

Original Research Article

Calretinin expression in molecular subtypes of invasive carcinoma breast

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ABSTRACT

Background: Breast cancer is a leading cause of cancer death in women worldwide. Breast carcinoma is currently managed by assessing clinicopathological features. Elucidation of molecular mechanisms of pathogenesis of breast carcinoma may lead to the development of new targeted therapies, particularly in triple negative cancers. Literature shows a few studies on the expression of calretinin in breast carcinoma particularly in basal like type and its prognostic significance. In this study, authors are trying to assess the expression of a new marker calretinin in different molecular subtypes of invasive carcinoma breast.

Methods: This study was done in 107 cases of invasive carcinoma breast specimens received in Department of Pathology, Government Medical college, Kottayam from December 2017 to May 2019.

Results: Among the molecular subtypes, Basal like tumours showed 68.4% of cases with high level and 31.6% of cases with low level calretinin expression which is comparable with the study by Farrag et al. All the other molecular subgroups showed predominantly low level of calretinin expression.

Conclusions: Different molecular subtypes of invasive carcinoma breast showed varied calretinin expression. High level calretinin expression was significantly associated with grade 3 (p value = 0.002), ER negativity (p = 0.004), PR negativity (p = 0.018) and Basal like molecular subtype (p : <0.001). This suggests that calretinin might play a role in pathogenesis of basal like breast carcinomas.

Keywords: Calretinin, Invasive carcinoma, Molecular subtype

INTRODUCTION

Breast cancer is the most common non-skin malignancy in women and is a leading cause of cancer death worldwide with an estimated 1.67 million new cases diagnosed in 2012.¹ It has now become the major cause of morbidity and mortality among females in urban India.² Breast cancer is considered as a highly heterogeneous disease, comprising a number of biologically distinct entities with specific pathological features and biological behaviors.³ Different breast tumor subtypes have been identified which differ in their risk factors, clinical presentation, histopathological features, outcome, and response to systemic therapies.²

Invasive breast carcinoma can be classified based on molecular and morphologic characteristics into several clinically important subgroups. This subcategorisation is mainly based on the histological type and grade. The majority of breast cancers belongs to invasive ductal carcinoma of no special type, or not otherwise specified (NST/NOS).³ According to Nottingham Grading System, tumour is graded by assessing tubule formation, nuclear pleomorphism and mitosis.⁴

IHC markers including ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are classically used for molecular subtyping of breast carcinoma. These are hormonal and growth factor

receptors which are known to mediate cell growth signaling.³

Breast tumors are grouped into four basic subgroups according to these markers, they are: Luminal A- [ER+|PR+]HER2-, Ki67- (tumors with either ER or PR positivity, and HER2 negativity, low levels of Ki67), Luminal B - [ER+|PR+]HER2-,Ki67+ (tumors with either ER or PR positivity, and HER2 negativity, high levels of Ki67), and [ER+|PR+]HER2+,Ki67+ (tumors with either ER or PR positivity, and HER2 positivity, high levels of Ki67), HER2/neu over expressing : ER-PR-HER2+ (tumors with ER and PR negativity, and HER2 positivity, also named HER2 positive), Triple negative : ER-PR-HER2- (tumors with ER, PR, HER2 negativity).^{3,5}

As per the previous studies, ER-PR-HER2- tumors are described as one of the most relevant subtypes among breast tumors considering the lack of targeted therapies and aggressive clinical behaviour. These tumors can again be clustered into two distinct molecular classes : the basal and non-basal triple negative tumors. Studies have showed that basal and non-basal tumors also differ in their behaviour, outcome and therapeutic response.³ According to previous studies by Dai X, Xiang L et al, CK5/6 can be used to differentiate basal from non-basal tumours.³

Studies have showed that triple negative breast tumors frequently exhibit atypical metastatic patterns, such as early visceral metastases, along with lack of targeted therapy. Furthermore, they arise through one of the least understood pathways. Hence, novel markers are to be identified to establish breast origin, to suggest prognosis and to find pathogenetic mechanisms. This study aims to assess the calretinin, a commonly used mesothelial marker expression in the molecular subtypes of invasive carcinoma breast. Calretinin is a 29 kD, intracellular, vitamin D- dependant calcium binding protein with multiple functional roles including intracellular calcium buffering, message targeting, and neoplastic proliferation of the cells expressing it.⁶

According to the study by Farrag MS et al, high levels of calretinin expression were found most commonly in grade 3 invasive ductal carcinomas and in ER- and PR-negative breast carcinomas. Among the molecular subtypes, the basal-like (BL) subtype was the one that most frequently expressed calretinin.⁷

METHODS

A Descriptive study conducted 18 months, December 2017 to May 2019. Department of Pathology, Govt. Medical College, Kottayam, Kerala, India

Among 107 cases of invasive carcinoma breast specimens which are received, and hormone receptor studies have been done in the Department of Pathology,

Government Medical college, Kottayam during the study period.

$$\text{Sample size } N = \frac{4pq}{d^2}$$

p= prevalence / proportion in previous study

q= 100-p

d= precision / allowable error

In the study 'Calretinin expression as a reliable prognostic marker in different molecular subtypes of breast carcinoma' by Mayada Saad Farrag et al, proportion of patients with high calretinin expression was found to be 32 %, and fixing the absolute precision as 9, sample size N is calculated as ⁵

$$N = \frac{4 \times 34 \times 68}{9 \times 9} = 107$$

Study tools

1. Instruments to take bits of tissues to be studied.
2. Reagents for tissue processing.
3. Instruments for making paraffin blocks and cutting thin sections from it.
4. Glass slides and cover slips for mounting.
5. Microscope
6. Eosin- Haematoxyline staining.
7. Antibody and other reagents for immunohistochemical studies.
8. Proforma to record details.

Inclusion criteria

All mastectomy / wide excision specimens, tru cut biopsies with adequate material, diagnosed as invasive carcinoma breast, in which hormone receptor study has been done.

Exclusion criteria

1. Cases which receive preoperative neoadjuvant chemotherapy or radiotherapy
2. Tru cut biopsies of carcinoma breast with inadequate material
3. Cases of Invasive carcinoma breast in which hormone receptor study has not been done.

Study procedure

Relevant clinical details of each cases were recorded from requests accompanying the samples. Gross examination of the specimen was done. Appropriate bits of tissues, representative of areas to be studied were taken. All specimens were fixed in formalin, processed and embedded in paraffin. 4 microns thick sections were stained with H & E for routine histological examination. Histological type and grade were assessed.

For immunohistochemistry 3-micron thick sections were cut and stained with ER, PR, Her2, Ki67, CK5/6 and

calretinin antibodies. Estrogen receptor - EP1 Rabbit monoclonal antibody, progesterone receptor - EP2 Rabbit monoclonal antibody, Her2/erb2-EP3 Rabbit monoclonal antibody, Ki67-GM001 Mouse monoclonal antibody, CK5/6 EP24/EP67 Rabbit monoclonal antibody and Calretinin Rabbit polyclonal antibody were used following the manufactures instructions.

ER and PR stains were considered positive if expression was observed in more than 1% of tumour cell nuclei. Scoring of HER2 immunostaining is done according to 2018 ASCO/CAP guidelines (Table 1).⁸

Table 1: IHC Scoring of Her2/neu.

Score	Criteria
0 (Negative)	no staining or membrane staining that is incomplete and is faint/ barely perceptible and present in less than or equal to 10% of the tumour cells.
1+ (Negative)	incomplete membrane staining that is faint/ barely perceptible and in >10% of tumour cells
2+ (Equivocal)	weak to moderate complete membrane staining observed in >10% of tumour cells, need HER2-gene amplification by FISH.
3+ (Positive)	circumferential membrane staining that is complete, intense and in >10% of cells

Scores 0 and 1+ are considered negative whereas score 3+ is considered positive. Since score 2+ needed FISH confirmation, such cases were excluded from this study. Ki67 expression was considered low with a score of <14% and high with a score of ≥14%. CK5/6 was considered positive in cases with any cytoplasmic and/ or membranous staining in tumour cells including weak and strong staining.⁵

Nuclear and/or cytoplasmic staining was considered as positive calretinin immunostaining.⁵ A combined score was used to assess the degree of immunoreactivity.

Table 2: Scoring of percentage of calretinin positive tumour cells.

Score	Percentage of tumour cells
0	0%
1	<25%
2	25 - 50%
3	>50%

The combined score consisted of percentage or distribution of positive tumour cells and intensity of staining. The percentage of tumour cells (Table 2) and intensity of staining (Table 3) were scored according to the following tables.

The complete overall score was calculated by multiplying the percentage score and intensity score, which ranged from 0 -9 and was classified as follows (Table 4).

Table 3: Scoring of intensity of calretinin staining.

Score	Intensity of staining
0	0
1	1
2	2
3	3

Table 4: Overall calretinin scoring.

Overall calretinin score	Staining
0	Negative
1-2	Weak staining
3-6	Moderate staining
9	Strong staining

Both negative and weak staining were considered as low level expression of calretinin whereas both moderate and strong staining were considered as high level expression of calretinin. High level and low level expression of calretinin immunostaining are depicted in figures 1,2,3,4,5 and 6.

Data management and analysis

The data was entered in Microsoft excel and further statistical analysis was done using SPSS software.

Consent

This study was conducted on the specimens of Carcinoma breast, received in the Department of Pathology. Hence implied consent is present.

RESULTS

The present study was conducted on 107 specimens of invasive carcinoma breast received in the Department of Pathology, Government Medical College, Kottayam, during a period of 18 months.

The mean age of the present study population was 55.69 years with age ranging from 32 years to 79 years. Most of the cases belonged to ≥ 40 age group. 106 cases were females with one case of male breast carcinoma. 68.2 % of cases were Grade 2 tumours. According to the histological type, invasive carcinoma breast cases in the present study were distributed as 99 (92.5%) cases of NST and 8 (7.5%) cases of other subtypes.

Molecular subtypes were distributed as 53 (49.5%) cases of Luminal B, 19 (17.8%) cases of Luminal A and 10 (9.3%) cases were of Her2 overexpressed type, 19 (17.8%) cases of basal like and 6 (5.6%) cases of unclassified type. Calretinin expression was high in 32

(29.9%) cases and low in 75 (70.1%) cases. No calretinin expression was found in normal breast tissue. The correlation of calretinin expression with histopathological

and immunohistochemical parameters is depicted in table 5.

Table 5: Correlation of histological, immunohistochemical parameters and calretinin expression.

Histopathological and immunological parameters	Calretinin Expression		p value
	High Level Cr Expression	Low Level Cr Expression	
Tumour grade	1	3 (15.8%)	16 (84.2%)
	2	19 (26%)	54 (74%)
	3	10 (66.7%)	5 (33.3%)
Histological subtype	NST	29 (29.2%)	70 (70.8%)
	Other subtypes	3 (37.5%)	5 (62.5%)
ER immunoreactivity	Negative	18 (47.4%)	20(52.6%)
	Positive	14 (20.3%)	55 (79.7%)
PR immunoreactivity	Negative	19 (43.2%)	25 (56.8%)
	Positive	13 (20.6%)	50 (79.4%)
Her2/neu	Negative	24 (27.9%)	62 (72.1%)
	Positive	8 (38.1%)	13 (61.9%)
Ki67	Low	4 (17.4%)	19 (82.6%)
	High	28 (33.3%)	56 (66.7%)
CK5/6	Positive	13 (68.4%)	6 (31.6%)
	Negative	1 (16.7%)	5 (83.3%)

Table 6: Calretinin expression in molecular subtypes of invasive carcinoma breast.

Molecular subtype	High level expression	Low level expression	Total	p value
Luminal A	2 (10.5%)	17 (89.5%)	19(100%)	0.053
Luminal B	13 (24.5%)	40 (75.5%)	53 (100%)	0.229
HER2 overexpressed	3 (30%)	7 (70%)	10 (100%)	1
Basal Like	13 (68.4%)	6 (31.6%)	19 (100%)	<0.001
Unclassified	1 (16.7%)	5 (83.3%)	6 (100%)	0.666
Total	32	75	107	

Among the molecular subtypes, Basal like type showed high level of calretinin expression in 68.4% (13) of cases which was significant with a p value of <0.001. Luminal A, Luminal B, HER2 overexpressed and unclassified subtypes showed low level expression of calretinin in 89.5%, 75.5%, 70% and 83.3% of cases respectively. The proportion of each of the molecular subtypes showing high and low levels of calretinin expression is depicted in the table 6.

Photomicrographs

Different cases of invasive carcinoma breast showing high and low levels of calretinin expression are shown in figures 1 to 6.

DISCUSSION

Hormonal receptors are now used for molecular classification of breast carcinoma, among which triple negative tumours are considered relevant considering the lack of targeted therapies, aggressive behavior and poor prognosis. Elucidation of molecular mechanisms of pathogenesis of this subgroup with additional markers may bring light to the mechanism of pathogenesis and development of novel therapies.

Calretinin is a useful marker for the diagnosis of malignant mesothelioma. It has multiple functional roles such as intracellular calcium buffering and message targeting. There are only a few studies describing the association of calretinin and breast carcinoma and its prognostic significance. This study aimed to determine calretinin (CR) expression in invasive breast carcinoma with respect to the molecular subtypes.

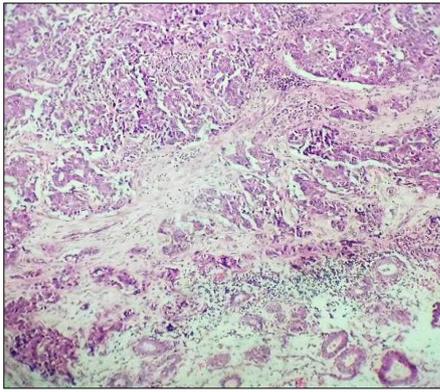


Figure 1: Invasive carcinoma breast H&E 10 X.

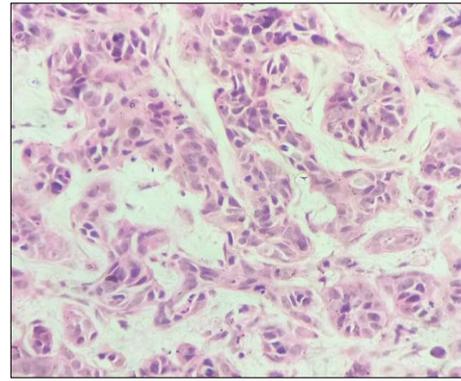


Figure 5 : Invasive carcinoma breast H&E 40 X.

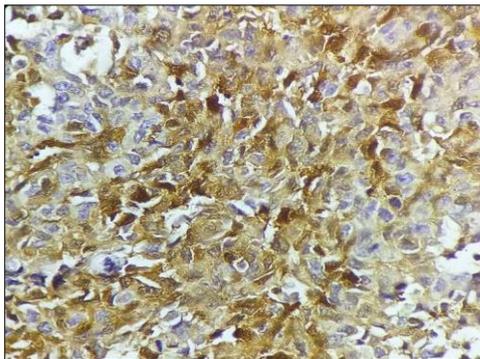


Figure 2: High level calretinin expression - 40 X.

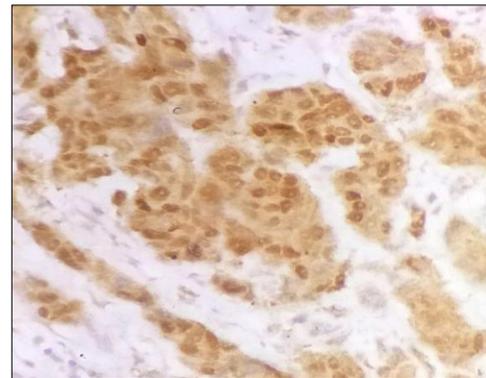


Figure 6: High level calretinin expression - 40 X.

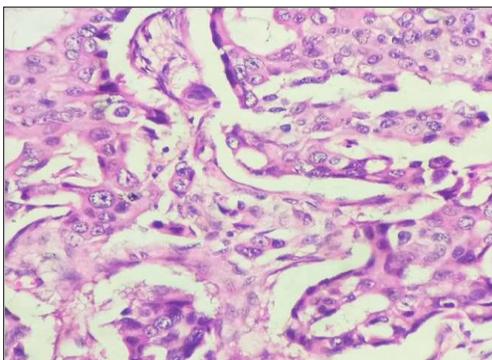


Figure 3 : Invasive carcinoma breast H&E 40 X.

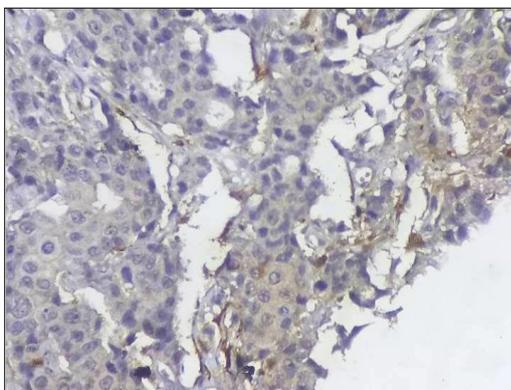


Figure 4: Low level calretinin expression - 40 X.

In the present study, out of 107 cases, high level expression of calretinin was seen in 32 (29.9%) of cases and low level expression in 75 (70.1%) of cases. This is in agreement with the study by Farrag et al which showed high level CR expression in 32% of cases. Among the histological types, Invasive carcinoma of NST showed high calretinin expression in 29 (29.2%) cases and 3 (37.5%) cases of other subtypes showed high calretinin expression in the present study which was also similar to the study by Farrag et al.⁵

Table 7: Calretinin expression and ER, PR immunoreactivity comparison with other studies.

Study	High level Cr expression	
	ER Negative	PR Negative
Present study	47.4%	43.2%
Farrag et al	45.9%	39.7%
Aboobacker D. K et al	57.1%	57.1%

With respect to the grade, high calretinin expression was seen in grade 3 tumours (66.7%, 10 cases) which was in agreement with studies by Farrag et al (61.9%) and Aboobacker DK et al (58.3%). High level calretinin expression was observed in 47.4% of ER negative and 20.3% of ER positive tumours.⁹ Similarly, high calretinin was observed in 43.2% of PR negative and 20.6% of PR positive tumours. Hence, high level of CR expression

was significantly associated with ER and PR negativity. This observation was in line with the observations by Farrag et al, Aboobacker DK et al (Table 7).

High level calretinin expression was found to be more in HER2/neu positive tumours (38.1%) as compared to HER2/neu negative tumours (27.9%). This was in agreement with the study conducted by Farrag et al and contrast to study done by Aboobacker D K et al. High level of calretinin expression was associated with high level expression of Ki67 in the present study, which was similar to the studies conducted by Farrag et al and Aboobacker D K et al.⁵ High level of CR expression was observed in carcinomas expressing basal markers (68.4%) in the present study. This was in line with the studies done by Farrag et al, Taliano et al, and Powell et al.^{10,11}

Different molecular subtypes of breast cancer showed varied calretinin expression. Among the basal like subtype, 68.4% of cases showed a significant high calretinin expression compared to 31.6% of cases with low level expression. Regarding other subtypes, 89.5% cases of Luminal A, 75.5% cases of Luminal B and 83.3% cases of unclassified tumours showed low level of calretinin expression. These observations were similar to the studies by Farrag et al and Taliano et al.

In the present study, only 30% of HER2 overexpressed tumours showed high level of calretinin expression whereas 70% of cases showed low level of calretinin expression. This was in agreement with the study by Taliano et al and in contrast to the study by Farrag et al. This disagreement with study by Farrag et al may be due to the decreased number of HER2 overexpressed tumours included in the study (Table 8).

Table 8: Calretinin expression in molecular subtypes - comparison with other studies.

Molecular subtype	High level Cr expression		
	Present study	Farrag et al ⁵	Taliano et al ¹¹
Luminal A	10.5%	9.5%	11.1%
Luminal B	24.5%	33.3%	12.7%
Her2 overexpressed	30%	59.3%	33.3%
Basal like	68.4%	70%	54.3%
Unclassified	16.7%	17.2%	30%

A few studies have been conducted to study the prognostic implications of calretinin in cancers. Farrag et al found a statistically significant correlation between high level calretinin expression and poor patient overall survival. Taliano et al demonstrated the association of calretinin expression with basal like tumours and that high level calretinin expression appeared to be a strong predictor of adverse prognosis. In the present study, the association of high level calretinin expression with higher

Ki-67 indices which has been confirmed as a prognostic factor, refers to the prognostic importance of calretinin.

CONCLUSION

Among the molecular subtypes, Basal like subtype showed significant high level of calretinin expression whereas the other subtypes showed low level of calretinin expression predominantly. The association between high level calretinin expression and basal like breast carcinoma suggests that calretinin might play a role in the pathogenesis of such high grade carcinomas. This hypothesis is supported by studies in colon cancer cell lines which showed that increased calretinin expression has been linked to increased resistance of these tumour cells to the apoptotic signals.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee Review Board, Govt Medical College, Kottayam, Kerala, India

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