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Original Article

Pathology Section

Evaluation of CD9 Expression of Tumour Cells and Stromal Immune Cells in Breast Carcinoma by Immunohistochemistry

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ABSTRACT

Introduction: Cluster of Differentiation 9 (CD9), known as Motility Related Protein (MRP-1) regulates cell adhesion, motility, migration and proliferation. Many studies have stated conflicting results on prognostic significance of invasive breast carcinomas with CD9 expression that had performed on tumour tissues.

Aim: To assess the inter-relationship of CD9 expression of tumour cells and stromal immune cells in breast carcinoma with clinicopathological parameters which include age, tumour size, grade, histological type, lymph nodes, tumour staging and molecular classification.

Materials and Methods: An observational prospective (July 2020 to June 2021) and retrospective (October 2019 to June 2020) study was done in 71 cases of resected primary invasive breast carcinoma over a period of one and half year at Sri Devaraj Urs Medical College, Karnataka, India. Immunohistochemistry (IHC) staining was done using CD9 antibody. Tumour cells (T-CD9) expression was evaluated by Immunoreactivity scoring (IS= Intensity score×Extent of staining). The stromal cells (S-CD9) expression was evaluated by percentage (%) of stromal area occupied by CD9 stained immune cells. Chi-square test was used as test of significance for qualitative data. The p-value of <0.05 was considered as statistically significant.

Results: Out of 71 cases, T-CD9 expression was noticed in 40 (56.34%) cases and IS <4 considered as negative was observed in 31 (43.66%) cases. However there was no association with age, tumour size, grade and molecular markers for the expression of both T-CD9 and S-CD9. Human Epidermal growth factor receptor 2 neu (HER2neu) negative was associated with T-CD9 expression (p-value=0.05). Hence, CD9 can be used as prognostic marker for Her2neu negative cases.

Conclusion: The CD9 expression was not significantly associated with tumour cells (T-CD9) and stromal cells (S-CD9) in breast carcinoma cases. However, it was significantly associated with Her2neu negative tumour cells. T-CD9 showed more positivity in Luminal A followed by triple negative, whereas S-CD9 showed more positivity in Luminal B. CD9 did not show association with any parameters except Her2neu negative.

Keywords: Invasive breast cancer, Molecular classification, Motility related protein-1, Nottingham prognostic index scoring, Stromal cells

INTRODUCTION

Breast cancer has become the most commonly diagnosed cancer worldwide surpassing lung cancer and is one of the leading sources of cancer associated deaths [1]. During 2020, there were 2.3 million women diagnosed with breast cancer with global mortality rate of 685,000 [1]. There were 1,78361 new cases and 90,408 deaths in India during 2020 [2]. The incidence rate of breast cancer in Kolar region was 6.41% in 2010 [3].

The CD9, otherwise known as Motility Related Protein (MRP-1) which is a member of tetraspanin family associated in various process that includes cell adhesion, cell motility, migration and proliferation as a result of interaction with integrins and other tetraspanins (CD8 and CD151), growth factor receptors and signalling molecules [4]. It is a cell membrane glycoprotein and it has four functional regions which are N and C terminal cytoplasmic domains, a small intracellular loop and 2 extracellular loops [4,5]. CD9 is extensively detected in various types of cancer cells and also in normal tissues [6]. Previous studies have showed that decreased expression of CD9 would be associated with poor prognosis in breast carcinoma and various other malignancies such as oesophageal carcinoma, oral squamous cell carcinoma and gastrointestinal stromal tumour [7-10]. Various studies have stated conflicting results on prognostic significance of invasive breast carcinomas with CD9 expression that was performed on tumour tissues [6,7]. The purpose of present study was to evaluate CD9 expression in tumour cells as well as stromal immune cells by performing IHC in invasive breast carcinoma cases and to evaluate the inter-relationship of tumour cells-CD9 and stromal cells-CD9 expression with clinicopathological parameters and with molecular classification.

MATERIALS AND METHODS

An observational prospective (July 2020 to June 2021) and retrospective (October 2019 to June 2020) study done in duration of one and half year at Sri Devaraj Urs Medical College at Tamaka, Kolar, India, was conducted.

Primary invasive breast carcinoma that had undergone surgical resection were collected from the archives of pathology in R.L. Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College, Tamaka, Kolar, Karnataka after obtaining the approval from Ethics Committee (DMC/KLR/IEC/694/2020-21).

Inclusion criteria: In the present study, mainly infiltrating ductal carcinoma cases and other histological types such as papillary carcinoma, invasive lobular carcinoma, mucinous carcinoma, medullary carcinoma, metaplatsic carcinoma, phyllodes tumour and mixed tumours were included in the study.

Exclusion criteria: All the cases of breast carcinoma patients who received neoadjuvant chemotherapy or any other malignancies and small biopsies were excluded from this study.

Sample size calculation: Sample size of 71 was based on CD9 expression in study by Baek J et al., (42.5%) with 95% confidence intervals with absolute error of 10% [11]. Sample size formula: $N=Z\alpha 2\ P\ (1-P)/d^2$, P=expected proportion i.e. 42.5%, $Z\alpha=1.96$ (95% confidence interval), D=absolute error (10%).

Study Procedure

All the Haematoxylin and Eosin (H&E) slides were rescreened by two authors to confirm the histological type, tumour grade, presence and absence of nodal metastasis and lymphovascular invasion. IHC slides of Estrogen Receptor (ER), Progesterone Receptor (PR), Her2 and Ki-67 were also screened. All the breast carcinoma cases were classified according to World Health Organisation (WHO) classification [12]. Tumour grading was done as per Modified Scarff-Bloom Richardson Histologic Grading [13].

Patients survival status was determined using Nottingham Prognostic Index (NPI) and was calculated using standard formula NPI = (0.2xS)+G+N

where S= Tumour Size in cm, G= Grade of tumour (grade 3=3, grade 2=2, grade 1=1), N=Number of lymph nodes involved (>4=3, 4-1=2, 0=1). All cases were categorised into three different prognostic groups: Good=2.00-3.40, moderate=3.41-5.40, poor=>5.40 [14]. IHC was done using principle of peroxidase-antiperoxidase method using tonsil as positive control. ER, PR was interpreted as per the Allred score and Her2neu were scored as per 2018 American Society of Clinical Oncology (ASCO) guidelines [15,16]. Ki-67 was scored using the standard guidelines [17]. To evaluate the CD9 expression IHC was performed by using CD9 antibody. In both tumour cells (T-CD9) as well as stromal cells (S-CD9), CD9 expression was elucidated. To elucidate the T-CD9 expression IS was used [Table/Fig-1]. This IS scoring is produced by taking intensity and extent of staining into consideration and is as follows [11,18]:

Intensity score: Evaluated as 0-3 [11,18]	Extent of staining: Percentage of positive tumour cells showing membranous to cytoplasmic staining.
0: Negative 1: Weak positive 2: Moderate positive 3: Strong positive	0: 0% cells show positivity 1: 1-25% cells show positivity 2: 26-50% cells show positivity 3: 51-75% cells show positivity 4: >75% cells show positivity
Tumour cells (T-CD9): Immunoreactivity scoring= Intensity score×Extent of staining score Ranges from 0-12.	

[Table/Fig-1]: Showing immunoreactivity scoring of T-CD9 expression.

On the basis of mean IS, cases were divided into positive and negative and the scoring is as follows: IS : <4: Negative, ≥4: Positive. The stromal immune cells (S-CD9) expression was evaluated by: Percentage (%) of stromal area occupied by CD9 stained immune cells. S-CD9 was assessed same as Tumour Infiltrating Lymphocytes (TILs) [19]. Molecular classification of all cases was done and T-CD9 and S-CD9 expression were analysed.

Molecular classification: By performing IHC on the tumour tissues of breast, cases were classified into five subtypes depending on the hormonal status as stated in 2011 St. Gallen consensus is as follows [Table/Fig-2] [20].

Molecular subtypes	Hormonal status
Luminal A	ER +, PR +, HER2-, Low Ki-67
Luminal B	ER +, PR +, HER2-/+, High Ki-67
HER2 overexpression	ER-, PR-, HER2 +
Triple Negative Breast Carcinoma (TNBCS)	ER-, PR-, HER2-
Basal like: Basal markers like CK5/6 positive in Triple Negative Breast Carcinoma (TNBCS)	

[Table/Fig-2]: Showing molecular classification of breast carcinoma.
ER: Estrogen receptor; PR: Progesterone receptor; HER2: Human epidermal growth factor receptor 2

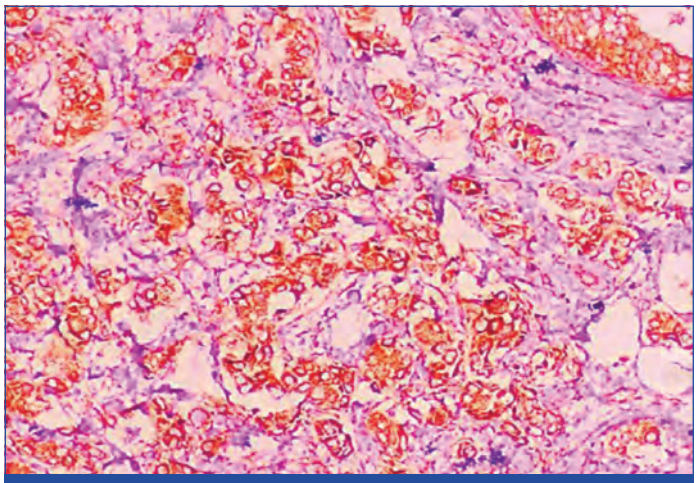
To assess the prognosis NPI scoring system and other clinicopathological parameters which include age, tumour size, grade, lymph node, pathological Tumour, Node and Metastases (TNM) staging, hormonal markers and molecular classification were used and evaluated the association of both T-CD9 and S-CD9 expression.

STATISTICAL ANALYSIS

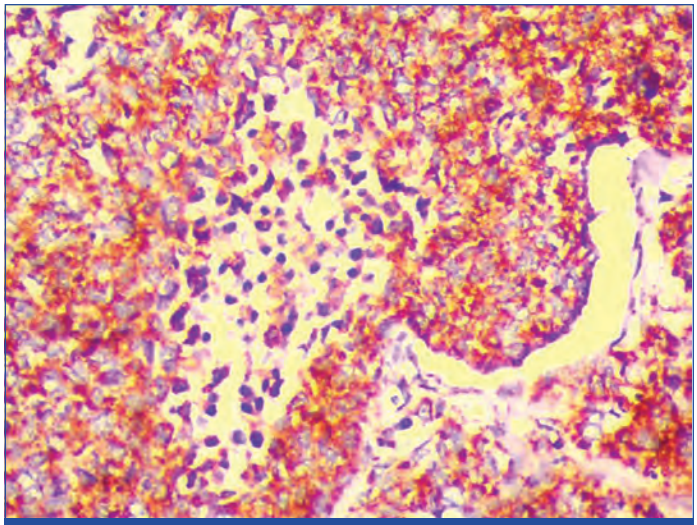
Data was entered into Microsoft excel data sheet and was analysed using Statistical Package for the Social Sciences (SPSS) 22.0 version software (IBM SPSS Statistics, Somers NY, USA). Categorical data was represented in the form of frequencies and proportions. Chi-square test or Fisher's exact test (for 2×2 tables only) was used as test of significance for qualitative data. The p-value (probability that the result is true) of ≤0.05 was considered as statistically significant.

RESULTS

Out of 71 cases of invasive breast carcinomas, CD9 expression was elucidated by IHC in T-CD9 and S-CD9 and these were interpreted and assessed separately. Of all cases, the intensity for T-CD9 expression was differed for every case that is 28 (39.4%) cases showed strong positivity [Table/Fig-3], moderate positivity was observed in 24 (33.8%) cases, 10 (14.1%) cases showed weak positivity and 9 (12.7%) cases were showed negative. Also this CD9 expression was noticed in normal breast ductal epithelial cells which were expressed as weak positivity. CD9 was expressed in both tumour cells and stromal immune cells [Table/Fig-4].



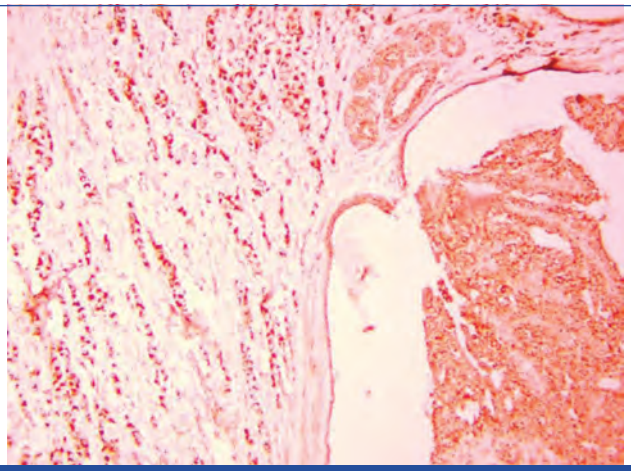
[Table/Fig-3]: Microscopic image of T-CD9 expression in membrane staining shows strong positivity (400X).



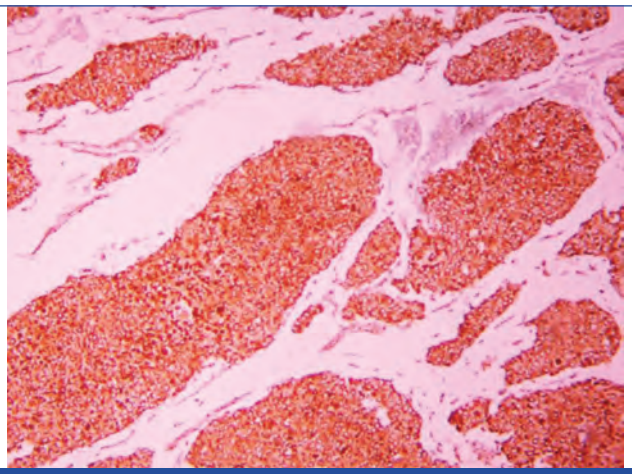
[Table/Fig-4]: Microscopic image showing both T-CD9 and S-CD9 expression in (400X).

In 71 cases, CD9 expression in tumour cells with IS >4 which considered positive was noticed in 40 (56.34%) cases and IS <4 which considered as negative was observed in 31 (43.66%) cases. Among 71 cases, 57 were of Infiltrating Ductal Carcinoma (IDC) type and 14 cases were of other histological types. Out of these 57 IDC cases, T-CD9 was expressed in 31 (54.4%) cases and 26 (45.6%) cases did not show any expression and among 14 other histological

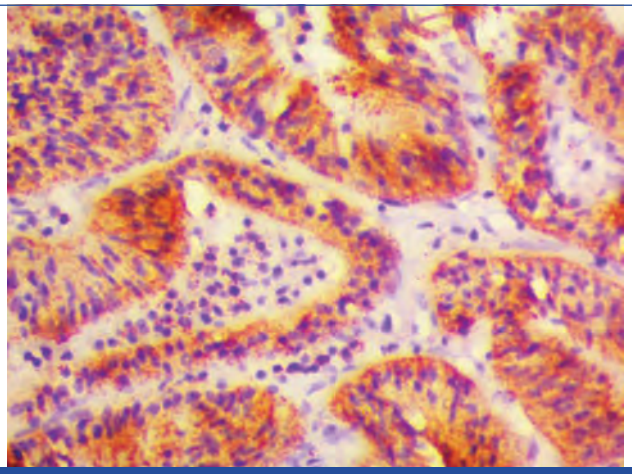
types, 9 (64.29%) cases showed T-CD9 expression and 5 (35.71%) cases did not show T-CD9 expression. Other histological types which showed T-CD9 expression were mixed carcinoma (IDC+ILC) [Table/Fig-5], mucinous carcinoma [Table/Fig-6], papillary carcinoma [Table/Fig-7], invasive lobular carcinoma [Table/Fig-8], medullary and metaplastic carcinoma [Table/Fig-9]. T-CD9 was not expressed in phyllodes tumour [Table/Fig-10]. Whereas, S-CD9 expression was seen in 43 (60.56%) cases and S-CD9 expression was not observed in 28 (39.44%) cases [Table/Fig-11]. However there was no association observed with clinicopathological data such as age, tumour size, tumour grade and molecular markers of ER, PR, and Ki67 for the expression of both T-CD9 and S-CD9 [Table/Fig-12,13]. But Her2neu molecular marker was associated with T-CD9 expression with p-value=0.05 [Table/Fig-13].



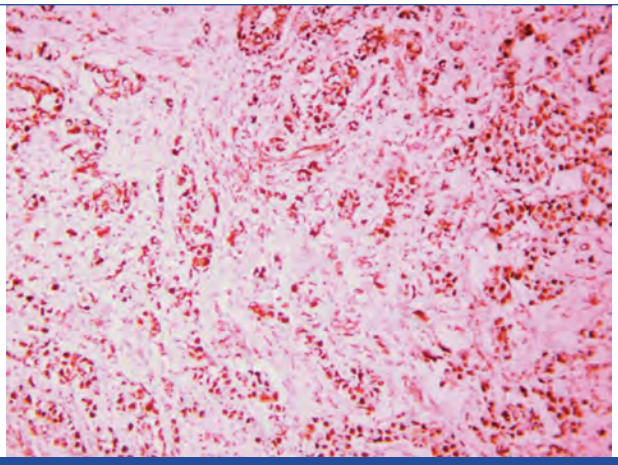
[Table/Fig-5]: Microscopic image of T-CD9 expression in mixed carcinoma (IDC+ILC) (100X).



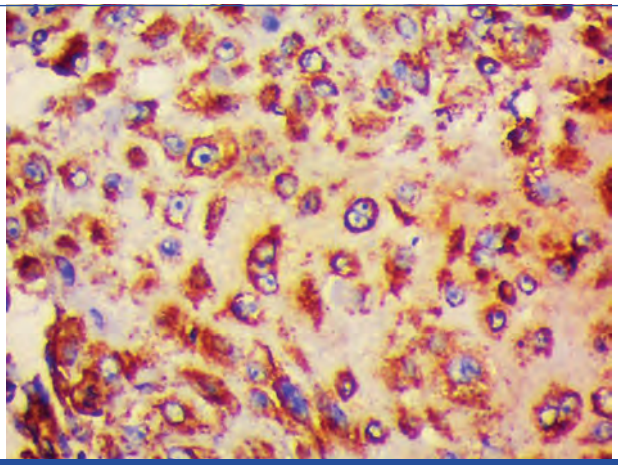
[Table/Fig-6]: Microscopic image of T-CD9 expression in mucinous carcinoma (100X).



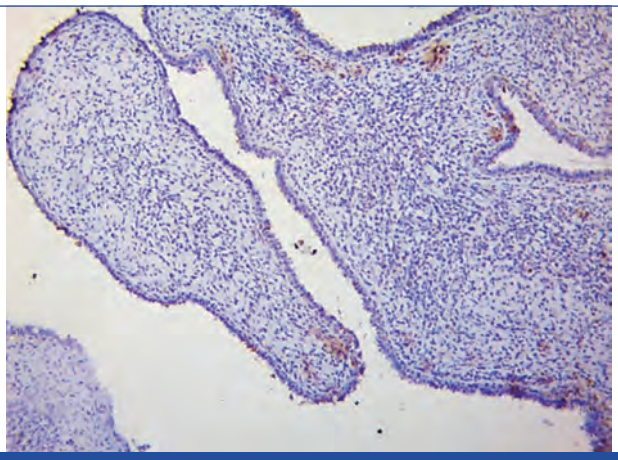
[Table/Fig-7]: Microscopic image of papillary carcinoma showing T-CD9 expression (400X).



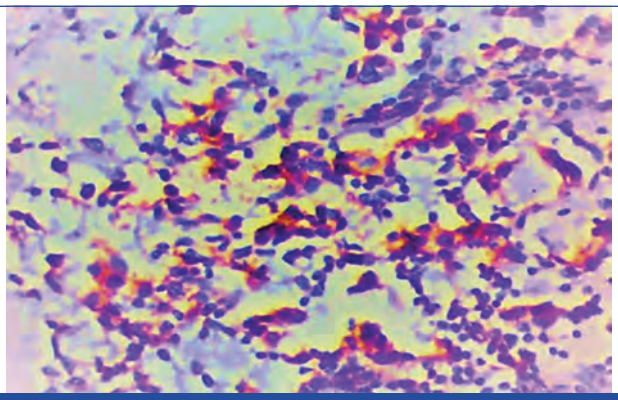
[Table/Fig-8]: Microscopic image of T-CD9 expression in invasive lobular carcinoma (100X).



[Table/Fig-9]: Microscopic image of metaplastic carcinoma showing T-CD9 expression (400X).



[Table/Fig-10]: Microscopic image showing phyllodes tumour which did not show T-CD9 expression (100X).



[Table/Fig-11]: Microscopic image of S-CD9 expression showing moderate intensity (400X).

Variables	T-CD9		p-value	S-CD9			p-value
	Negative	Positive		<10%	10-40%	>40%	
	No. of cases (%)	No. of cases (%)		No. of cases (%)	No. of cases (%)	No. of cases (%)	
Age group (years)							
≤50	13 (52)	12 (48.0)	0.326	7 (28)	11 (44)	7 (28)	0.249
>50	18 (39.1)	28 (60.9)		22 (47.8)	16 (34.8)	8 (17.4)	
Tumour size (cm)							
≤2	3 (27.3)	8 (72.7)	0.327	5 (45.4)	2 (18.2)	4 (36.4)	0.241
>2	28 (46.7)	32 (53.3)		24 (40)	25 (41.7)	11 (18.3)	
Tumour grade							
1	18 (50)	18 (50)	0.479	17 (47.2)	10 (27.8)	9 (25)	0.197
2	10 (41.7)	14 (58.3)		8 (33.3)	13 (54.2)	3 (12.5)	
3	7 (63.6)	4 (36.4)		6 (54.5)	3 (27.3)	2 (18.2)	
Histological type							
*IDC	26 (45.6)	31 (54.4)	0.280	22 (38.6)	21 (36.8)	14 (24.6)	0.384
*ILC	-	1 (100)		1 (100)	-	-	
Mixed	3 (60)	2 (40)		1 (20)	4 (80)	-	
Medullary	-	1 (100)		1 (100)	-	-	
Metaplastic Ca	-	1 (100)		-	-	1 (100)	
Mucinous	-	2 (100)		1 (50)	1 (50)	-	
Papillary Ca	-	2 (100)		1 (50)	1 (50)	-	
Phyllodes	2 (100)	-		2 (100)	-		
Lymph node status							
Positive	12 (35.29)	22 (64.71)	0.172	13 (38.2)	12 (35.3)	9 (26.5)	0.571
Negative	19 (51.35)	18 (48.65)		16 (43.2)	15 (40.6)	6 (16.2)	
TNM stage							
IA	2 (50)	2 (50)	0.795	2 (50)	1 (25)	1 (25)	0.314
IIA	9 (45)	11 (55)		5 (25)	8 (40)	7 (35)	
IIB	12 (52.2)	11 (47.8)		12 (52.2)	9 (39.1)	2 (8.7)	
IIIA	4 (28.6)	10 (71.4)		5 (35.7)	5 (35.7)	4 (28.6)	
IIIB	2 (50)	2 (50)		3 (75)	-	1 (25)	
IIIC	2 (33.3)	4 (66.7)		2 (33.3)	4 (66.7)	-	

[Table/Fig-12]: T-CD9 and S-CD9 expression with age, tumour size, grade, histology, nodes and stage.
The Chi-square statistic test was used. *IDC-Invasive Ductal Carcinoma, * ILC- Invasive Lobular Carcinoma.

Variables	T-CD9		p-value	S-CD9			p-value
	Negative	Positive		<10%	10-40%	>40%	
	No. of cases (%)	No. of cases (%)		No. of cases (%)	No. of cases (%)	No. of cases (%)	
NPI score							
Excellent	4 (40)	6 (60)	0.462	4 (40)	3 (30)	3 (30)	0.814
Good	6 (35.3)	11 (64.7)		7 (41.2)	7 (41.2)	3 (17.6)	
Moderate	12 (46.2)	14 (53.8)		9 (34.6)	12 (46.2)	5 (19.2)	
Poor	11 (61.1)	7 (38.9)		10 (55.6)	5 (27.8)	3 (16.6)	
ER							
Negative	16 (53.3)	14 (46.7)	0.231	12 (40)	14 (46.6)	4 (13.4)	0.283
Positive	16 (39)	25 (61)		17 (41.5)	13 (31.7)	11 (26.8)	
PR							
Negative	15 (48.3)	16 (51.7)	0.62	12 (38.7)	14 (45.2)	5 (16.1)	0.484
Positive	17 (42.5)	23 (57.5)		17 (42.5)	13 (32.5)	10 (25)	
Her 2neu							
Negative	16 (36.3)	28 (63.7)	**0.05**	21 (47.7)	15 (34.1)	8 (18.2)	0.318
Positive	16 (59.3)	11 (40.7)		8 (29.7)	12 (44.4)	7 (25.9)	
Ki-67 (%)							
<15%	29 (45.3)	35 (54.7)	0.901	26 (42.6)	22 (36.1)	13 (21.3)	0.676
>15%	3 (42.9)	4 (57.1)		3 (27.3)	5 (45.5)	2 (18.2)	
Molecular typing							
Her 2+	7 (50)	7 (50)		7 (50)	5 (35.7)	2 (14.3)	

Luminal A	11 (34.37)	21 (65.63)	0.247	15 (46.8)	8 (25)	9 (28.2)	0.331
Luminal B	6 (66.7)	3 (33.3)		2 (22.2)	5 (55.6)	2 (22.2)	
*TNBCs	5 (31.2)	11 (68.8)		5 (31.3)	9 (56.3)	2 (12.5)	

[Table/Fig-13]: T-CD9 and S-CD9 expression with molecular classification.
(Significant association of T-CD9 Expression observed in Her 2 neu negative cases. The Chi-square statistic test was used. The p-value is 0.05).
*TNBCs: Triple Negative Breast Carcinoma

DISCUSSION

Breast carcinoma is one of the most common cancers worldwide [1]. Because of the extension of the tumour tissue and heterogeneous nature of the breast tumour metastases, the morbidity is increasing in breast carcinoma cases. The major concern about the breast carcinoma is its metastatic spread. To invade into mesenchymal stromal cells, breast cancer cells depend on CD9.

Many studies demonstrated that decreased expression of CD9 correlated with a poor prognosis in breast carcinomas [7]. In a study done by Rappa G et al., it was reported that the invasion of breast cancer cells into mesenchymal stromal cells was reduced with CD9 lacking breast cancer cells and this study suggested that by inhibiting CD9, metastases and invasion can be reduced [21].

In this study, CD9 was expressed in both tumour cells and stromal immune cells and their expression patterns have different association with clinicopathological features of patients with invasive breast carcinoma. In the present study, among 71 cases T-CD9 positive expression was observed in 40 cases. Out of these 31 cases were of invasive ductal carcinoma and nine cases were other histological types which include two mixed carcinoma cases (IDC+ILC), two mucinous carcinomas, two papillary carcinoma, one each of ILC, medullary and metaplastic carcinoma types. Remaining 31 cases showed negative T-CD9 expression. Among these 26 were IDC type and five cases were of other histological type which include three cases of mixed carcinomas (ILC +Mucinous, IDC+ Mucinous, IDC+ILC) and two phyllodes tumours.

In the study, done by Jamil F et al., it was noted that strong CD9 expression was seen in all normal and benign epithelial cells and 40% of ductal carcinoma in situ and IBCs. The rest of the tumour cases exhibited weak CD9 expression. There was no significant association of CD9 expression with pathological parameters and also with molecular markers such as tumour grading, lymph node metastases and ER, PR respectively. They had stated that CD9 marker could not serve as a significant prognostic marker in invasive breast carcinoma cases [22]. Present study showed similar results as the expression of T-CD9 marker was not significantly associated with clinicopathological parameters and with molecular markers except Her2neu negative marker.

The expression of CD9 showed weak intensity in benign ductal epithelial cells and normal breast tissues in this study. In this study the prognostic significance of CD9 expression was also compared with NPI scoring system and did not show any significant association [14].

The study done by Adams S et al., showed the expression of stromal immune cells in invasive breast carcinoma cases had better prognostic significance especially with molecular classification that includes mainly triple negative breast carcinoma cases [23]. In contrary to their study, present study did not show any significant association for stromal immune cells (S-CD9) with any clinicopathological parameters and molecular classification.

In the study done by Khomo S et al., CD9 was expressed more in chemotherapy resistant cases, disseminated spread and also in recurrent cases of lung carcinoma. This could be used for targeted therapy. When coming to metastases of breast carcinomas especially to the bone with comparison of primary breast carcinoma cases and visceral metastases, the expression of CD9 was considered significantly more in bone metastases [24].

In the study done by Kischel P et al., both prostate carcinomas and osteotropic breast carcinoma cases CD9 was excessively expressed [25].

Limitation(s)

Present study was a unicentric study done in a smaller sample size. The sample size of other histological types was also less. IHC for CD9 was not performed on metastatic lymph nodes.

CONCLUSION(S)

The immunostaining for CD9 expression was not significantly associated with Tumour cells (T-CD9) and Stromal immune cells (S-CD9) in breast carcinoma cases. However, it was significantly associated with Her2neu negative for T-CD9. Hence, CD9 can be used as prognostic marker for Her2neu negative cases. CD9 expression for tumour cells showed more positivity in Luminal A followed by triple negative whereas S-CD9 showed more positivity in Luminal B. Assessment of CD9 expression depends on many variables like genetic makeup and variability, tumour microenvironment and molecular pathogenesis in our geographical population. However, furthermore follow-up studies, genetic studies and compartment specific studies of CD9 expression in invasive breast carcinomas are needed for prognostic significance as there are no studies reported in India.

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