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To study the expression of cytokeratin 5 and 6 in triple negative breast carcinoma: An immunohistochemical study

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Abstract
Introduction: Breast cancer is the second most common cancer developing in women worldwide Triple Negative Breast Carcinomas (TNBC) are regarded as one of the most malignant phenotypes, Immunohisto-chemically these tumors are endocrine receptor negative, HER2 –receptor negative, with expression of basal markers - Cytokeratin 5 /6, carrying a poor prognosis. Effective evaluation of these tumor markers helps in efficient treatment of the patient with all these markers carrying a prognostic significance. The current study helps in identifying Cytokeratin 5/6 as a potential multifaceted biomarker and in identifying novel therapeutic agents for Triple Negative Breast Carcinomas.
Materials and Methods: The present immunohistochemical study was conducted on 40 histologically proven cases of triple negative breast carcinoma (TNBC) which were further subjected to Cytokeratin 5/6 expression to determine prognostic and therapeutic value.
Result: Cytokeratin 5/6 expression was seen in 16 cases of TNBC, out of 40 histologically proven cases of TNBC. The maximum number of patients of triple negative breast carcinoma were in the age group of 41-50 years comprising 40% of the total cases. % of the total cases. Maximum number of cases of triple negative breast carcinoma were of grade III, constituting 60% followed by grade II
Conclusion: According to the current study, Cytokeratin 5/6 positivity has got direct relationship with the grade of the tumour, lymph nodal status and lymphovascular invasion in TNBC cases. TNBC have a grave clinical outcome and decreased 5 year survival rate. Thus, it is highlighted that all TNBC patients should be stained by CK 5/6 and if found positive for CK 5/6 expression it, implies basal like phenotype and aggressive intervention is required. Cytokeratin 5/6 carries a diagnostic as wells as therapeutic importance.

Keywords: TNBC-Triple negative breast carcinoma. CK-Cytokeratin

Introduction
Breast cancer is the second most common cancer developing in women worldwide, accounting up to 25. 1% of all cancer [1]. The age adjusted incidence rate of carcinoma of the breast has been found to be as high as 41 per 100,000 women in Delhi, followed by lowest in Thiruvananthapuram District. In Punjab, Amritsar district records highest number of cases of breast carcinoma every year [2].
Triple-negative breast carcinomas (TNBC), accounting for 12 – 25% are defined as Tumours showing lack of expression of estrogen receptor, progesterone receptor and Her-2/ neu expression. These Tumours are associated with poor prognosis. Two sub types of triple-negative breast cancers have been described: basal and non-basal. Basal type TNBC exhibits p53 mutation, high histological grade and expresses basal cytokeratin (CK5/6, CK14 and CK17) along with epidermal growth factor receptor (EGFR) over-expression. Non basal type TNBC is characterised by negative expression of ER, PR and Her-2/ neu and is negative for CK 5/6 (basal markers) [3].
However, in the recent years there has been considerable development in determining diagnostic as well as prognostic modalities for breast carcinoma, in order to reduce the mortality and morbidity.
Cytokeratin 5 is commonly found in outermost layer of skin in humans and is encoded by the KRT5 gene and pairs with the type I keratin K14. Cytokeratin 5 and 6 (CK5/6) are type 2 medium sized neutral polypeptides that are part of the cytokeratin family of polypeptides.

CK5/6 is a sensitive marker for squamous differentiation and is expressed in both benign and malignant tumours found in the epithelium, squamous mucosa, and myoepithelium. Its expression in TN breast cancer is correlated with poor prognosis, high grade differentiation and axillary lymph node metastasis. Gene expression profiling is the gold standard for the identification of basal type TNBC [4].

Cytokeratins are diagnostic tools of pathology, most importantly in the detection of tumours. Primary tumours and metastases of a given carcinoma share the same pattern of cytokeratins, that distinguishes them from other types of carcinomas and hence allowing differentiation between the different tumours. For example, Mesotheliomas and Adenocarcinomas (originating in glandular tissue) can be distinguished by detection of Keratin 5 [7].

The present study has provided varying opinions on the role of CK 5/6 in TNBC and correlation of CK5/6 expression with prognosis, clinical outcome and chemo-sensitivity in various cases.

According to GLOBOCAN 2020, female breast cancer has surpassed lung cancer and is the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), followed by lung (11.4%), and stomach (5.6%) cancers. Mortality rates of breast carcinoma was higher in developing countries (15. 0 per 100,000) than developed countries (12. 4 per 100,000) [5].

Table 1: Features of the molecular subtypes of breast cancer [6]

Molecular subtype	Frequency.	Cell of origin	Er/pr/ her2	Prolife ration rate	Histo-logic Grade	Prognosis
Luminal A	50- 60%	Luminal epithelial cell	ER and/or PR +ve, Her- 2 –ve	Low – Ki - 67 < 14%	Low	Excellent
Luminal B	10- 20%	Luminal epithelial cell	Her-2/ neu expression variable a)Her-2- ve, ER and/or PR +ve b) Her- 2+ve, ER and/or PR +ve	a) High- Ki-67>14%	Intermediate/ High	Intermediate/ Bad
HER-2 overexpressing	10- 15%	Late luminal progenitor cell	Her-2 +ve ER - ve and PR –ve	High	High	Bad
Normal breast-like	5- 10%	Luminal epithelial cell	ER - ve/+ve Her-2 -ve	Low	Low	Intermediate/ good
Triple negative/ Basal type	10- 20%	Basal/myoepithelial cell/ bipotent progenitor	ER -ve, PR -ve, and Her-2 -ve	High	High	Bad

Immunohistochemistry (IHC) has been established as a powerful diagnostic tool which is suitable for immunophhenotyping of tumours. As compared to gene expression profiling, IHC is more feasible logistically for routine diagnostic histology laboratories.

The basal subtype of breast tumours specifically has been characterised by Nielsen *et al*. in their study which used the markers such as ER, HER2, the basal cytokeratin 5/6 (CK5/6) and epidermal growth factor receptor (EGFR, also known as HER1) to detect basal-like subtype of breast tumours. Basal tumours were identified immunohistochemically as ER negative, HER2- negative, and CK5/6 and/or EGFR- positive. [8]

Breast carcinomas exhibiting basal epithelium cytokeratins, constitute a tumour subgroup which shows different immunophenotypical, morphological and genetical features. This group is associated with poor prognosis.[9]

Hence with this study, we have assessed the histological grade in triple negative breast carcinoma and further found out Cytokeratin 5 and 6 expression in triple negative breast carcinoma. Also, Cytokeratin 5 and 6 expression with histological grade and other parameters eg -age, size and lympho-vascular invasion etc. has also been assessed

Materials and Methods

The present study was conducted on 40 histologically proven cases of triple negative breast carcinoma (TNBC) in the Department of Pathology, Sri Guru Ram Das Institute of Medical Sciences and Research, Sri Amritsar. Routinely ER, PR and HER-2neu examination is being done in the department on all the breast carcinomas from which triple negative breast carcinomas were taken from the archives. The tissues were formalin fixed and paraffin embedded and were then stained for haematoxylin and eosin for histopathological typing and grading. All the cases were then subjected to immunohistochemistry for CK5/6 expression. (Ck5/6 (primary antibody: monoclonal mouse antihuman Ck 5/6 clone D5/16B4)

For CK 5/6: Membranous staining and/or both cytoplasmic and membranous staining was taken as positive. Tumours were classified based on intensity of staining and proportion of cells showing cytokeratin expression.

Staining Index = Staining Intensity X Proportion of Immuno-positive Cells. These values were then multiplied and combined into a final score as:

Table 2: Staining intensity

Score	Description
0	No staining
1+	Weak staining
2+	Moderate staining
3+	Strong staining

Table 3: Proportion of immune-positive cells

Score	Description
0	Less than 1% positivity
1+	1-10% tumour cells are positive
2+	10 to 50% tumour cells are positive
3+	More than 50% tumour cells are positive

Specimens with staining index value of 1-9 were considered as positive whereas staining index value of zero was considered to be negative.

Table 4: Showing age distribution

Age (Years)	No of cases	Percentage
≤40	2	5%
41-50	16	40%
51-60	12	30%
61-70	8	20%
>70	2	5%
Total	40	100%

Table 5: Showing size of the tumour

Size	No of cases	Percentage
<2	1	2.5%
2-5	37	92.5%
>5	2	5.0%
Total	40	100%

Table 6: Showing correlation of the histopathological grades of the carcinoma with lymph node status

Histopathological Grade	Lymph-node status				Total no of cases	
	Lymph node positivity		Lymph node negativity			
	n	%	n	%	n	%
Grade I	0		0		0	0%
Grade II	7	43.8%	9	56.2%	16	40.0%
Grade III	19	79.2%	5	20.8%	24	60.0%
Total	26	65%	14	35%	40	100%

Chi Square value: 5.293, p value: 0.021

Table 7: Showing number of cells positive for ck5/6

CK 5/6 -% of positive cells	No of cases	Percentage
0 (0%)	24	60.0%
1+ (1-10%)	5	12.5%
2+ (11-50%)	10	25.0%
3+ (>50%)	1	2.5%
Total	40	100%

Table 8: Showing grade of the tumour

Histopathological grade	No of cases	Percentage
Grade I	0	0%
Grade II	16	40%
Grade III	24	60%
Total	40	100%

Table 9: Showing lymph-node status

Status of lymph node	No of cases	Percentage
Reactive (n0)	14	35%
N1 (1-3)	6	15%
N2 (4-9)	12	30%
N3 (>9)	8	20%
Total	40	100%

Table 10: Showing correlation of cytokeratin 5/6 with age

Age (Years)	CK 5/6 -% of positive cases				Total no of cases	
	Positive		Negative			
	n	%	n	%	n	%
≤40	1	6.2%	1	4.2%	2	5%
41-50	6	37.5%	10	41.7%	16	40%
51-60	3	18.8%	9	37.5%	12	30%
61-70	4	25.0%	4	16.7%	8	20%
>70	2	12.5%	0	0.0%	2	5%
Total	16	100%	24	100%	40	100%

Chi Square value: 1.600, p value: 0.808

Table 11: Showing correlation of cytokeratin 5 and 6 with tumour grade

	CK 5/6 -% of Positive Cases				Total no of cases	
	Positive		Negative			
Grade	n	%	n	%	n	%
Grade I	0	-	0	-	0	-
Grade II	3	18.8%	13	81.3%	16	40%
Grade III	13	54.2%	11	45.8%	24	60%
Total	16	19%	24	81%	40	100%

Chi Square value: 5.017, p value: 0.025

Table 12: Showing correlation of ck5/6 with lymph node status of the tumour

Lymph node status	CK 5/6 -% of positive cases				Total no of cases	
	Positive		Negative			
	n	%	n	%	n	%
Reactive	0	0%	14	100%	14	35%
Metastatic	16	61.5%	10	38.5%	26	65%
Total	16	40%	24	60%	40	100%

Chi Square value: 8.759, p value: 0.003

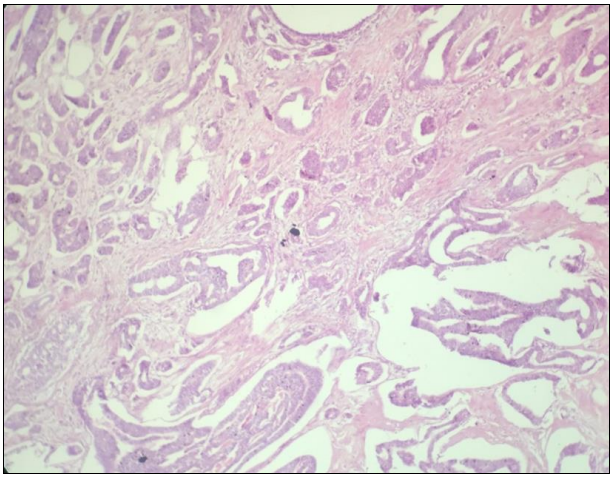

Fig 1: Microphotograph showing infiltrating ductal carcinoma grade ii (h & e: 100x)

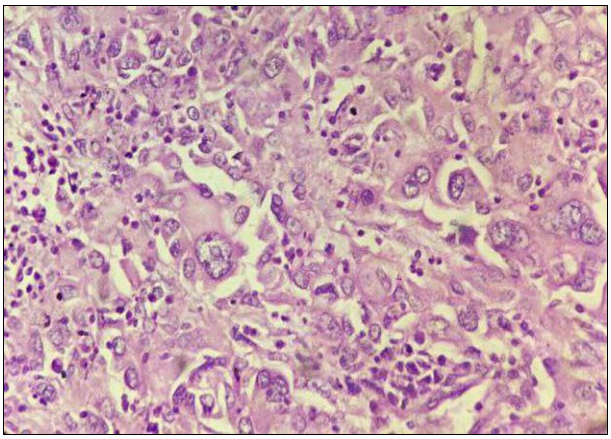

Fig 2: Microphotograph showing infiltrating ductal carcinoma grade ii (h & e: 100x)

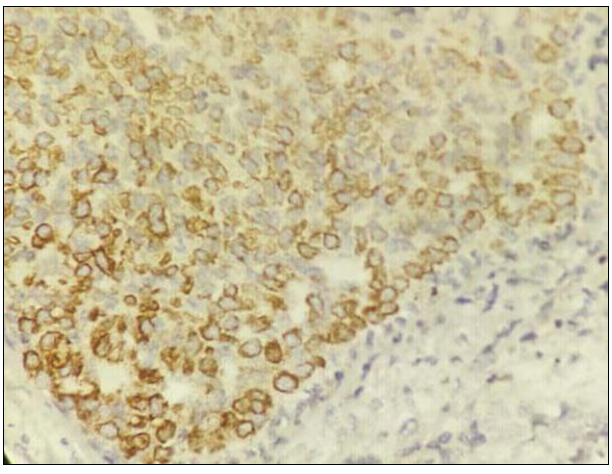

Fig 3: Microphotograph showing moderate positivity of cytokeratin 5/6 (Magnification 400 x)

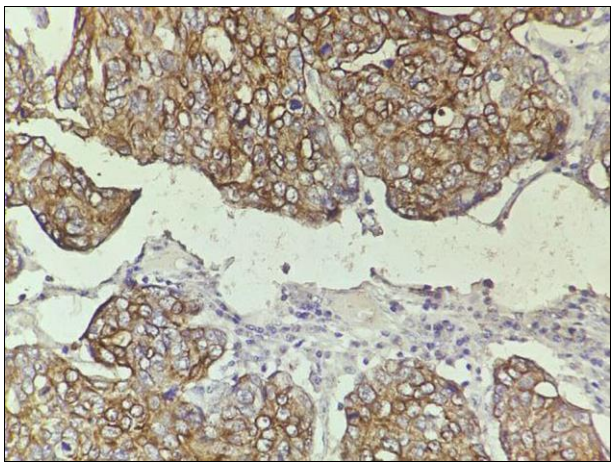

Fig 4: Microphotograph showing strong positivity of cytokeratin 5/6 (magnification 400 x)

Results and Observations

In the present study conducted on 40 cases of triple negative breast carcinoma in the Department of Pathology, Sri Guru Ram Das Institute of Medical Sciences and Research, Sri Amritsar, the mean age of the patients was found to be 53.95 years with maximum number of the patients in the age group of 41-50 years comprising 40% of the total. Tumour size varied from as small as 1.5 cm to as large as 6 cm and 24 cases were of grade III and 16 were of grade II. Lymphovascular invasion was seen in 26 cases (65%) whereas it was absent in rest of the 14 cases (35%) and a highly significant correlation between tumour grade and lymph node status was observed. As the tumour grade increased, lymph node involvement by metastatic carcinomatous deposits increased.

CK 5/6 expression was observed in 16 cases (40%) and the remaining 24 cases (60%) showed negative immunoexpression for CK 5/6. Cytoplasmic and membranous staining was taken as positive staining pattern. In the above study, in 61- 70 year age group, CK 5/6 positivity was 25% cases were positive. However, no significant correlation between CK5/6 positivity and age of the patient was found.CK 5/6 positivity was found to have a direct correlation with tumor grade. With increase in tumor grade, CK 5/6 positivity increased. Grade II cases reported CK 5/6 positivity of 54.2%.

A linear direct relationship was found between CK 5/6 positivity and lymph node metastasis. All the 16 cases with metastatic lymph nodes showed CK 5/6 positivity whereas cases with reactive lymph nodes were negative for CK 5/6 immuno expression.

Discussion

Triple-negative breast cancer are distinct because of their aggressive clinical behaviour and characteristic clinico pathologic and prognostic factors. Cytokeratin 5/6 are basal markers and their positive expression in breast carcinoma is associated with aggressiveness of the tumour, poor prognosis and decreased 5 - year survival rate.

In this study we have studied the significance of expression of basal cytokeratins - CK 5/6 in triple negative breast cancer

In the current study, age of the patients with TNBC were found in the age group 41-50 years comprising 40% of the total. The mean age was found to be 53.95 years. The findings concur with studies conducted by Sood *et al.* [10] and Rao *et al.* [11] in different parts of the country, where mean age was found to be – 45.18 years and 41- 50 year age group to be the most common age group for triple negative breast carcinoma respectively. In our study, right side and left side were involved equally with each side constituting 50%.

However, in contrast to the present study, Al-Ahwal1 [2] in a study of 260 cases of breast carcinoma reported a slightly higher incidence (51.9%) in the right breast whereas Amer 13 observed left sided involvement to be more common than right sided breast cancer.

The study conducted reported tumour size varying from 1.5 cm to 6 cm with maximum number of cases varying from 2 - 5 cm, comprising 93% of the total cases. These findings corroborated with the results from the studies done by other researchers where maximum number of cases had tumour size between 2-5 cm [10-11].

In the present study, maximum cases were of Grade III - 60% followed by Grade II cases. Similar observations were noted in other studies where grade III tumour cases out numbered grade II cases [14-15].

Axillary lymph node metastasis was seen in 65% cases. Similar trends were seen in studies done by Mohammadizadeh *et al*., [16]; Suhani *et al.* [17] who reported involvement of lymph node metastasis in 73% and 59.2% cases.

In present study no definite correlation between tumour size and lymph node status was observed. However various studies in past show a definite correlation between tumour size and lymph node metastasis.

The above study shows linear correlation between lymph node status and tumour grade was found to be statistically significant. (p=0.021) which is similar to observations reported by Huang *et al.* [18] and Singhai *et al.* [19] in their studies and reported that lymph node metastasis showed an increasing trend with increase in tumour grade respectively.

CK 5/6 expression was noted in 40% cases of triple negative breast carcinoma

These findings corroborated with results from studies by Mohammadizadeh *et al.* [16] and Yadav *et al.* [20], reporting CK 5/6 positivity in TNBC as 47.8% and 59% cases respectively. Similar observations were also recorded by Sutton *et al.* [21] and Pintens *et al.* [22] in their respective studies observing CK 5/6 positivity in TNBC cases to be 62% and 60% respectively

No significant association was found between CK 5/6 expression and the size of tumour. The above findings were in concordance to indian studies by Sood *et al*, [10]; Rao *et al.* [11] However, in contrast Herranz *et al.* [23] reported significant correlation between tumour size and CK 5/6 positivity, with greater the tumour size, more CK 5/6 positivity.

According to the present study CK 5/6 expression in TNBC was statistically correlated with histological grade. (p value – 0.025).

Furthermore, as the histological tumour grade increased, CK 5/6 expression increased. Similar observations were noted in work done by da Silva *et al.* [15] who found CK 5/6 expression in 74.8% grade III cases and 53.6% grade II cases of TNB Calong with corroboration with Indian studies by Sood *et al.* [10] and Rao *et al.* [11].

CK5/6 positivity was associated with lymph node involvement with 16 cases showing lymph node metastasis showed CK 5/6 positivity comprising 62% whereas cases with reactive hyperplasia of lymph node were negative for

CK5/6, similar to studies done by indian authors Das *et al*. [24]; Suhani *et al*. [17]

Conclusion

Thus, it is concluded that Cytokeratin 5/6 positivity has got direct relationship with the grade of the tumour, lymph nodal status and lymphovascular invasion. It is highlighted that all TNBC patients should be stained for CK 5/6 and If found positive for CK 5/6 expression it implies basal like phenotype and aggressive intervention is required. Furthermore, after CK5/6, these tumours should be subjected to BRCA 1 mutation testing and EGFR receptor study so that if found positive, use of monoclonal antibody – Cetuximab which is targeted against EGFR is recommended. Thus with CK 5/6 molecular testing, we can help to provide prognostic information and better treatment modalities and this approach can also guarantee more concordance with gene expression based studies.

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Author's Contribution

Not available

Conflict of Interest

Not available

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