

Correlation between cyclin D1 expression and standard clinicopathological variables in invasive breast cancer in Eastern India

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Abstract

Introduction: Breast cancer is the leading oncogenic threat in South-East Asian women showing an inexplicable biological aggressiveness. High expression of cyclin D1, a key molecule in breast cancer pathogenesis, has been shown by previous studies in the Western world to be associated with favorable tumoral characteristics. Apart from determining the correlation between cyclin D1 expression and standard clinicopathological variables in invasive breast cancer in Eastern India, questions that we aimed to answer through this study included: Is there a significant regional difference in expression patterns of this protein? And if yes, can it possibly account for the epidemiological differences in breast cancer occurrence and biological behavior? Finally, is testing for overexpression of this protein in regions with limited resources beneficial? **Materials and Methods:** The present study was carried out on 110 previously untreated, female patients with primary breast carcinoma. Cyclin D1 expression was determined by immunohistochemistry using specific anti-cyclin D1 monoclonal antibodies. **Results:** Overexpression of cyclin D1 was found in 78 of 110 cases (70.9%). High expression of cyclin D1 showed a significant negative correlation with tumor size ($P = 0.023$) and tumor grade ($P = 0.045$). Estrogen receptor and progesterone receptor positive cases showed a significantly positive correlation with cyclin D1 overexpression ($P = 0.026$ and 0.046 , respectively). Interestingly, cyclin D1 positivity showed a strong correlation with the type of surgical procedure performed ($P = 0.002$). **Conclusion:** Cyclin D1 overexpression in breast cancer is associated with less aggressive tumoral characteristics. Furthermore, its potential epidemiological role and utility as a prognostic marker have been discussed.

Key words: Breast cancer, cyclin D1, immunohistochemistry, prognostic factor

Introduction

More than 400,000 women die of breast cancer each year. Although breast cancer is considered to be a disease of the developed world, the incidence of breast cancer in developing countries has increased at an alarming rate over the last 40 years. It is estimated that 1.7 million women will be diagnosed with breast cancer in 2020 - a 26% increase from current levels, mostly in the developing world. Breast cancer is already the leading cause of cancer in South-East Asian women.^[1] In India, almost 100,000 women are diagnosed with breast cancer every year, and a rise to 131,000 cases is predicted by 2020.^[2]

The lack of breast cancer awareness, absence of proper screening programs, social barriers to early diagnosis and treatment along with misconceptions about cancer treatment and outcomes, while contributing to the dismal statistics mentioned above do not entirely account for it. Although the regional influence in breast cancer with relation to age at presentation, clinicopathological features, and outcome of treatment has been widely reported, the biological aggressiveness of breast cancer in these regions in terms of affecting younger females, poor histologic grade, low rates of hormone receptor positivity and overall poor survival remain unexplained.

Thanks to the advances in breast cancer research, a number of proto-oncogenes have been described, abnormalities in the structure and activity of which may contribute to the development and progression of breast cancer.

Cyclin D1 is one such proto-oncogene. Originally cloned as an oncogene responsible for parathyroid adenomas, its role in the development of human breast cancers has been well documented following the observation that transgenic mice overexpressing this cyclin in their breast tissue were prone to mammary adenocarcinomas.^[3] Subsequently, alterations in

cyclin D1 has been the subject of intense study in Western females with breast cancer leading to its proposal as a putative prognostic marker of potential clinical utility.^[4]

At present few studies exist on the clinicopathological correlation of cyclin D1 expression in breast cancer in developing countries like India. The objective of this prospective study was two-fold: First, to determine the prevalence of cyclin D1 overexpression in patients with invasive breast cancer and to assess its correlation with standard clinicopathological variables in an effort to elucidate the epidemiological differences in breast cancer occurrence and biological behavior. Second, to evaluate the utility of cyclin D1 testing as a prognostic marker in countries with limited resources.

Materials and Methods

Case selection

This study was carried out in the Department of General Surgery in a Tertiary Care Teaching Hospital in Eastern India. A total of 110 participants were included in the study. The participants consisted of female patients with primary breast carcinoma. Male patients with breast cancer were excluded from this study because breast cancer in male patients are rare, probably a different entity, and there may be a different expression of hormone receptors.^[5] Also excluded from the study were breast cancer patients who had previously received radiotherapy, chemotherapy or hormonal therapy, since systemic therapy may alter the expression of cyclin-D1, human epidermal growth factor receptor 2 (Her-2/neu) receptors and steroid hormone receptors.^[6]

Clinical status was determined by available clinical data in the form of history taking using a structured questionnaire, physical examination and imaging techniques (including ultrasound, mammography, X-ray and isotope bone scan as was deemed appropriate). The initial cancer diagnoses were based on the review of pathologic slides obtained mainly by core needle biopsy.

Written consent was taken from all participants, and the study was approved by the Institutional Ethics Committee.

Specimen preparation

The fresh surgical specimens were fixed in 10% buffered

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formalin, processed, and embedded in paraffin using the standard protocol. Routine staining with hematoxylin and eosin for diagnosis and histological typing was performed according to the World Health Organization criteria and graded according to the Elston and Ellis modification of Scarff-Bloom-Richardson system.^[7] All specimens were evaluated without the knowledge of clinical data.

Immunohistochemistry

Cyclin D1 expression was determined by immunohistochemistry (IHC) using specific anti-cyclin D1 monoclonal antibodies. IHC was the preferred method in our study because of its wide availability, easy preservation of stained slides, use of the familiar light microscope and relatively low cost, which is an important consideration in developing countries with limited resources. Sections (3 µm thick) of each block were mounted on superfrost/plus slides, air dried overnight, and heated to 60°C for 1 h to promote section adherence. Cut sections were stored at 4°C. Prior to staining, the sections were dried overnight, deparaffinized in xylene and rehydrated in an alcohol series (100%, 70% and 40%). Antigen unmasking was 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 was quenched with 3% hydrogen peroxide at room temperature for 10 min, followed by two phosphate buffered saline (PBS) washes (pH = 7.4). Nonspecific epitopes were locked with 1 normal bovine serum albumin in PBS (room temperature for 20 min, no PBS wash). Sections were then incubated with 1:50 anti-cyclin D1 in 2% DSA/PBS (4°C overnight). After two PBS washes, primary antibody binding was detected with 1:200 biotinylated goat antimouse secondary antibody and an avidin-biotin-peroxidase (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 were lightly counterstained with Harris hematoxylin.

Cyclin D1 immunostaining is nuclear (brown color deposits) with occasional faint cytoplasmic staining. Only cyclin D1 nuclear staining intensity was considered for analysis after evaluation of 500 cells in the tumor areas at medium and high magnification by light microscopy in all cases. The following scoring system was used: 1+, weak staining; 2+, moderate staining; 3+, strong staining. All tumors demonstrating cyclin D1 nuclear staining were considered as being positive for cyclin D1 overexpression, irrespective of the intensity of staining (+1, +2, +3). Estrogen receptor (ER) and progesterone receptor (PR) status was determined by IHC using rabbit monoclonal antibody (ER, clone SP1; PR, clone SP2; Labvision USA) from paraffin embedded histopathology specimens. ER and PR positivity were defined as the presence of 10% or more positively stained nuclei in ten high-power fields.

The intensity of Her-2/neu membrane staining was scored as 0, 1+, 2+ or 3+ (according to the standardization of the particular laboratory concerned). Tumors with 2+ or 3+ scores were

classified as positive for Her-2/neu overexpression, whereas tumors with 0 or 1+ scores were considered as negative. All specimens were evaluated without the knowledge of clinical data.

Statistical analysis

All statistical analyses were performed with SPSS software version 11.0.1 for Windows (SPSS Inc., Chicago, IL, USA). The Chi-square test (χ^2) was used to examine the categorical variables and the association between cyclin D1 status and other clinicopathological variables. Both univariate and multivariate analysis were done to examine the relation of each prognostic factor with cyclin D1 status. The frequency of cyclin D1 expression according to joint ER/PR status and the distribution of the hormone receptor status (ER/PR) according to cyclin D1 were also calculated. All statistical tests were two-sided. $P < 0.05$ were considered as significant.

Table 1: Patient and tumor characteristics

Characteristics	Number of patients (%)
Age (years)	
<35	23 (20.9)
35-49	38 (34.5)
>49	49 (44.6)
Tumor size	
T1 (<2 cm)	33 (30)
T2 (2-5 cm)	45 (40.9)
T3 and T4 (>5 cm)	32 (29.1)*
Number of positive nodes	
<3	26 (23.6)
>3	60 (54.6)
Negative	24 (21.8)
Tumor grade	
I	29 (26.4)
II	48 (43.6)
III	33 (30)
Breast cancer type	
Ductal carcinoma	91 (82.7)
Lobular carcinoma	19 (17.3)
Type of surgery	
Wide local excision	28 (25.4)
MRM	62 (56.4)
Palliative mastectomy	20 (18.2)
OCP use	
Yes	63 (57.3)
No	47 (42.7)
ER status	
+	49 (44.5)
-	61 (55.5)
PR status	
+	32 (29.1)
-	78 (70.9)
HER-2/neu	
+	47 (42.7)
-	63 (57.3)
Cyclin D1	
1+	30 (27.3)
2+	35 (31.8)
3+	13 (11.8)
Negative	32 (29.1)

*T3=7 cases, T4=25 cases with 22 cases showing fungating skin involvement with or without chest wall involvement. All 25 cases were uniformly >5 cm in size.

MRM=Modified radical mastectomy, OCP=Oral contraceptive, ER=Estrogen receptor, PR=Progesterone receptor, HER-2=Human epidermal growth factor receptor 2

Table 2: Correlations between expression of cyclin D1 and standard clinicopathological variables

Variable	Grouping	Positive for cyclin D1				Negative for cyclin D1 (%)	χ^2	P
		1+	2+	3+	Total (%)			
Age	<35	5	8	4	17 (73.9)	6 (26.1)		0.762
	35-49	14	10	4	28 (73.7)	10 (26.3)		
	>49	11	17	5	33 (67.3)	16 (32.7)		
Tumor size	T1 (<2 cm)	7	15	5	27 (81.8)	6 (18.2)	7.281	0.026
	T2 (2-5 cm)	16	12	6	34 (75.6)	11 (24.4)		
	T3 and T4 (>5 cm)	7	8	2	17 (53.1)	15 (46.9)		
Number of positive nodes	<3	6	9	5	20 (76.9)	6 (23.1)	1.174	0.556
	>3	17	18	5	40 (66.7)	20 (33.3)		
	Negative	7	8	3	18 (75)	6 (25)		
Tumor grade	I	5	10	7	22 (75.9)	7 (24.1)	6.215	0.045
	II	13	20	5	38 (79.2)	10 (20.8)		
	III	12	5	1	18 (54.5)	15 (45.5)		
Type of tumor	Ductal	26	28	8	62 (68.1)	29 (31.9)	1.970	0.160
	Lobular	4	7	6	16 (84.2)	3 (15.8)		
Type of surgery	WLE	5	10	9	24 (85.7)	4 (14.3)	12.52	0.002
	MRM	22	21	3	46 (74.2)	16 (25.8)		
	PM	3	4	1	8 (40)	12 (60)		
OCP use	Yes	18	20	10	48 (76.2)	15 (23.8)	1.994	0.158
	No	12	15	3	30 (63.8)	17 (36.2)		
ER status	+	14	18	8	40 (81.6)	9 (18.4)	4.926	0.026
	-	16	17	5	38 (62.3)	23 (37.7)		
PR status	+	9	8	10	27 (84.4)	5 (15.6)	3.967	0.046
	-	21	27	3	51 (65.4)	27 (34.6)		
HER-2/neu	+	9	12	8	29 (61.7)	18 (38.3)	3.372	0.066
	-	21	23	5	49 (77.8)	14 (22.2)		

MRM=Modified radical mastectomy, ER=Estrogen receptor, PR=Progesterone receptor, WLE=Wide local excision, PM=Palliative mastectomy, OCP=Oral contraceptive, HER-2=Human epidermal growth factor receptor 2

Results

The clinical and pathologic characteristics of the 110 participants with primary breast cancer are described in Table 1. Table 2 shows the association analysis between cyclin D1 expression and the clinicopathological variables that were under consideration.

Patients were staged as per the AJCC staging system for breast cancer, 2002.

Based on TNM staging, 25.45% of the patients (28 cases) were treated with wide local excision (WLE), followed by radiation therapy; 56.36% (62 cases) with modified radical mastectomy (MRM) involving a level I and II axillary clearance and 18.19% cases (20 cases) with palliative mastectomy (PM) for fungating breast cancer with systemic metastases.

Overexpression of cyclin D1 was found in 78 of 110 cases (70.9%). The majority of the cases stained 2+ (moderately positive). However, multivariate analysis failed to reveal a significant difference in the staining pattern among the cyclin D1 positive cases. The mean age of the study population was 46.6 years (range, 26–71 years). The maximum number of cases, that is, 49 cases (44.6%) were in the ≥ 50 year age group. Though the majority of the cyclin D1 positive cases were more than 50 years of age, this did not reach statistically significant levels ($P = 0.762$).

Majority of the breast tumors, that is, 78 (70.9%) were < 5 cm in size. Tumors > 5 cm in size were grouped together, irrespective of the chest wall and skin involvement. There was a significant negative correlation between cyclin D1

overexpression and tumor size ($P = 0.023$). Among the low and moderate grade (grade I and grade II) tumors, 77.9% highly expressed the protein as compared to only 54.5% of high grade (grade III) tumors. Hence, tumor grade was seen to have a significant negative correlation with cyclin D1 ($P = 0.045$). ER and PR positive cases showed a significantly positive correlation with cyclin D1 overexpression ($P = 0.026$ and 0.046 , respectively). Interestingly, cyclin D1 positivity showed a strong correlation with the type of surgical procedure performed ($P = 0.002$) with 85.7% WLE specimens staining positive for cyclin D1 when compared to only 40% of the PM specimens.

There was no significant correlation between high expression of cyclin D1 and lymph node status ($P = 0.556$), type of breast cancer ($P = 0.160$), oral contraceptive use ($P = 0.158$) and Her-2/neu overexpression ($P = 0.066$).

Discussion

Cyclin D1 belongs to a group of cell cycle regulatory proteins called cyclins that together with cyclin-dependent kinases, orchestrate the orderly progression of cells through various phases of the cell cycle. Complex and still poorly understood variations in cyclin D1 levels through the cell cycle are essential for the continued proliferation of a cell. The cyclin D1 gene, designated as *CCND1* or *PRAD1*, located on human chromosome band 11q13, is an established oncogene, overexpression of which is commonly found in multiple types of human cancer.^[8] However, the exact mechanism by which cyclin D1 exerts its neoplastic effect remains to be clearly defined. Furthermore, there is limited and conflicting data on

the clinical significance of cyclin D1 in human invasive breast carcinomas.

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.^[9] Although the cause of this early occurrence remains to be elucidated,^[10-12] a younger age at diagnosis has been associated with larger tumor size, higher grade and low levels of hormone receptors thereby portending a poor prognosis in this age group. In our own study population, the mean age was 46.6 years (range, 26–71 years) with 20.9% of the participants being <35 years of age. However, consistent with the findings of Kenny *et al.*^[13] and Michalides *et al.*,^[14] we found no significant correlation between age and cyclin D1 overexpression. This underlines the fact that high cyclin D1 expression may be genetically predetermined.

The prevalence of cyclin D1 overexpression in our study population was 70.9%. This was considerably higher than western studies such as those carried out by Michalides *et al.*^[14] and Zukerberg *et al.*,^[15] probably resulting from the difference in methods used for the detection of cyclin D1 overexpression (IHC vs. gene amplification techniques).

Although our study was limited by the fact that we had not evaluated cyclin D1 status by gene amplification techniques such as fluorescence *in situ* hybridization (FISH) and reverse transcription polymerase chain reaction (RT-PCR) because of financial constraints, the use of a method (IHC) with wider availability and relatively low cost augured well with the objective of our study. Moreover, the intensity of the staining (1+, 2+, 3+) considered individually and in combination did not affect the final results of our study. In addition, several published studies have pointed to the disparity between the rates of cyclin D1 nuclear expression and gene amplification as measured by IHC and FISH/Southern blotting, respectively. The frequency of cyclin D1 expression is approximately 3 times greater than gene amplification.^[16,17] Therefore, IHC can identify tumors in which the gene is overexpressed without an apparent increase in copy number. Staining with the monoclonal antibody consequently provides a more rapid assay for the amplification of cyclin D1 and a more accurate indication of dysregulated expression of cyclin D1.^[16]

In keeping with the aggressive nature of breast cancer and late presentation of the disease seen in this part of the world, 70% of the patients had a tumor size >2 cm at presentation, 78.2% had clinically positive axillary nodes with 54.6% having >3 palpable nodes and 73.6% of the excised tumors demonstrated a moderate to high histologic grade.

There was a definite positive correlation between tumor size and cyclin D1 expression, independent of the other variables, with tumors <2 cm overexpressing cyclin D1 more frequently than tumors 2–5 cm in size, which in turn had higher expression of the protein than tumors >5 cm in size. Our findings paralleled that of Pelosio *et al.*^[18] but significantly deviated from studies carried out independently by Diest *et al.*^[19] and Kenny *et al.*^[13] who failed to show a similar correlation. One reason for this could be the difference in the method of categorizing the tumor sizes with Diest *et al.* and

Kenny *et al.* using a tumor size cutoff value of 2.5 cm and 2 cm respectively, instead of the T₁₋₃ grouping applied by both Pelosio *et al.* and us.

Consistent with the findings of earlier western studies, high expression of cyclin D1 was also noted in a significant majority of patients with low to moderate tumor grade^[18,19] and those with positive ER/PR status.^[13,14,18,19] And although lymph node status failed to show a statistically significant correlation with cyclin D1 overexpression, we found a strong correlation with the type of surgery that was carried out for the treatment of these tumors. Most patients undergoing WLE for early, prognostically favorable tumors showed cyclin D1 positivity as compared to those undergoing PM for advanced, prognostically poor tumors. Moreover, among tumors >5 cm in size, T3 tumors that were subjected to MRM had significantly higher levels of cyclin D1 expression as compared to T4 tumors with fungating/ulcerating skin involvement that were treated with PM. However, further studies showing similar cyclin D1 expression rates in different parts of the developing world will be required to corroborate this finding in order to delineate its true significance, if any as additional studies reveal the precise role of cyclin D1 in breast cancer pathogenesis.

In the light of the above findings, it would be reasonably safe to conclude that cyclin D1 overexpression in breast cancer is a favorable prognostic marker associated with less aggressive tumoral characteristics.

However, having said that, there are a few concerns that need to be expressed. Firstly, although we did observe a few cases of tumor recurrence, both locoregional and distant, during the follow up period, occurring mostly in originally cyclin D1 negative cases, they were not included in the study since only a fraction of the patients actually turned up in the follow-up clinic (a common phenomenon in this part of the world and therefore one that should be taken into consideration when electing a putative prognostic marker with implications on survival). This lack of follow up data resulted in our inability to compare tumor recurrence and survival of cases with known favorable tumor characteristics (small size, low-grade etc.) who overexpressed cyclin D1 with those who did not. Consequently, the actual significance of cyclin D1 overexpression as a favorable prognostic marker in human breast cancer independent of existing known prognostic markers could not be determined by this study.

Secondly, our findings were largely congruent with those of previously carried out western studies.^[13,14,18,19] And albeit the tumoral characteristics of our study population were in keeping with the aggressive nature of invasive breast cancer normally seen in this part of the world (*vide supra*), the high prevalence of cyclin D1 overexpression in our study (70.9%) would imply that molecular alterations in cyclin D1 is not likely to be a major participant in accounting for the epidemiological differences in breast cancer occurrence and biological behavior.

Our third concern regards the utility of cyclin D1 testing. This study, as well as many others, shows a significant correlation of high cyclin D1 expression with tumor size, tumor grade and hormone receptor status. On the other hand, the evidence regarding the correlation between cyclin D1 overexpression and

survival is limited and contradictory.^[8] Accordingly, without an effective therapy specifically targeting cyclin D1 currently in existence, the benefit of testing for high expression of this protein simply for its prognostic significance is questionable.

We conclude that till such time additional studies are done to elucidate the exact role of cyclin D1 on tumor pathogenesis and patient survival and definite therapies targeting it are developed, cyclin D1 testing especially in developing countries with limited resources should by and large be done on an experimental basis only.

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