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Significance of Cyclin D1 Overexpression and Amplification in Ductal Hyperplasia, Carcinoma *in situ* and Invasive Carcinoma in Egyptian Female Breast

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Abstract: Cyclin D1 is involved in regulating the transition of G1 to S-phase in the cell cycle. Amplification and overexpression of the cyclin D1 gene have been reported to be implicated in breast carcinoma and are suggested to play important roles in breast carcinogenesis. In the present study, we tried to evaluate the correlation between cyclin D1 expression and gene amplification and analyze the correlations between cyclin D1 overexpression with clinicopathological features in different breast lesions. Cyclin D1 gene amplification and protein overexpression were assessed in 20 cases of ductal hyperplasia without atypia, 20 cases of atypical ductal hyperplasia, 24 cases ductal carcinoma *in situ* and 114 cases of invasive carcinoma. Cyclin D1 overexpression was found in 0, 30, 58.3 and 63.2%, respectively. While gene amplification was detected in 0, 0, 16.7 and 15.8%, respectively. In ductal carcinoma *in situ*, no-significant correlations between either cyclin D1 overexpression or amplification and any of clinicopathological features. In cases of invasive carcinoma, cyclin D1 overexpression and amplification showed a strong direct correlation with expression in both hormonal receptors. There was a significant correlation between cyclin D1 expression and good prognostic parameters, including low histological grade ($p = 0.04$) and small tumor size ($p = 0.003$). There was a strong correlation between cyclin D1 overexpression and histological tumor type ($p = 0.008$). In conclusion, amplification and overexpression of cyclin D1 in atypical ductal hyperplasia, ductal carcinoma *in situ* and invasive carcinoma suggests its role in early and late stages of breast cancer.

Key words: Ductal hyperplasia, ductal carcinoma *in situ*, invasive breast carcinoma, cyclin D1, immunohistochemistry, fluorescence *in situ* hybridization

INTRODUCTION

Breast cancer is the most common malignancy among Egyptian females accounting for 37.6% of all malignancies in women (Parkin *et al.*, 2002). Breast cancer in Egyptian patients is biologically more aggressive than that encountered in the West. This is explained partly by the predominance of premenopausal patients and partly by the late presentation of patients at an advanced stage (El-Bolkainy *et al.*, 2005).

It was found that genetic alterations play an important role in the development of invasive carcinoma. Preinvasive lesions indicate the presence of intermediate stages in the

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development of invasive carcinoma in some cases but we still have little understanding of what genetic/epigenetic events are likely to be associated with the earliest phases of the disease (Heffelfinger *et al.*, 2000). Cyclin D1 is claimed to be one of the genes that are known to be involved in preinvasive breast lesions (Naidu *et al.*, 2002).

Gene amplification is a well-defined cause of oncogene activation during tumor development (Luo *et al.*, 2006). Amplification of chromosome locus 11q13 has been reported at high frequencies in a wide variety of human cancers, such as bladder (Zaharieva *et al.*, 2003), lung (Shibata *et al.*, 2005), breast (Elsheikh *et al.*, 2008) and ovarian (Brown *et al.*, 2006) carcinomas.

The cyclins are a family of key regulatory proteins that control the progression of human cells through critical transition points in the cell cycle. Several classes of cyclins have been identified, displaying sequential expression in different phases of the cell cycle. The D-type of cyclins control the transition through the G1 phase enabling the entry into S phase playing a pivotal role in the regulation of progression from the G1 to S phase through the formation of active enzyme complexes with cyclin dependent kinases Cdk4 and Cdk6 (Esteva and Hortobagyi, 2004).

Cyclin D1 overexpression has been reported in invasive breast cancer with expression rates between 35 to 90% of cases. Overexpression may occur with or without CCND1 gene amplification, which is observed in about 5-20% of tumors (Gillett *et al.*, 1994; Barnes, 1997; Naidu *et al.*, 2002; Bieche *et al.*, 2002; Molland *et al.*, 2004; Stendahl *et al.*, 2004; Jirstrom *et al.*, 2005; Ahnstrom *et al.*, 2005; Butt *et al.*, 2005; Reis-Filho *et al.*, 2006) and therefore qualifies as one of the most commonly overexpressed proteins in breast cancer (Ormandy *et al.*, 2003; Butt *et al.*, 2005). Though CCND1 amplification correlates well with the overexpression of the protein (Mrhalova *et al.*, 2002; Jirstrom *et al.*, 2005), high expression of cyclin D1 is not always secondary to gene amplification implying that other mechanisms contribute to maintain cyclin D1 overexpression.

CCND1 amplification has been investigated in breast cancer by Southern blotting (Zukerberg *et al.*, 1995; Cuny *et al.*, 2000), fluorescent *in situ* hybridization (FISH) (Gillett *et al.*, 1994; Stendahl *et al.*, 2004; Jirstrom *et al.*, 2005) and real-time polymerase chain reaction-based methods (Bieche *et al.*, 2002).

Recently, cyclin D1 overexpression and CCND1 amplification, have received great attention due to data from clinical trials implicating cyclin D1 overexpression in resistance to tamoxifen treatment in postmenopausal (Jirstrom *et al.*, 2005) and in premenopausal breast cancer patients (Stendahl *et al.*, 2004).

Estrogen receptor status has been used in predicting the response to adjuvant tamoxifen therapy for more than 20 years and has remained the most powerful molecular marker for treatment decision. ER-positive invasive ductal carcinoma (IDC) have a 40-70% response rate in tamoxifen treatment (Fitzgibbons *et al.*, 2000). It is not known what makes the difference between responders and non-responders towards therapeutic agents such as Trastuzumab or tamoxifen. It has been suggested that cyclin D1 can bind directly to and activate estrogen receptors independently from estrogen (Neuman *et al.*, 1997).

Until now, the prognostic value of cyclin D1 protein has been controversial, with studies reporting both a positive and negative role in breast cancer, whereas amplification of the CCND1 gene is predominantly related with worse outcome in ER positive patients (Sutherland and Musgrove, 2004).

To the best of our knowledge, this is the first report of simultaneous evaluation of cyclin D1 protein and gene amplification status in both of preinvasive and invasive breast

cancer using immunohistochemistry (IHC) and FISH in Egypt, together with studying the correlations between both of them and clinicopathological features with hormone receptor status.

MATERIALS AND METHODS

Patient's Selection

For ductal hyperlasia (DH), atypical ductal hyperlasia (ADH) and ductal carcinoma *in situ* (DCIS) cases, excisional biopsies were taken with full history of the patients from Minia University Hospital and private clinic of the co-author, in the period between 2006 and 2009. Paraffin embedded formalin fixed blocks were prepared for the present study. Twenty cases of DH, 20 cases of ADH without invasive or *in situ* carcinoma that were accidentally found beside benign breast lesions. ADH was diagnosed using the criteria previously described (Page and Rogers, 1992). Twenty-four cases of DCIS without any associated invasive carcinoma were investigated. No lobular carcinoma *in situ* (LCIS) cases were found. The histological subtypes were divided into comedo and non-comedo types. The histologic grade was also classified into well, intermediately and poorly differentiated as the previously classified (Holland *et al.*, 1994).

One hundred fourteen cases with invasive carcinoma (IC) were selected for this study. All cases undergone modified radical mastectomy. We were found adjacent DCIS in 42 cases. Tumors were graded according to a modified Bloom-Richardson scoring system (Elston and Ellis, 1991). Tumor staging was performed according to the TNM system of the International Union against Cancer (Hermanek and Sobin, 1992). Tumor size and lymph node status were categorized according to Carter *et al.* (1989) and Recht *et al.* (2001).

Regarding DH cases, the mean age of the patient was 42.5 ± 7.47 years (range 30-55 years) and the median of the cases was 42 years. In cases of ADH, the mean age of the patient was 46.7 ± 5.57 years (range 39-54 years) and the median of the cases was 46.5 years. Regarding DCIS cases, the mean age of the patient was 45.75 ± 7.64 years (range 35-60 years) and the median of the cases was 47.5 years. In invasive cases, the mean age was 51.43 ± 8.59 years (range 35-69 years) and the median of the cases was 51 years.

Immunohistochemical Staining

It has been shown in previous studies that for cyclin D1, the use of the rabbit monoclonal antibody SP4, would give reproducible results (Cheuk *et al.*, 2004; Reis-Filho *et al.*, 2006; Elsheikh *et al.*, 2008). The SP4 antibody is a rabbit monoclonal antibody raised against a synthetic peptide from C-terminus of human cyclin D1, which was deemed specific to cyclin D1, identifying a single 36 kDa band on Western blot analysis (Reis-Filho *et al.*, 2006). In addition, the SP4 clone was reported to be at least as specific as other monoclonal antibodies against cyclin D1, but is reported to be more sensitive than other clones (Loden *et al.*, 2002; Cheuk *et al.*, 2004; Reis-Filho *et al.*, 2006). In contrast to other antibodies, the rabbit anticyclin D1 monoclonal antibody shows a strong correlation with CCND1 gene amplification. Therefore, to determine cyclin D1 protein expression we chose this antibody for employing the protocol described previously (Cheuk *et al.*, 2004).

Immunohistochemistry for Cyclin D1 was Performed as follows

First, the sections were dewaxed and hydrated through graded alcohol then the slides are lowered into boiling EDTA buffer (pH 8.0) in a pressure cooker and boil for 3 min under full pressure and cooling down slides in a sink of cold running tap water.

The slides then rinsed in Tris-buffer saline for 3 min and the endogenous peroxidase was blocked with 3% hydrogen peroxide for 3 minutes and incubated with 1:10 dilution of

cyclin D1 rabbit monoclonal antibody (clone SP4, LabVision) for 1.5 h and washed with distilled water and incubated with EnVisionanti-rabbit labeled polymer (Dako, Denmark) for 1 h.

Lastly the slides were rinsed with three changes of Tris-buffer saline, 5 min each, incubated with diaminobenzidine solution (Dako, Denmark) for 4 min and checked with microscope for optimal staining then washed thoroughly with distilled water and incubate with 0.5% copper sulphate solution in saline for 6 min.

At last, washing the slides in running tap water and counterstaining nuclei with hematoxylin, dehydrating, clear and mount.

For Hormone Receptors Status

ER and PR expression were evaluated on formalin-fixed and paraffin embedded sections after enhanced microwave antigen retrieval using an anti-estrogen receptor antibody (ER ID5, DAKO) and an anti-progesterone receptor antibody (PR 1A6, DAKO). Staining was performed by a standard streptavidin-biotin-peroxidase technique using DAB for visualization and hematoxylin for nuclear counterstaining.

Evaluation of Staining Results

Scoring of the Cyclin D1 in DH, ADH and DCIS

Due to the restricted number of these lesions, variations in cyclin D1 expression were not seen. Therefore, cases were stratified into positive or negative. Overexpression of cyclin D1 was defined as cells greater than 10% with moderate/strong nuclear staining. This cut-off value was chosen in accordance with the available literature (Fiche *et al.*, 2000; Oh *et al.*, 2001; Lebeau *et al.*, 2003). Faint nuclear staining or cytoplasmic staining was not considered significant.

Scoring of the Cyclin D1 in IC

Scoring of the cyclin D1 reactivity was performed using the Allred method (Harvey *et al.*, 1999; Reis-Filho *et al.*, 2006). With this method, the intensity of the immunohistochemical reaction was recorded as 0, negative (no staining of any nuclei even at high magnification); 1, weak (only visible at high magnification); 2, moderate (readily visible at low magnification) or 3, strong (strikingly positive even at low power magnification). The proportion of tumor nuclei showing positive staining was also recorded as either: 0, no staining; 1, <1% nuclei staining; 2, 1-10%; 3, 11-33%; 4, 34-66% and 5, 67-100% nuclei staining. The proportion and intensity scores were subsequently added to obtain a total score, which ranged from 0 to 8. Tumors were categorized into four groups: negative 0, weak (total scores 1-2), moderate (total scores 3-5) and strong (total scores 6-8). Only nuclear staining was considered specific.

Scoring of the Hormone Receptors

The slides in which more than 10% of tumor cells were stained were scored as positive. Staining intensity was not evaluated (Nishimura *et al.*, 2007).

FISH Study

FISH was performed on 4 µm paraffin sections according to previously reported protocol (Lebeau *et al.*, 2001, 2003). In brief, the slide-mounted tissue sections were air-dried and baked overnight at 56°C. Slides were dewaxed in xylene for 10 min ×3, followed by immersion in 100% ethanol for 5 min ×2. Air-dried tissue sections were subsequently covered with

1 M NaSCN (Sodium Isothiocyanate) and placed in an oven for 30 min at 80°C. Afterwards slides were washed in Aqua bidest and treated in a pepsin solution (8 mg/mL H₂O; pH 2.0) for 30 min at 37°C. Slides were washed in Aqua bidest and air dried. CCND1 gene copies were determined by using a spectrum orange-labelled probe purchased from Vysis (Vysis SA, Maurens Scopont, France). Cyclin D1 probe was prepared as follows (1 µL probe, 2 µL purified water, 7 µL LSI Hybridization Buffer). Prepared probes were centrifuged after preparation. Slides were incubated at 80°C for 20 min. Twenty microliter of the probe in appropriate hybridisation buffer (Vysis SA) were applied to the sections and incubated 10 min at 80°. Hybridisation was carried out overnight at 37°C in a moist chamber. Washes were performed at 42°C three times in 0.1× SSC (sodium chloride/sodium citrate) and Post Hybridization Wash Buffer (PHWB) for 1 min each. Tissue sections were counterstained with DAPI/Vectashield.

Scoring Criteria

Slides were evaluated for CCND1 gene amplification according to previously published criteria (Lebeau *et al.*, 2001) using a Bausch and Lomb fluorescence microscope (Balplan research illuminator) equipped with appropriate filters (Vysis, Inc., Downers Grove, IL). The cyclin D1 gene to chromosome/centromere ratio was measured in at least 60 nuclei from the tumor cells and an average score was taken. More than two copies of cyclin D1 for each chromosome was considered to be a positive sign for cyclin D1 gene amplification

Statistical Analysis

Statistical analyses were performed using the SPSS Version 17 for Windows (SPSS Inc., Chicago, IL) program package. The two-sided Chi-squared (χ^2) test was used to compare categorical variables, if the sample size was large. Fischer's exact test was used when the sample size was small. Student t test was used to compare means of patients ages. The significance level was considered at 0.05.

RESULTS

Cases of DH and ADH

In cases of DH, the expression of cyclin D1 was not found. Among ADH cases, overexpression of cyclin D1 was found in six cases (30%), While CCND1 gene amplification was not identified (Table 1; Fig. 1a, b). We found no significant correlation between cyclin D1 and ER expression in spite that all cases that were positive with cyclin D1 also were positive for ER ($p = 0.29$).

Cases of DCIS

In the DCIS cases, cyclin D1 overexpression was seen in 14 cases (58.3%) and no staining in ten cases (41.7%). while, CCND1 gene status was amplified in four patients (16.7%) and non-amplified in 20 (83.3%) patients. We found that the four amplified cases showed strong cyclin D1 protein expression, but p-value was not statically significant ($p = 0.31$) (Table 1; Fig. 1c, d, e).

Correlation of Cyclin D1 Overexpression and CCND1 Gene Amplification with Clinicopathological Features of DCIS cases

Table 2 shows the association between cyclin D1 expression and clinicopathological features of DCIS. Non-significant correlations between cyclin D1 expression and amplification with any of clinicopathological features could be detected.

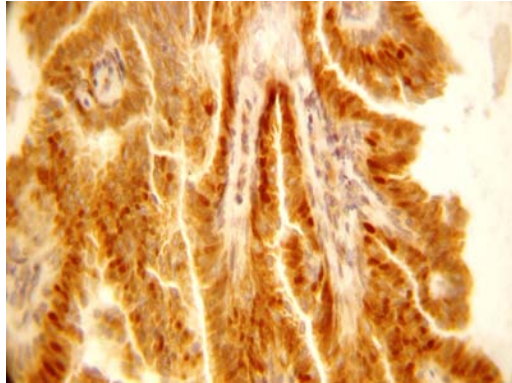


Fig. 1a: Cyclin D1 overexpression in ADH

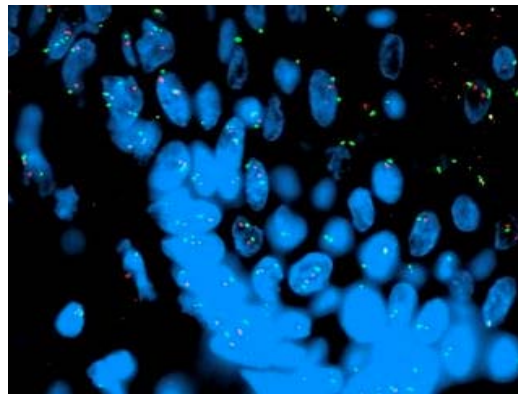


Fig. 1b: Fluorescent *in situ* hybridization signals in ADH showing non-amplified cyclin D1 gene. (Cyclin D1, Spectrum Orange, Centromere 11 Spectrum Green)

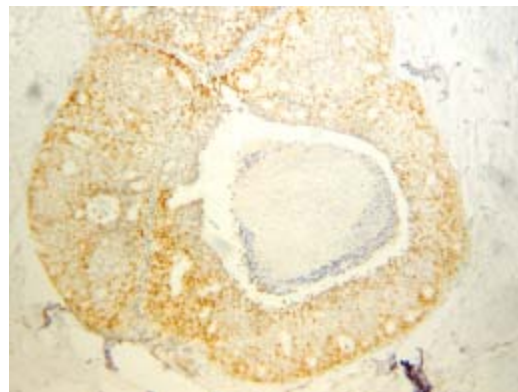


Fig. 1c: Cyclin D1 overexpression in comedo DCIS

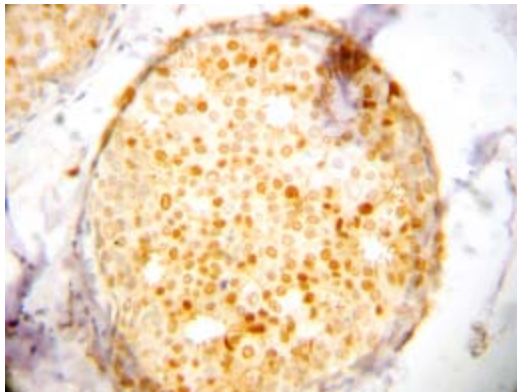


Fig. 1d: Cyclin D1 overexpression in non- comedo DCIS

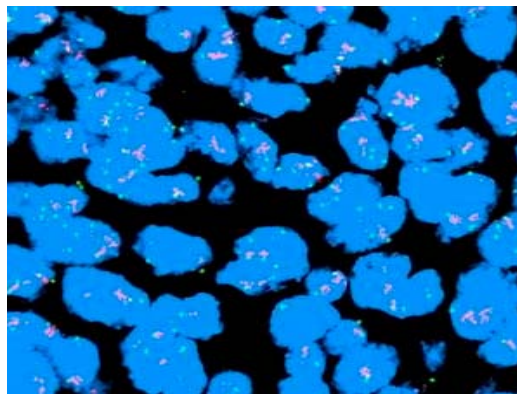


Fig. 1e: Fluorescent *in situ* hybridization cyclin D1 signals in DCIS showing Cyclin D1 gene amplification clusters (Cyclin D1 gene/CEP 11 ratio = 2)

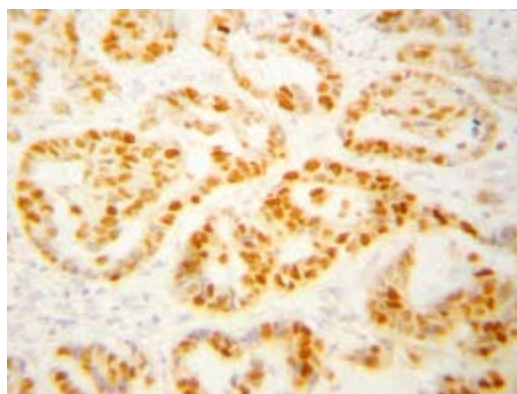


Fig. 1f: Cyclin D1 overexpression in IDC grade I, strong expression

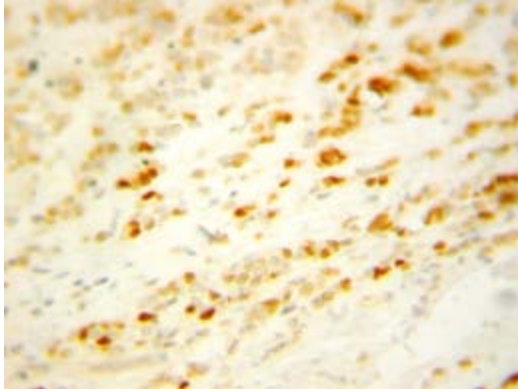


Fig. 1g: Cyclin D1 overexpression in IDC grade III, weak expression

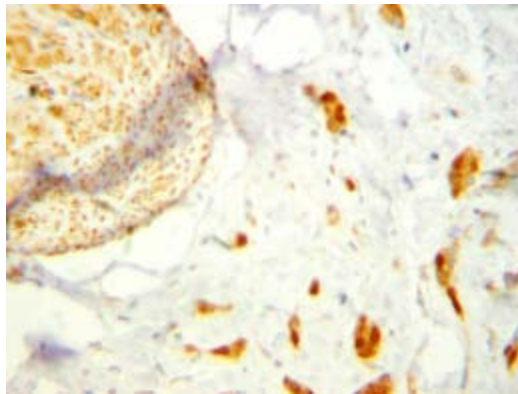


Fig. 1h: Cyclin D1 overexpression in IDC with DCIS component, showing same expression pattern

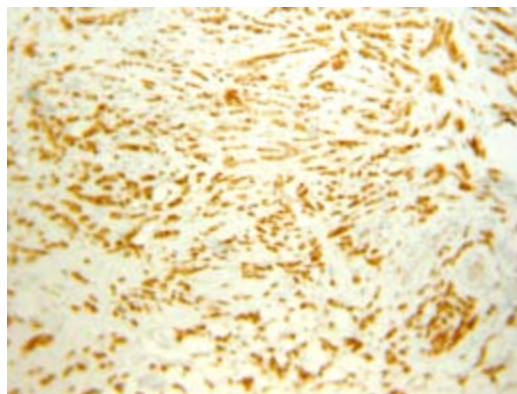


Fig. 1i: Cyclin D1 overexpression in IL, strong expression

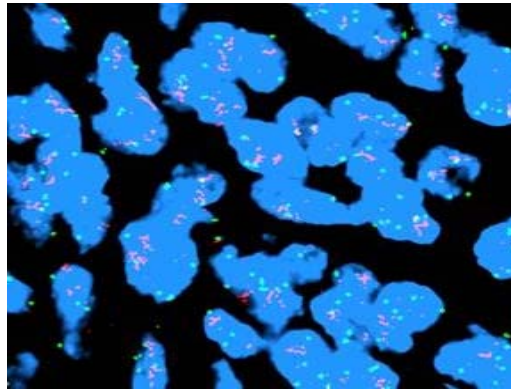


Fig. 1j: Fluorescent *in situ* hybridization cyclin D1 signals in IC, showing cyclin D1 gene amplification clusters and multiple scattered amplification signals

Table 1: Cyclin D1 overexpression and CCND1 gene amplification in different breast lesions

Breast lesion	No. of cases	Cyclin D1 overexpression		CCND1 gene amplification	
		-ve (%)	+ve (%)	CCND1 non-amplified (%)	CCND1 amplified (%)
DH	20	20(100)	0(0)	20(100)	0(0)
ADH	20	14(70)	6(30)	20(100)	0(0)
DCIS	24	10(41.7)	14(58.3)	20(83.3)	4(16.7)
IC	114	42(36.8)	72(63.2)	96(84.2)	18(15.8)

Cases of IC

Cyclin D1 expression as detected by immunohistochemistry was negative in 42 (36.8%) cases and positive in 72 (63.2%) of cases (Table 1; Fig. 1f-i). Positive cases were categorized into three groups; weak 24 (21.1%), moderate 28 (24.6%) and strong 20 (17.5%). We noticed that positive cases for cyclin D1 maintain the same expression score in *in situ* component as well as invasive component.

CCND1 amplification as detected by FISH was amplified in 18 patients (15.8%) from these cases we found four cases with *in situ* component and non-amplified in 96 patients (84.2%) of invasive breast cancer (Table 1; Fig. 1j).

Correlation between CCND1 Gene Amplification and Cyclin D1 Protein Expression in IC

An excellent correlation between CCND1 amplification and cyclin D1 overexpression was found ($p = 0.01$). Most tumors with CCND1 amplification showed moderate or strong cyclin D1 protein expression. Ten out of 18 tumors with CCND1 amplification showed strong cyclin D1 expression, whereas 6/18 showed moderate expression (Table 3).

Correlation of Cyclin D1 Expression with Clinicopathological Parameters in IC

Table 4 shows the association between cyclin D1 expression and different clinicopathologic parameters. Cyclin D1 expression showed a strong direct correlation with expression of ER ($p = 0.03$) and PR ($p = 0.01$). There was a significant correlation between moderate/strong cyclin D1 expression and good prognostic parameters, including low histological grade ($p = 0.04$) and small tumor size ($p = 0.003$). There was a strong correlation

Table 2: Correlation of cyclin D1 overexpression and CCND1 gene amplification with DCIS clinicopathological features

Clinicopathological features	cyclin D1 expression			CCND1 gene amplification		
	- ve (%)	+ve (%)	p-value	CCND1 non-amplified (%)	CCND1 amplified (%)	p-value
Age						
≤50	8(80)	10(71.4)	0.63	16(80)	2(50)	0.45
>50	2(20)	4(28.6)		4(20)	2(50)	
Nuclear grade						
Low	4(40)	2(14.3)	0.51	2(10)	0(0)	0.54
Intermediate	2(20)	2(14.3)		6(30)	0(0)	
High	4(20)	10(71.4)		12(60)	4(100)	
Type						
Non-comedo	4(40)	4(28.6)	0.57	8(40)	0(0)	0.42
Comedo	6(60)	10(71.4)		12(60)	4(100)	
ER status						
Negative	6(60)	4(28.6)	0.31	10(50)	0(0)	0.31
Positive	4(40)	10(71.4)		10(50)	4(100)	
PR status						
Negative	8(80)	4(28.6)	0.12	12(60)	0(0)	0.22
Positive	2(20)	10(71.4)		8(40)	4(100)	

Test of significance: Fisher's exact test; p-value ≤0.05 are considered significant

Table 3: Correlation between CCND1 gene amplification and cyclin D1 protein expression in IC

Cyclin D1 expression categories	Cyclin D1 expression*	CCND1 non-amplified	CCND1-amplified
Negative weak	0	42	0
	1	10	0
	2	12	2
Moderate	3	6	0
	4	6	2
	5	10	4
Strong	6	6	4
	7	4	2
	8	0	4

p = 0.01 with χ^2 -test; *Allred scoring system

between cyclin D1 overexpression and histological type (p = 0.008). Positive cyclin D1 staining was seen in 12/14 of lobular carcinomas (85%), most of the cases show strong expression 8/12 cases (Fig. 1i) while all medullary carcinomas were completely negative.

Correlation of CCND1 Gene Amplification with Clinicopathological Parameters in IC

CCND1 gene amplification showed a significant correlation with expression of ER (p = 0.04) and PR (p = 0.01). Fourteen out of eighteen tumors showing CCND1 amplification also showed positive ER and PR expression. Non-significant correlations between CCND1 gene amplification and any of clinicopathological features were found as shown in Table 5.

DISCUSSION

Cyclin D1 gene amplification and overexpression has been reported in breast cancers. *In situ* hybridisation studies suggest that cyclin D1 overexpression occurs at the transition from *in situ* to invasive cancer (Fiche *et al.*, 2000), while immunohistochemical studies indicate that cyclin D1 overexpression increases progressively from ADH, to DCIS and to IC (Heffelfinger *et al.*, 2000; Lebeau *et al.*, 2003). Cyclin D1 overexpression was present in 64% of cases of DCIS and in only 14% of cases of ADH (Gillett *et al.*, 1998). Gradual increase in cyclin D1 overexpression from DH to ADH to DCIS and finally to IC with significant differences not always being seen between the lesions

Table 4: Correlation of cyclin D1 expression with different clinicopathological parameters in IC

Clinicopathological features	-ve (%)	Cyclin D1 expression			p-value
		Weak (%)	Moderate (%)	Strong (%)	
Age					
≤50	22(52.4)	4(16.7)	12(42.9)	10(50)	0.22
>50	20(30.3)	20(30.3)	16(57.1)	10(50)	
Grade					
GI	0(0)	2(8.3)	2(7.1)	6(30)	0.04
GII	20(47.6)	10(41.7)	20(71.4)	12(60)	
GIII	22(52.4)	12(50)	6(21.4)	2(10)	
Tumor Size					
<2 cm	2(4.8)	0(0)	4(14.3)	10(50)	0.003
2-5 cm	22(34.4)	22(34.4)	14(50)	6(30)	
>5 cm	18(42.9)	2(8.3)	10(35.7)	4(20)	
Lymph node status					
-ve L. nodes	8(19)	2(8.3)	4(14.3)	12(60)	0.07
1-3 + ve L. nodes	20(47.6)	8(33.3)	12(42.9)	4(20)	
>3 +ve L.nodes	14(33.3)	14(58.3)	12(42.9)	4(20)	
Stage					
T1	2(4.8)	4(16.7)	2(7.1)	4(20)	0.42
T2	18(42.9)	10(41.7)	20(71.4)	8(40)	
T3	22(52.4)	10(41.7)	6(21.4)	8(40)	
Histopathological types					
IDC	30(71.4)	22(91.7)	26(92.9)	12(60)	0.008
ILC	2(4.8)	2(8.3)	2(7.1)	8(40)	
MC	10(23.8)	0(0)	0(0)	0(0)	
ER status					
Negative	32(76.2)	14(58.3)	10(35.7)	6(30)	0.03
Positive	10(23.8)	10(41.7)	18(64.3)	14(70)	
PR status					
Negative	34(81)	14(58.3)	18(64.3)	4(20)	0.01
Positive	8(19)	10(41.7)	10(35.7)	16(80)	

Test of significance: chi-square test; p-value ≤0.05 are considered significant

(Alle *et al.*, 1998; Mommers *et al.*, 1998; Shoker *et al.*, 2001). Our own findings agree with these studies.

We examined preinvasive breast lesions for the expression of cyclin D1 to determine the beginning of invasion alterations in the expression levels and to correlate these changes with the clinicopathological feature. Our study demonstrated high expression of cyclin D1 in 58.3% in DCIS cases. This is in line with other studies (Alle *et al.*, 1998; Gillett *et al.*, 1998; Oh *et al.*, 2001; Lebeau *et al.*, 2003), who reported that cyclin D1 expression in DCIS range from 48 to 64%. The frequency of CCND1 amplifications as defined by FISH in the present study was 16.8% in DICS. It was reported by others that CCND1 was amplified in 33% and 18% of DICS respectively (Simpson *et al.*, 1997; Fiche *et al.*, 2000). In accordance with previous studies, we did not find a correlation between cyclin D1 overexpression or CCND1 amplification and any of clinicopathological features in DCIS cases (Vos *et al.*, 1999; Umekita and Yoshida, 2000; Oh *et al.*, 2001; Lebeau *et al.*, 2003).

In the present study, the rate of cyclin D1 overexpression in cases of IC is 63.2% that was correspond to reported results that were 64% cyclin D1 overexpression in IC (Lee *et al.*, 2007). However, there is a wide range of cyclin D1 expression in breast cancer, varying between 35-90% (Hwang *et al.*, 2003; Sutherland and Musgrove, 2004; Bilalovic *et al.*, 2005; Cho *et al.*, 2008; Elsheikh *et al.*, 2008). These might have resulted from different criteria for defining cyclin D1 overexpression and immunostaining techniques.

In this study, we found that, expression of cyclin D1 did not increase with the progression from DCIS to IC. Similarly, there was no change in expression from low-grade to

Table 5: Correlation of CCND1 gene amplification with different clinicopathological parameters in IC

Clinicopathological features	CCND1 gene amplification		p-value
	CCND1 non-amplified (%)	CCND1 amplified (%)	
Age			
≤50	18(39.6)	10(55.6)	0.29
>50	58(60.4)	8(44.4)	
Grade			
GI	6(6.3)	4(22.2)	0.11
GII	50(52.1)	12(66.7)	
GIII	40(41.7)	2(11.1)	
Tumor Size			
<2 cm	10(10.4)	6(33.3)	0.19
2-5 cm	56(58.3)	8(44.4)	
>5 cm	30(31.3)	4(22.2)	
Lymph node status			
- ve L. nodes	20(20.8)	6(33.3)	0.71
1-3 + ve L. nodes	38(39.6)	6(33.3)	
>3 +ve L.nodes	38(39.6)	6(33.3)	
Stage			
T1	8(8.3)	4(22.2)	0.45
T2	48(50)	8(44.4)	
T3	40(41.7)	6(33.3)	
Histopathological types			
IDC	76(79.2)	14(77.8)	0.40
ILC	10(10.4)	4(22.2)	
MC	10(10.4)	0(0)	
ER status			
Negative	58(60.4)	4(22.2)	0.04
Positive	38(39.6)	14(77.8)	
PR status			
Negative	66(68.8)	4(22.2)	0.01
Positive	30(31.3)	14(77.8)	

Test of significance: chi-square test and Fisher's exact test; p-value ≤ 0.05 are considered significant; ILC: Invasive lobular carcinoma; MC: Medullary carcinoma

high-grade DCIS. Cyclin D1 overexpression is elevated in both DCIS and IC to similar levels and is increased above normal levels in preneoplastic hyperproliferative lesions implying that molecular alterations leading to cyclin D1 overexpression occur relatively early during breast carcinogenesis (Alle *et al.*, 1998).

In our study, CCND1 amplifications was 15.8% in IC, which was similar to that reported by Worsley *et al.* (1996), Loden *et al.* (2002) and Jirstrom *et al.* (2005) using FISH, or using chromogenic *in situ* hybridization (CISH) (Reis-Filho *et al.*, 2006). We have identified a strong significant correlation between cyclin D1 protein expression and CCND1 amplification (p = 0.01). Interestingly, all cases with CCND1 gene amplification showed either moderate or strong cyclin D1 expression. Similar results were reported before in a series of 485 cases of IC, an excellent correlation between cyclin D1 overexpression and CCND1 amplification (p = 0.001). All cases with CCND1 gene amplification showed either moderate (42%) or strong (58%) cyclin D1 expression. In our study, we found non-significant correlations between CCND1 gene amplification and any of clinicopathological features (Elsheikh *et al.*, 2008). The same finding was reported by Worsley *et al.* (1996) using FISH technique and using CISH Reis-Filho *et al.* (2006).

Other reported discordance between the amplification of the cyclin D1 gene and the overexpression of cyclin D1 protein in breast cancers which may be due to several different pathways, including the ER, c-myc and fibroblast growth factor receptor pathways (Fu *et al.*, 2004; Butt *et al.*, 2005; Arnold and Papanikolaou, 2005).

As with previous immunohistochemical studies, the localization of the cyclin D1 protein was predominantly confined to the nucleus of the breast cancer cells (Hwang *et al.*, 2003; Bilalovic *et al.*, 2005) and the intensity and percentage of staining varied within the individual tumor and from cell to cell within the same tumor signifying that cyclin D1 overexpression is regulated in a cyclical manner. These observations support the original suggestion that cyclin D1 protein is not constantly expressed and must be degraded or expelled from the nucleus before progression into the S-phase (Baldin *et al.*, 1993).

In this study, we found that, cyclin D1 overexpression and CCND1 amplification are associated with positive hormone receptor status. Similar correlation was reported by other studies (Hwang *et al.*, 2003; Al-Kuraya *et al.*, 2004; Jirstrom *et al.*, 2005; Reis-Filho *et al.*, 2006; Elsheikh *et al.*, 2008). In DCIS and ADH, this relationship is apparently not of the same significance. Two studies reported a significant association (Vos *et al.*, 1999; Oh *et al.*, 2001), whereas others (Gillett *et al.*, 1998; Lebeau *et al.*, 2003) like the present study failed to find a statistically significant association. In the present study, despite those cases with positive cyclin D1 expression showed hormone receptor positivity there was no significant correlation between both of them.

A potential functional link between ER expression and cyclin D1 expression is supported by evidence that cyclin D1 is a major downstream target of estrogen action and plays a pivotal role in estrogen-induced mitogenesis in breast cancer cells (Planas-Silva and Weinberg, 1997). This supports the finding that cyclin D1 is an ER-regulated gene (Sutherland *et al.*, 1995). The disparity between the amplification of the cyclin D1 gene and the overexpression of cyclin D1 protein in breast cancers (Barnes, 1997) may therefore be due to the dysregulation of ER that occurs in the majority of these lesions (Shoker *et al.*, 2000, 2001). These data along with our result support the concept that high ER expression may help in the maintenance of high levels of cyclin D1 protein expression. Given the high prevalence of ER expression in breast cancers, it is not surprising that cyclin D1 overexpression is frequently due to upregulation by ER than as a result of CCND1 gene amplification and that anti-oestrogen therapy may offset some of these upregulating effects (Elsheikh *et al.*, 2008).

In this study, a significant correlation was observed between cyclin D1 overexpression and smaller size of the tumor ($p = 0.003$), well-differentiated carcinomas ($p = 0.04$), positive ER ($p = 0.009$) and PR ($p = 0.01$) all these features known to be associated with a good prognosis.

Until now, the prognostic value of cyclin D1 expression on breast cancer outcome has been controversial. Some studies have reported that cyclin D1 overexpression indicates a better prognosis in breast cancers (Hwang *et al.*, 2003; Bilalovic *et al.*, 2005; Cho *et al.*, 2008) while others reported it to be of no prognostic value (Worsley *et al.*, 1996; Lee *et al.*, 2007), at the same time, Ahnstrom *et al.* (2005) reported that cyclin D1 overexpression is associated with a poor prognosis in breast cancer. This difference may be due to the use of different antibodies, different thresholds for cyclin D1 positivity and different methods for the analysis of cyclin D1 expression by Western blotting, IHC and FISH. This is also in agreement with studies in which cyclin D1 was predominantly expressed in well differentiated, low-grade and slow growing breast cancers (Naidu *et al.*, 2002; Hwang *et al.*, 2003; Stendahl *et al.*, 2004). In other tumors, like lung cancer and colorectal cancer, expression of cyclin D1 correlated with a worse outcome and a positive correlation with proliferative markers. This indicates that cyclin D1 activities might be not only diverse but also tissue specific (Fu *et al.*, 2004).

In this study, there was a strong correlation between cyclin D1 overexpression and histological type ($p = 0.008$). Positive cyclin D1 staining was seen in 12 of 14 lobular

carcinomas (85%) most of cases show strong expression 8/12 cases. This finding was in line with other studies (Van Diest *et al.*, 1997; Soslow *et al.*, 2000; Naidu *et al.*, 2002; Reis-Filho *et al.*, 2006) who reported that, overexpression was found in 73 to 90% of lobular cancer, which also usually show low nuclear atypicality, low proliferation and positive steroid receptor status and are associated with an intermediate prognosis (Ellis *et al.*, 1992).

As regard medullary carcinoma, all five cases were completely negative for cyclin D1 expression or CCND1 amplification. Similar result by IHC and CISH were reported (Van Diest *et al.*, 1997; Elsheikh *et al.*, 2008). This may be due to its high nuclear atypicality, high proliferation and poor prognosis (Ellis *et al.*, 1992). On contrary to our results, a study reported 27% (4/15) of the medullary carcinomas was positive (Naidu *et al.*, 2002). So, overexpression of cyclin D1 is confined to specific phenotypes, implying different roles in different subtypes of the disease.

In conclusion, Overexpression and amplification of cyclin D1 in preinvasive alone or with adjacent invasive lesions and invasive carcinomas suggest that the gene may play an important role in early and late stages of breast carcinogenesis. Our results demonstrate a strong correlation between CCND1 amplification and its protein expression in invasive breast cancer. Our data are in agreement with the results of previous studies showing that cyclin D1 is not associated with poor prognosis. However, this study has a limitation due to lack of follow up data and treatment information. Therefore survival analysis could not be conducted. Further studies are needed to clarify the function of cyclin D1 as a prognostic factor.

ABBREVIATIONS

ADH	: Atypical Ductal Hyperplasia
CISH	: Chromogenic in situ hybridization
DH	: Ductal Hyperplasia without atypia
FISH	: Fluorescence <i>in situ</i> hybridization
IDC	: Invasive duct carcinoma
ILC	: Invasive lobular carcinoma
MC	: Medullary carcinoma
DCIS	: Ductal carcinoma <i>in situ</i>
ER	: Estrogen receptor
IC	: Invasive carcinoma
IHC	: Immunohistochemistry
LCIS	: Lobular carcinoma <i>in situ</i>
PR	: Progesterone receptor

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