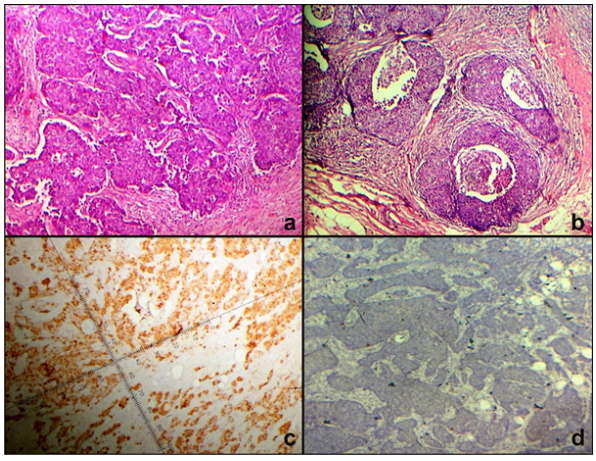

Figure1

Figure 1 a. photomicrography of IDC-NOS showing high grade tumour cells (H and E, x400) b. tumour cells exhibiting comedo necrosis (H and E, x400), c. CK5/6 immunostaining showing 3+ positivity (x100), d. CK 5/6 immunostaining showing negative reaction (X100)

Table 1: Clinicopathological findings

Clinicopathological Variables	CK5/6 positive n=28(40%)	CK5/6 negative n=42(60%)	Total cases n=70
Age (years)			
Mean Age	51.7	54.04	54.5
Histopathological Diagnosis			
IDC NOS	22 (79%)	38 (90%)	60 (86%)
Medullary Carcinoma	0	4 (10%)	4 (6%)
Mucinous Carcinoma	2 (7%)	0	2 (3%)
Papillary Carcinoma	2 (7%)	0	2 (3%)
Tubulolobular Carcinoma	1(3.5%))	0	1 (1.5%)
Lobular carcinoma	1(3.5%)	0	1(1.5%)
Tumor Size			
Average Size(cm)	5.5	4.5	5
Tumor Necrosis			
Present	16 (57%)	8 (19%)	24 (34%)
Absent	12 (43%)	34 (81%)	46 (66%)
LymphnodeInfilteration			
Present	14 (50%)	8 (19%)	22(31%)
Absent	14 (50%)	34 (81%)	48(69%)
Grade of tumor			
Grade I	0	16 (38%)	16 (23%)
Grade II	16 (57%)	14 (33%)	30 (43%)
GradeIII	12 (43%)	12 (29%)	24 (34%)

On statistical analysis, significant positive correlationwas found between the tumour necrosis, lymph node metastasis and the tumour grade with CK5/6 positivity. Correlation of patient age and tumour size with CK 5/6 positivity was not statistically significant(Table 2).

Table 2: correlation of clinicopathological prognostic markers with CK5/6 positivity.

Clininopathological Variables	CK5/6 positive n=28(40%)	CK5/6 negative n=42(60%)	P Value
Age of patient			
<=50 years	4	16	0.059
>50 years	24	26	

Tumor Size			
< 2cm	0	4	0.178
2 - 5 cm	12	20	
>5 cm	16	18	
Tumor Necrosis			
Present	16	8	0.002
Absent	12	34	
LymphnodeInfilteration			
Present	14	8	0.014
Absent	14	34	
Grade of tumor			
Grade I	0	16	0.027
Grade II	16	14	
GradeIII	12	12	

Discussion:

Cytokeratin (CK) is an intermediate filament protein, which reflects the epithelial cell type. It is used for the fingerprinting of various carcinomas in day to day practice by using IHC. CK5/6, a high molecular weight cytokeratin is frequently employed in the panel of markers used to differentiate benign and malignant lesions of the breast[7]. It is also the commonly used surrogate marker to identify basal phenotype inbreast carcinoma[3,8].The term basal in normal breast indicates myoepithelial cells which are basally located and a sub group of luminal cells which express high molecular weight cytokeratin [7]. In the normal resting breast tissue, the luminal cells express CK 8/18, CK 7, CK 19, the basal/ myoepithelial cells express CK 5/6, CK14, CK 17 and smooth muscle actin. A small subset of stem cells which are dispersed in the inner layer of ductal system express CK 5, whichmay differentiate into myoepithelial or glandular cells [7].

The word basal has been linked to breast carcinoma for more than 30 years. Basal cytokeratin expression in breast carcinoma was first demonstrated in 1982[1]. It is only after the microarray based GEP by Perou and colleges in 2000 that the basal like phenotype of breast carcinoma gained importance in terms of prognostic significance and treatment strategy [1,10].

Currently the molecular subtyping of breast carcinoma is widely used for the overall patient management. It differentiates patients having breast malignancies with respect to clinical, biological, prognostic and therapeutic implications [7,11]. GEP is the gold standard for molecular phenotyping of breast carcinomas[1].There is a remarkable discrepancy between the immunohistochemical and GEP based molecular classification [1,3]. Use of GEP in the daily diagnostic practiceis cumbersome and limited,due to the high cost [1].CK5/6 identifies basal like phenotype in upto 81 % of invasive breast carcinomas by usingimmunohistochemistry[1,8].Majority (76.88%) of the basal like breast tumours defined by GEP are triple negative by immunohistochemistry while the percentage of triple negative tumours which are positive for basal cytokeratins may vary from 40 to 80%[3,12]. But the term "basal like phenotype" and "triple negative" tumours are not synonymous. They differ in prognosis and possibly in chemotherapeutic sensitivity [1, 5,12].

Expression of basal cytokeratin in breast carcinoma is not uncommon[13]. Basal like carcinomas represent 8 to 37 % of all breast cancers[1]. Incidence of CK5/6 positive tumours varies widely in the literature[14]. In a study by Kafil Akhtar1 et al.,27% of the breast carcinomas were CK5/6 positive. In the present study, the CK5/6 positive tumours accounted to 40% of the total cases studied. The higher percentage may be attributed to the smaller sample size. The most common histological type in various studies in literature is IDC-NOS [11,12]. It was also the most common type in the present study constituting 85.7% of cases.

Many studies done previously have shown that basal like phenotype of breast carcinoma are common in younger age group41 to 50 years [5,6,12,15].Likewise, in the present study the mean age of CK 5/6 positive cases(51.7years) was lesser compared to CK5/6 negative cases(54.4years). In study by Kafil Akhtar1 et al,. the mean age for CK5/6postive cases was 53 years and that for CK5/6 negative cases was 57.7years 16]. Present study did not show statistically significant association between age and CK5/6 positivity (p=0.06), so did the study by Mohammadizadeh F et al., and Rao et al., [14,15].

Among various prognostic factors in breast carcinoma, the most important once are lymphnode status, tumour size and tumour grade[13]. Tumour size was larger in CK5/6 positive tumours (5.5cm) compared to the CK5/6 negative tumours (4.5cm). This showed association between tumour size and CK5/6 positivity, but it was not statistically significant. Our findings were in agreement with the study by AE rahem et al., and Kafil Akthar et.al., [6,16]. In generalbasal like tumors tend to be larger with more than 2cm in size [15].

Various studies specify basal like phenotype to be histologically high grade tumours [5,16, 17]. On the contrary in the present study, the most commongrade was grade 2(57.14%) followe by grade 3(42.8%) and none of the tumours were grade1. Our findings were in accordance with the study by Sood et at.,in which the commonest histological grade was grade 2 (47.22%) followed by grade 3(38.8%) and grade 1(13.89%)[12]. Though CK5/6is commonly expressed in high grade IDC, they are also seen in hybrid medullary carcinoma, and prognosticallyfavoured secretory and adenoid cystic carcinoma [5,18]. Probably this explains the predominance of grade 2 cases over grade3 in the present study. This study also showed a statistically significant association(p=0.02) between pathological grades with CK5/6 positivity and so did the study by Sood et al., [12].

In the present study, significant positive association was found between tumour necrosis and CK5/6 positivity. (p=0.002) Similar findings were also seen in study by Rao et al.,[15]. In study by Livasy CA etal., basal phenotype was identified by GEP using cDNA micro array which showed necrosis in the form of centraltumour necrosis and geographical necrosis in 22% and 74% respectively[19].Hence necrosis is a common feature of CK5/6 positive tumours. The present study also showed statistical positivity of necrosis with CK5/6 positivity.

Present study demonstrated higher percentage of lymph node positivity(50%) in CK5/6 positive cases as against lymph node positivity(19%) in CK5/6 negative cases, and was found to be statistically significant(p=0.014) Many studies in the literature also showed higher percentage of lymphnode cases among basal CK positive tumours, though they did not demonstrate statistical significance[1,12,15,16]. In a study by Van De Rijn M et al,. statistical significance was observed similar to the present study [13]. In a study by Abd El-Rehim DM et.al., positive association was seen between distant metastasis and CK5/6 positivity [6]. There are conflicting data in the literature regarding the lymph node metastasis and CK5/6 positivity[15].Present study is one of the few studies which demonstrated positive association between lymph node metastasis and CK5/6 positivity. Many studies in the literature reveals that basal cytokeratins are independent prognostic markers in node negative patients [6,13]. While few studies also demonstrated no significant difference in prognosis of lymph node positive and negative cases in basal phenotype while few studiesfavour positive association of Lymph node and distant metastasis with CK5/6 positivity among basal phenotype breast carcinomas [12,14,16,20].

Conclusion:

Basal phenotype of breast carcinoma is a distinct, yet heterogenous disease associated with aggressive behaviour and reduced disease free survival. CK5/6 is most commonly used marker to identify basal phenotype of breast carcinoma. It may not identify all the basal like tumours as identified by the GEP. But never the less CK5/6 as a single marker successfully identifies those tumours with poor outcome which is apparent in the present study. CK5/6 positive tumours tend to occur in younger patients compared to CK5/6 negative tumours. These tumours are usually larger in size. CK 5/6 positivity is

commonamongst IDC-NOS, which may be due to the higher occurrence of this histological type. Necrosis is a frequent finding in CK5/6 positive tumours. These tumours have higher chances of lymph node metastasis and tend to be relatively of higher histological grade.Hence IHC study of CK5/6must be included along with ER PR and HER2 as a routine in breast carcinoma as it has prognostic and therapeutic implications like the triple markers.

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