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Original Research Paper

Pathology

STUDY OF CYCLIN D1 AND KI-67 EXPRESSION IN INVASIVE BREAST CANCER AND ITS CORRELATION WITH TUMOR GRADING

Dr. Mayank Kumar Singh	Professor & Head, Department Of Pathology, MLB Medical College, Jhansi, UP.
Dr. Rajesh Kumar Bhatt*	Junior Resident, Department Of Pathology, MLB Medical College, Jhansi, UP. *Corresponding Author
Dr. Dvijendra Nath	Professor, Principal & Dean, Govt. Medical College, Jalaun, UP
Dr. Chhaya Shevra	Assistant Professor, Department Of Pathology, MLB Medical College, Jhansi, UP

ABSTRACT

BACKGROUND : Breast cancer (BC) is the most common cancer and leading cause of death in women. In India, almost 100,000 women are diagnosed with BC every year and a rise to 131,000 cases is predicted by 2020. It is associated with significant morbidity as it affects patients at a younger age.

MATERIAL AND METHODS : The prospective study was conducted on 40 formalin-fixed embedded tumor specimens of female breast lump of cancer patients. The TNM classification classes T1 to T4 were used to evaluate the tumor size T1: ≤ 2 cm, T2: > 2 cm but ≤ 5 cm, T3: > 5 cm and T4: tumor of any size. Haematoxylin Eosin Staining was done for all specimens and were classified according to WHO.

RESULTS : The maximum numbers of cases 12 (30%) were in premenopausal age group of 31-40 years. Maximum 19 (47.50%) cases were of tumor size >5 cm (T3). Out of these 26 positive cases, 6 (15%) cases have shown strong (+3) positivity while 9(22.50%) cases were show intermediate (+2) positivity and 11(27.50%) cases were weak (+1) positive for cyclin D1 expression. Out of 30 positive cases, 7 (17.50%) cases have shown strong (+3) positivity, 14 (35.0%) cases shown intermediate (+2) positivity, while 9 (22.50%) cases were shows weak (+1) positivity for Ki67 expression.

CONCLUSION : Cyclin D1 overexpression was inversely related to histological grades and Ki67 expression was directly related to tumor grades of breast carcinoma. There is no significant statistical relationship were found between cyclin D1 and Ki67 expression in breast carcinoma cases.

KEYWORDS : Cyclin D1, Ki-67, immunohistochemistry, over expression, Breast carcinoma

INTRODUCTION

Breast cancer is one of the most common carcinomas in women. Its incidence in India is rising and it is associated with significant morbidity as it affects patients at a younger age and most of the cases are detected at an advanced stage.[1]

Breast cancer is a heterogeneous disease and is currently divided into subtypes in accordance with the status of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [2]. These subtypes display significant diversity in regard to the clinical behavior, outcome and response to therapy[3,4]. One of these subtypes, triple-negative breast cancer (TNBC), which is characterized by a lack of ER, PR and HER2 expression, accounts for 10% to 20% of all breast cancers, and has a high probability of early tumor relapse after diagnosis, increased propensity to develop brain metastases, and rapid risk of death after tumor relapse[5,6].

Cyclin D1 is one of the main regulatory molecules of the cell cycle[7]. It belongs to the family of D-type cyclins, which regulate cell cycle progression from G1 to S phase by regulating the activity of cyclin-dependent kinases (CDKs)[8].

Binding of cyclin D1 to CDK4 and CDK6 induces hyperphosphorylation of retinoblastoma protein (Rb). Recent findings have revealed further roles of Cyclin D1 in promoting cell cycle progression through CDK-independent mechanisms such as interaction with and modulation of transcription factor activities[9].

The Ki-67 protein was originally defined by 359 kDa monoclonal antibody Ki-67, obtained by immunising mice with the nuclei of the Hodgkin's lymphoma cell line L248. Ki-67, a nuclear DNA binding protein which is expressed in all the vertebrates is a widely used marker of proliferation used for grading tumours[10]. Ki-67 labelling index as determined by immunohistochemistry (IHC) analysis on paraffin embedded section and percent positive frequency is indicative of patient outcomes. High Ki-67 index generally shows poor prognosis in clinical conditions[11]. Recently, Ki-67 has drawn increasing attention as an attractive prognostic, prediction and potential therapeutic target in subtypes of breast cancer. Ki-67 is present in all proliferating cells, and its role as a proliferation marker attracts considerable interest.

The present study aims to find the frequency of Cyclin D1 and Ki-67 overexpression in invasive breast cancer and its correlation with tumor grading.

MATERIAL AND METHODS

The prospective study was conducted on 40 formalin-fixed paraffin embedded tumor specimens of female breast lump of cancer patients received in the Department of Pathology, MLB Medical College Jhansi from May 2019 to October 2020. Informed consent was taken from the patients and approval of the Ethical Committee was taken for the use of the tumor specimens and patient medical records.

The diagnosis was re-evaluated according to the WHO classification in the course of grading and immunohisto chemical staining. The TNM classification classes T1 to T4 were used to evaluate the tumor size T1: ≤ 2 cm, T2: > 2 cm but ≤ 5 cm, T3: > 5 cm and T4: tumor of any size, with direct extension to chest wall or skin. Haematoxylin Eosin Staining was done for all specimens. All H&E stained tissue sections were classified according to WHO and then histological grading were done.

Inavasive ductal carcinomas and all other invasive tumors are graded based on an assessment of tubule/gland formations, nuclear pleomorphism and mitotic counts.

Feature	Score
Tubule and gland formation	
Majority of tumor (>75%)	1 point
Moderate degree (10-75%)	2 points
Little or more (<10%)	3 points
Nuclear pleomorphism	
Small, regular uniform cell	1 points
Moderate increase in size and variability	2 points
Marked variation	3 points
Mitotic counts	
Dependent on microscopic field area	1 to 3 points

In present study we count mitosis by using microscope of field diameter of 0.65 mm and field area of 0.332 mm^2. The three values are added together to produce score of 3 to 9, which the grade in assigned as follows-

Grade I	Well differentiated	3-5 points
Grade II	Moderately differentiated	6-7 points
Grade III	Poorly differentiated	8-9 points

Immunohistochemistry Staining Proceduce Of Cyclin D1

Cyclin D1 expression was determined by immunohisto chemistry (IHC) using specific anti-cyclin D1 monoclonal antibodies. Sections (3-4 µm thick) of each block will be mounted on superfrost/plus slides, air dried overnight, and heated to 60°C for 1 h to promote section adherence. Cut sections will be stored at 4°C. Prior to staining, the sections will be dried overnight, deparaffinized in xylene and rehydrated in an alcohol series (100%, 70% and 40%). Antigen unmasking will accomplished using a high-temperature technique by boiling under pressure in a 0.1 M citrate buffer (pH = 6) at 116°C for 2 min, then cooling for 20 min in a water bath. Endogenous peroxidase activity will quenched with 3% hydrogen peroxide at room temperature for 10 min, followed by two phosphate buffered saline (PBS) washes (pH = 7.4). Nonspecific epitopes will be locked with 1 normal bovine serum albumin in PBS (room temperature for 20 min, no PBS wash). Sections will be then incubated with 1:50 anti-cyclin D1 in 2% DSA/PBS (4°C overnight). After two PBS washes, primary antibody binding will be detected with 1:200 biotinylated goat antimouse secondary antibody and an avidin-biotin-peroxidise (HRP) and diaminobenzidine color detection system, used according to the manufacturer's instructions. The monoclonal antibody to cyclin D1 demonstrated no cross-reactivity with cyclin D2 and D3 using immunoblotting techniques. Sections will lightly counter stained with Harris hematoxylin.

The intensity and distribution of cyclin D1 immunoreactivity will be semiquantitatively scored using the Allred scoring method. The intensity of immunohistochemical reaction by light microscopy will be recorded as 0 (negative) when no staining of the nuclei was seen even at high magnification, 1+ (weak) if staining was visible only at high magnification, 2+ (moderate) when staining was readily visible at low magnification and 3+ (strong) if staining was strikingly positive even at low power magnification.

IHC staining procedure for Ki 67

Immunohistochemical study was performed as prescribed by manufacturer instructions (Dako, CA, USA). Formalin fixed paraffin embedded tissue sections were deparaffinized, and were heated in Target Retrieval Solution. Sections were then incubated in 10% normal goat or rabbit serum to reduce non-specific antibody binding. Tissue sections were then incubated with Ki67 (clone MIB-1, dilution 1:00; Dako). The slides were treated with streptavidinperoxidase reagent, and were incubated in PBS diaminobenzidine and 1% hydrogen peroxide v/v, followed by counterstaining with Mayer's hematoxylin. Positive and negative controls for each marker were used according to the supplier's data sheet (Dako).

The Ki-67 immunostaining is also give nuclear brown color deposits. The Ki-67 status was defined based on the intensity and the percentage of nuclear stain. Negative Ki-67 was defined as less than 1% tumor cells are showing nuclear brown deposits, and positive Ki-67 when greater than 1% tumor cells shows nuclear positivity. Patients with positive Ki-67 were divided into 3 groups:

1-5%cells positive- **Weak** staining
6-14% cells positive- **Intermediate** staining
≥ 15% cells positive –**Strong** expression of Ki-67 staining.

All statistical analyses were performed with SPSS software for Windows. The Chi-square test ($\chi 2$) was used to examine the categorical variables and the association between cyclin D1 and Ki67 status and other clinicopathological variables. Both univariate and multivariate analysis will be done to examine the relation of each prognostic factor with cyclin D1 and Ki67 status. All statistical tests were two-sided. P <0.05 were considered as significant.

RESULTS

The maximum numbers of cases were in premenopausal age group of 31-40 years that is 12 (30.0%) cases, followed by 9 (22.50%) cases in the menopausal age group 41-50 years. Majority of case are above 31 years of age. Maximum 19 (47.50%) cases were of tumor size >5 cm (T3) followed by 17 (42.50%) cases of size ranging between 2-5 cm (T2) and only 4 (10.0%) cases were of tumor size <2 cm (T1).

Table – 1 : Distribution of cases according to histological grading

Histological grade	No. of cases	Percentage
Grade I	12	30.0%
Grade II	23	57.50%
Grade III	5	12.50%
Total	40	100%

Maximum 23 (57.50%) cases were belongs to histological grade 2, followed by 12 (30.0%) cases were of grade 1, and only 5 (12.50%) cases were fall in grade 3.

Out of 40 cases, only 19 cases were submitted with lymph nodes. Out of these 19 cases with lymph nodes, 15 (78.94%) cases were positive for malignancy and 4 (21.06%) case were found negative for malignancy.

Table – 2: Distribution of cases according to Cyclin D1 and Ki-67 overexpression

	Cyclin D1 expression		Ki67 expression	
	No. of cases	**Percentage**	**No. of cases**	**Percentage**
Weak (+1)	11	27.50%	9	22.50%
Intermediate (+2)	9	22.50%	14	35.0%
Strong (+3)	6	15.0%	7	17.50%
Negative	14	35.0%	10	25.0%
Total	**40**	**100%**	**40**	**100%**

Out of these 26 positive cases, 6 (15%) cases have shown strong (+3) positivity while 9(22.50%) cases were show intermediate (+2) positivity for cyclin D1 expression and 11(27.50%) cases were weak (+1) positive for cyclin D1 expression in our study.

Out of 30 positive cases, 7 (17.50%) cases have shown strong (+3) positivity for Ki67 expression and maximum, 14 (35.0%) cases shown intermediate (+2) positivity for Ki67 immunostaining., while 9 (22.50%) cases were shows weak (+1)positivity for Ki67 expression.

Table – 3 : Correlation of histologic grading with Cyclin D1 and Ki67 Expression

Histologic grading	Cyclin D1		Ki 67	
	Positive	**Negative**	**Positive**	**Negative**
1 (12)	7 (58.3%)	5(41.7%)	7 (58.3%)	5 (41.7%)
2 (23)	17 (73.9%)	6 (26.1%)	19 (82.6%)	4 (17.4%)
3 (5)	2 (40%)	3 (60%)	4 (80%)	1 (20%)
Total	26	14	30	10
P value	Chi square statistic is $\chi2=2.4112$. The *p*- value is 0.29951, for cyclin D1. *The results are **Not** significant at p <0.05*		Chi square statistic is $\chi2=2.5546$. The *p*- value is 0.27879 for Ki67. *The results are **Not** significant at p <0.05*	

12 cases of histological grade I, only 7(58.3%) were shows immunostaining positivity for cyclin D1 and Ki 67 each. while 5 (41.7%) cases of Grade I were negative for cyclin D1 expression and Ki67 expression.

Out of 23 of histologic grade II cases, 17(73.9%) were positive for cyclin D1 expression and 19(82.6%) were positive for Ki67expression, while 6 (26.1%)cases and 4 (17.4%)cases of Grade II were negative for cyclin D1 and Ki67 expression respectively.

Out of 5 cases of histologic grade III, 2 (40%) cases have shown overexpression for cyclin D1, while 4 (80%) were positive for Ki 67 expression on the other hand 3 (60%) cases and 1(20%) cases of histological grade III were negative for cyclin D1 and Ki67 expression respectively.

The above data was calculated for significance, no significant relation was found between Cyclin D1 and Ki67 expression with Tumor Grades of invasive breast cancer as the *p*- value is 0.29951 and 0.27879, for cyclin D1 and Ki67 respectively, which is >0.05.

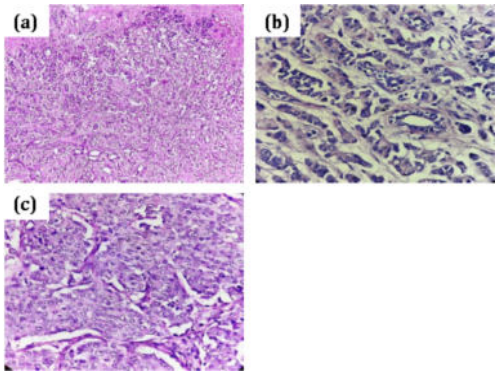


Fig 1 : Photomicrograph showing invasive breast carcinoma (a) Histological Grade 1 (H&E, 100X) (b) Histological Grade 2 (H&E, 400X) (c) Histological Grade 3 (H&E, 400X)

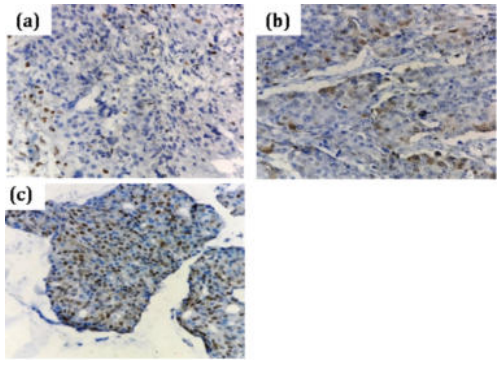
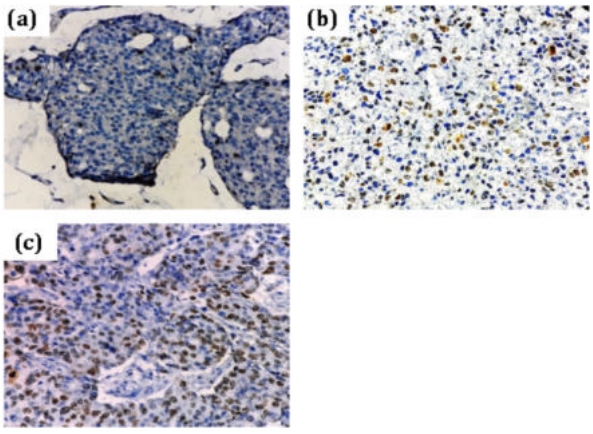


Fig 2 : Photomicrograph showing Cyclin D1 (a) Weak (+1) nuclear positivity (IHC, 400X) (b) Intermediate (+2) nuclear positivity (IHC, 400X) (c) Strong (+3) nuclear positivity (IHC, 400X)



Photomicrograph showing Ki67 (a) Weak (+1) nuclear positivity (IHC, 400X) (b) Intermediate (+2) nuclear positivity (IHC, 400X) (c) Strong (+3) nuclear positivity (IHC, 400X)

DISCUSSION

Breast cancer (BC) is the most common cancer and leading cause of death in women[12]. It is a heterogeneous disease encompassing several pathological and molecular subtypes characterized by different outcomes and responses to a given treatment[13]. In India, almost 100,000 women are diagnosed with BC every year and a rise to 131,000 cases is predicted by 2020[14].

40 formalin fixed paraffin embedded tumor specimen of malignant breast lesions were included in study to determine the relationship of cyclin D1 overexpression and Ki67 expression with well-known clinicopathologic prognostic determinants like age, tumor size, lymph node status, histological type and tumor grade in invasive breast carcinoma.

Breast cancer has been known to occur in a much younger age group in Asian populations when compared to the West with as many as 26% of breast cancer patients being younger than 35 years in some studies.***(Mathew A, et al, 2004)*** [15] Although the cause of this early occurrence remains to be elucidated, a younger age at diagnosis has been associated with larger tumor size, higher grade and low levels of hormone receptors thereby prevailing a poor prognosis in this age group.

In our study of 40 cases, maximum 12 (30.0%) cases were in the age group of 31-40 years followed by 9 (22.50%) cases in age group 41-50 years, which shows majority in age above 31 years with mean age 47.7 years which is consistent with the previous study by ***Santanu Sarkar, et al, 2019***[16] in which mean age of the study population was 46.6 years (range, 26–71 years). The maximum number, 49 (44.6%) cases was in ≥50 year of age group. However this was also consistent with the findings ***of Kenny et al.(1999)***[17] and ***Michalides et al.,(1996)***[18] we found no significant correlation between age and cyclin D1 overexpression. Our results are consistent with **DeSantis *et al.*** [19], who reported increased incidence of breast cancer by increasing age. Our results also consistent with **Nelson *et al.***[20].

We observed that maximum 19 (47.50%) cases were of tumor size >5 cm (T3) followed by 17 (42.50%) cases were of tumor size between 2-5 cm (T2) and only 4 (10.0%) cases were ≤2 cm (T1) in tumor size. This is in accordance with the study of **Lengare PV *et al*** (2020), 50 cases distributed according to tumor size with <2 cm (pT1) was 12%, 2-5 cm (pT2) in 58%, >5cm (pT3) in 24% and only 6% were categorized as pT4.

In our study, maximum 23 (57.50%) cases belonged to histological grade II, 12 (30.0%) cases of grade I and 5 (12.50%) cases categorized as histological grade III. Similar results were found by ***Peurala et al, 2013***[21], who showed 30

(29.4%) cases in histological grade I, 35 (34.3%) cases were in histological grade II and 37 (36.3%) cases were of histological grade III type.

In our study of 40 cases of breast carcinoma, only 19 cases had undergone radical mastectomy, was submitted with lymph nodes. Out of these 19 cases, 15 (78.94%) were positive for tumor invasion and 4 (21.06%) were negative for tumor invasion. According to ***Wartgotz and Norris (1990)*** about one third of the reported cases had lymph node metastasis.

There were no significant correlations between cyclin D1 and Ki67 expression with lymph node status, tumor size, or DNA ploidy. Interestingly, cyclin Dl overexpression was especially found in tubular, mucinous, and invasive cribriform cancers. These are well differentiated tumor types with relatively low nuclear a typicality, low proliferation, positive steroid receptor status, and favorable prognosis ***(Ellis IO, et al, 1992)***[22].

Our study of 40 cases of breast carcinoma reveals that 26 (65.0%) cases have cyclin D1 overexpression, while 14 (35.0%) cases are negative for cyclin D1 expression. Out of these cyclin D1 positive cases, 6(15%) have shown strong positivity, 9 (22.50%) were shows intermediate positivity for cyclin D1 while 11(27.50%) cases have shown weak positivity for cyclin D1 immunostaining. This is accordance with the study of ***Paul J. van Diest, et al, 1997***[23] where cyclin Dl overexpression was significantly negatively correlated with histological grade, overexpression of cyclin Dl was found in 87 of 148 cases (59%). Similar results are also found in the study of ***Bartkova et al (1994)*** [24] and ***Zhang et al (1994)*** [25] where cyclin D1 was overexpressed in 59% of cases of breast carcinoma.

In accordance with the results of cyclin D1 overexpression which is somewhat higher than in the study by ***Michalides et al.***(1996) [18]. Cyclin D1 overexpressin is also higher than the amplification of cyclin D1 gene, found in 19% of cases in a previous study ***(Schuuring E et al, 1992)*** [26]. This implies that there may be overexpression of cyclin Dl in the absence of amplification of gene, as has been found by others [11,19,26,27].

This could be due to a mutation-induced longer half-life of the protein or a translocation or as a result of increased hormone sensitivity. As to the latter, cyclin Dl overexpression was indeed strongly correlated to estrogen receptor positivity, as in a previous study ***(Michalides R, et al, 1996)***[18].

In a study of 102 breast carcinoma cases by ***Peurala et al, 2013***[21], who showed Ki67 expression were negative in 21 (20.6%) cases and strongly positivity was shown by 24(23.5%), intermediate 20 (19.6%) and weak expression was shown in 37(36.3%) cases of invasive breast carcinoma, similar results observed in our study of 40 cases, 30 (75.0%) cases have shown positivity for Ki-67 expression and 10 (25.0%) cases were negative for Ki-67 expression. From 30 cases of Ki67 positive expression, 7 (17.5%) cases have shown strong positivity, 14 (35.0%) cases have shown intermediate positivity while 9(22.50%) cases were shown weak positivity for Ki67 expression.

CONCLUSION

Cyclin D1 overexpression was inversely related to histological grades that is overexpression is more intense in lower grades of breast carcinoma and Ki67 expression was directly related to tumor grades that is overexpression seen in tumor of higher grades.

There is no significant statistical relationship were found between cyclin D1 and Ki67 expression in breast carcinoma cases tumor grades in our study. Hence Cyclin D1 and Ki 67 immunostaining can be used as prognostic marker routinely for invasive breast carcinoma, but further more studies with bigger sample size are needed to confirm the statistical significant.

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