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Original Article

Evaluation of CCND1 amplification and CyclinD1 expression: diffuse and strong staining of CyclinD1 could have same predictive roles as CCND1 amplification in ER positive breast cancers

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Abstract: CCND1 is amplified in around 10-20% of primary breast cancers and preferentially occurs in ER positive tumors. Though CCND1 amplification was reported predicting poor response of adjuvant tamoxifen treatment and poor prognosis in ER positive breast cancers, there were controversial data regarding the predicting value of CyclinD1 protein overexpression. In this study, we detected CyclinD1 expression using immunohistochemistry and CCND1 gene copy number using fluorescence in situ hybridization (FISH) in 355 invasive breast cancers with foci ductal carcinoma in situ (DCIS). CCND1 amplification was founded in 52 (14.6%) cases all of which showed moderate to strong CyclinD1 expression. However, majority of CCND1- tumors exhibited mild to moderate CyclinD1 staining. There were identical alterations in DCIS and the invasive lesions within the same tumor. CCND1 amplification was positively correlated with ER, PR and lymph node status (P<0.001) while negatively correlated with HER-2 amplification and p53 status (P<0.05). The majority of the CCND1 amplification/high CyclinD1 breast cancers were luminal B type while basal-like type often lost the expression of this protein. The ROC curve analysis showed that a cut-off point at which the immunostaining score of CyclinD1 is 6.5 could predict CCND1 gene amplification in breast cancer. This study indicated loss expression of CyclinD1 might be an important event in the tumorigenesis in basal-like breast cancers. Further, we confirmed an optimal cut-off point of immunostaining scores of CyclinD1 protein which could be used to predict the status of CCND1 gene and identify a subgroup of ER positive breast cancers with poor response to endocrine agents.

Keywords: Cyclin D1, CCND1, breast cancer, predictive markers, target therapy

Introduction

Breast cancer represents a collection of tumors with different behavior and clinical outcome, which is reflection of the biological heterogeneity and difference of genetic changes. Gene expression studies have led to the identification of a novel classification of breast cancer including luminal A, luminal B, HER-2-positive and basal-like subtypes [1]. Even in individual subtype, the tumors may have different pathological features, clinical outcome and therapy response.

About 70% of breast cancers are estrogen receptor (ER)-α positive and endocrine therapy represents a major treatment in this settings. Although tamoxifen treatment reduce recurrence rate by 50%, approximately one-third of patients will relapse during or after tamoxifen therapy and even more so patients with advanced breast cancer [2]. Acquired or de novo resistance to tamoxifen, thus, remains a major challenge in providing effective treatments for these patients. Therefore, it is imperative to identify biomarkers that can predict endocrine therapy response, so that alternative therapeutic strategies may be selected.

CCND1 gene, located on chromosome 11q13, encodes the key cell cycle G1 regulatory protein CyclinD1 which promotes the phosphorylation of retinoblastoma protein (Rb) and other substrates by binding to cyclin-dependent kinase

Table 1. CyclinD1 protein expression in relation to different clinicopathological variables and biological markers

Variables	Patients No (%)	CyclinD1 expression categories				P
		Negative	Weak	Moderate	Strong	
	355	69	73	132	81	
Age						0.372
<35 y	46 (13.0)	9	8	14	15	
≥35 y	309 (87.0)	60	65	118	66	
Size						0.775
≤2 cm	83 (23.4)	15	14	34	20	
2-5 cm	231 (65.1)	48	48	85	50	
>5 cm	41 (11.5)	6	11	13	11	
Grade						<0.01
I	26 (7.3)	5	4	6	11	
II	218 (61.4)	35	38	97	48	
III	111 (31.3)	29	31	29	22	
Lymph node						0.620
0	169 (47.6)	37	37	61	34	
1	186 (52.4)	32	36	71	47	
Subtypes						<0.01
Luminal A	91 (25.6)	11	13	39	28	
Luminal B	149 (42.0)	18	25	63	43	
HER-2	81 (22.8)	23	25	26	7	
Basal like	34 (9.6)	17	10	4	3	
ER						<0.01
Negative	123 (34.6)	45	38	31	9	
Positive	232 (65.4)	24	35	101	72	
PR						<0.01
Negative	152 (42.8)	46	41	49	16	
Positive	203 (57.2)	23	32	83	65	
HER-2						<0.01
Negative	222 (62.5)	38	40	81	63	
Positive	133 (37.5)	31	33	51	18	
Ki67						0.032
Low	129 (36.3)	23	17	53	36	
High	226 (63.7)	46	56	79	45	
P53						0.035
Negative	286 (80.6)	49	55	113	70	
Positive	69 (19.4)	20	18	19	11	

4/6 (CDK4/6) to make cells proliferation rapidly [3]. Overexpression of CyclinD1 has been observed in many human tumors. CyclinD1 also has CDK-independent function and may activate ER mediated transcription independently of oestrogen and potentially modify oestrogen/anti-oestrogen response [4]. CCND1 is amplified in around 10-20% while CyclinD1 overexpression has been reported up to 90% of all primary breast cancers [5-9], implying the elevated expression of CyclinD1 is not always secondary to gene amplification though CCND1 amplification correlates well with CyclinD1 overexpression. CCND1 gene amplification and CyclinD1 overexpression preferentially occurs in ER positive breast cancers [7, 10-13]. CCND1 amplification was reported predicting poor response of adjuvant tamoxifen treatment in ER positive breast cancers and therefore is associated with poor prognosis in this group of patients [13-17]. But there were controversial data regarding the prognostic value of CyclinD1 overexpression [7, 13, 18-22], which was considered owing to antibody disparities and methodological discrepancies [13, 19]. However failing to define "CyclinD1 overexpression" may contribute more to the conflicting findings. In this study, CCND1 amplification and CyclinD1 overexpression were assessed on a tissue microarray (TMA) in a cohort of 355 breast cancers containing both invasive carcinoma and ductal carcinoma in situ (DCIS) components using fluorescence in situ hybridization (FISH) assay and immunohistochemical analysis respectively. We aimed to compare the CCND1/CyclinD1 status in both components for each tumor and investigate the associations of CCND1 amplification and CyclinD1 expression with clinicopathological features. More importantly we hope to get a cut-off point of CyclinD1 overexpression comparable to the level with gene amplification, which could refine a group of ER positive breast cancers that exhibit different biological behavior and response to tamoxifen treatment.

Results

Characteristics of patients

The ages of patients at the time ofthe diagnosis ranged from 23 to 76 years (mean 52 years). The tumor size ranged from 0.5 to 11 cm in the

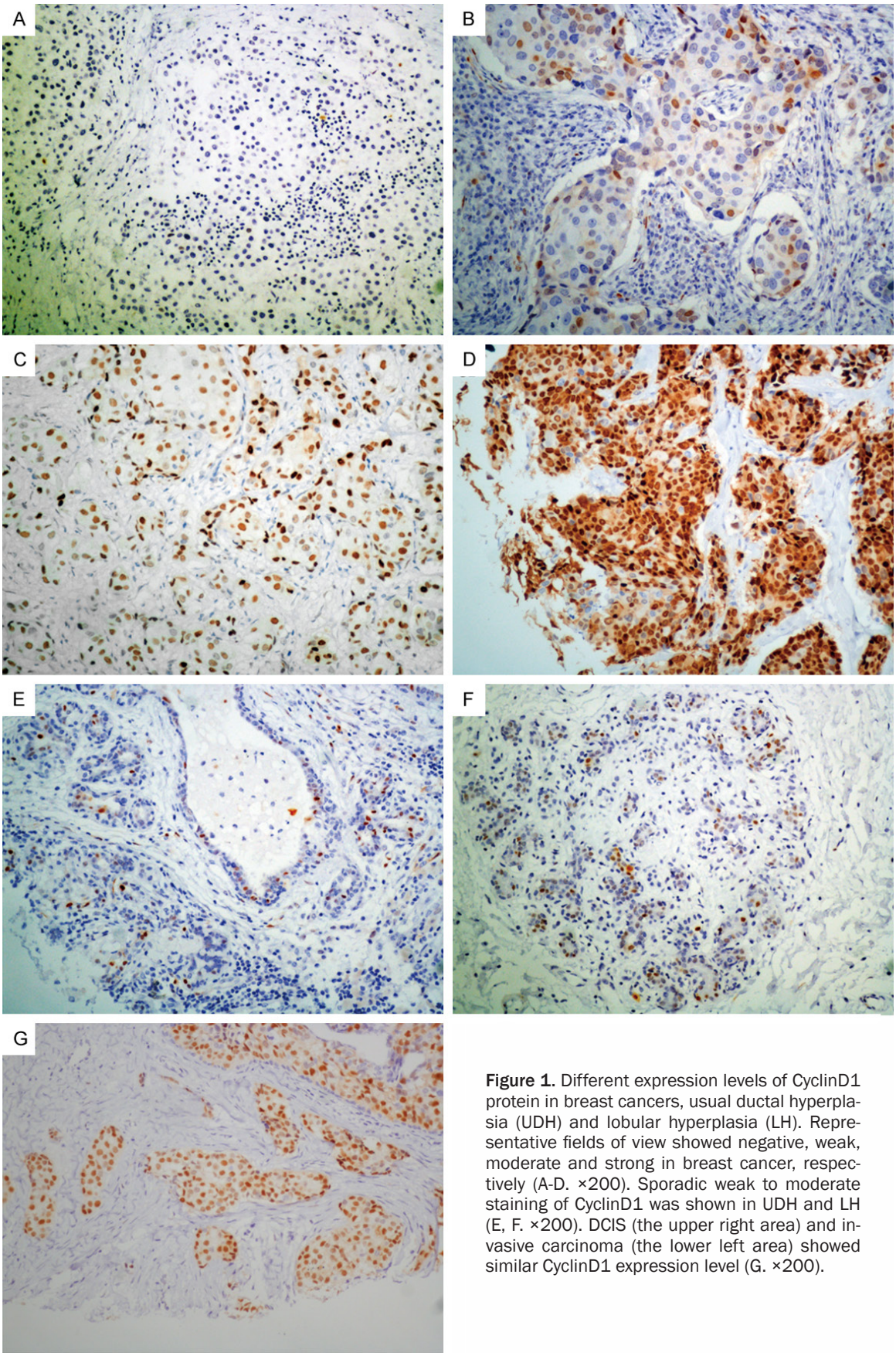

Figure 1. Different expression levels of CyclinD1 protein in breast cancers, usual ductal hyperplasia (UDH) and lobular hyperplasia (LH). Representative fields of view showed negative, weak, moderate and strong in breast cancer, respectively (A-D. ×200). Sporadic weak to moderate staining of CyclinD1 was shown in UDH and LH (E, F. ×200). DCIS (the upper right area) and invasive carcinoma (the lower left area) showed similar CyclinD1 expression level (G. ×200).

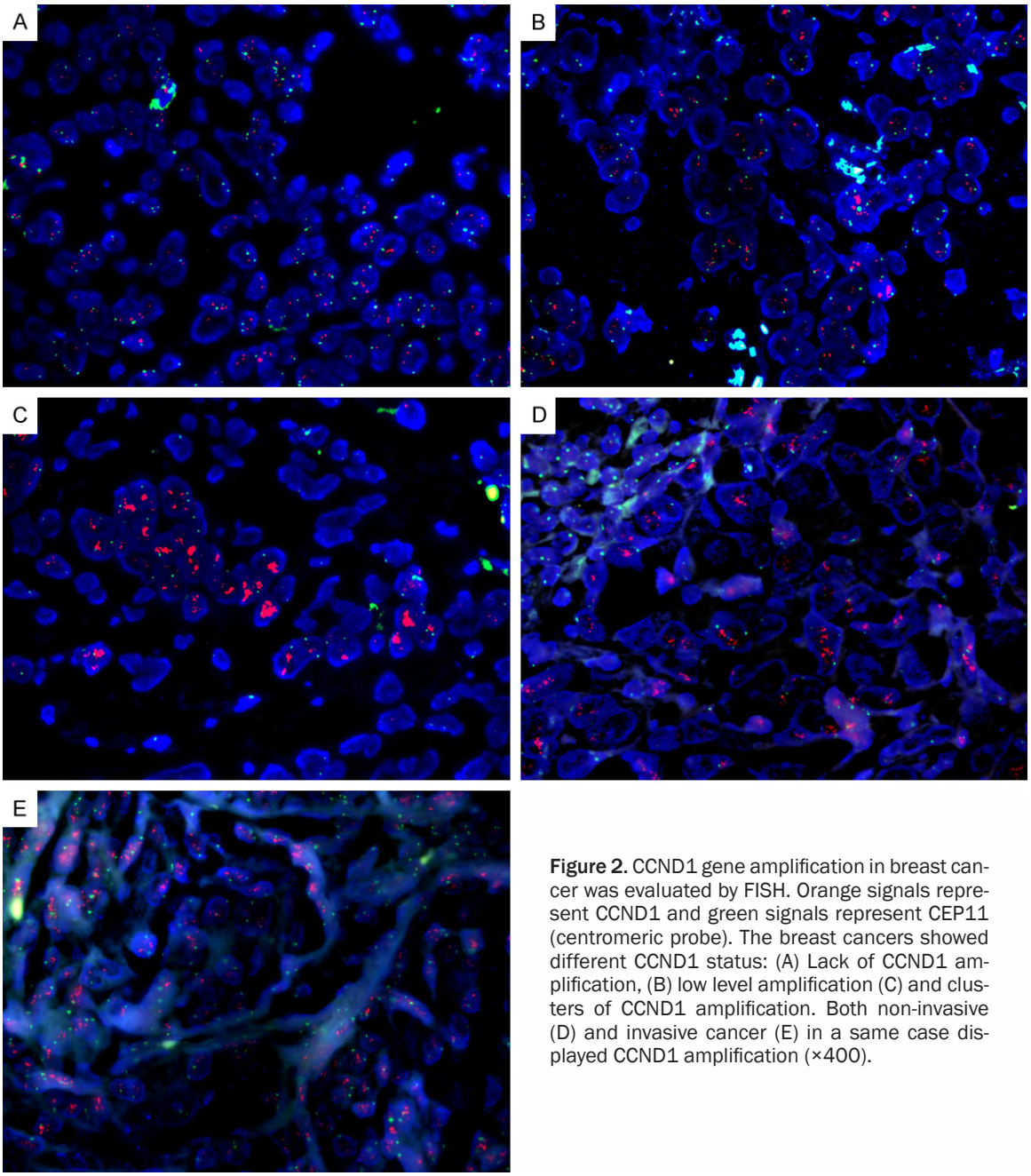

Figure 2. CCND1 gene amplification in breast cancer was evaluated by FISH. Orange signals represent CCND1 and green signals represent CEP11 (centromeric probe). The breast cancers showed different CCND1 status: (A) Lack of CCND1 amplification, (B) low level amplification (C) and clusters of CCND1 amplification. Both non-invasive (D) and invasive cancer (E) in a same case displayed CCND1 amplification (×400).

greatest dimension (mean 2.6 cm). 186 patients had lymph node positive disease. ER, PR, HER-2, ki67 and CK5/6 data were used as surrogates for molecular subtyping according to 13th St Gallen International Breast Cancer Conference Expert Panel recommendations [23]. Accordingly 91 cases (25.6%) were classified as luminal A,149 (42.0%) were luminal B, 81 (22.8%) were HER-2 positive, and 34 (9.6%) were basal like. Patients and tumor characteristics of 355 cases are summarized in **Table 1**.

Relationship between CyclinD1 expression and clinicopathological variables

As shown in **Figure 1**, various nuclear staining intensities of CyclinD1 were observed, and cytoplasmic staining was also shown in some cancer cells. Tumors were categorized into four groups of which the frequency was summarized in **Table 1**. High CyclinD1 expression was found in 213 (60.0%) cases including 132 moderately and 81 strongly positive cases. We compared

Table 2. CCND1 gene amplification status in relation to clinicopathological variables and biological markers

Variables	Patients No (%)	CCND1 amplification		P
		Non-amplified	Amplified	
	355	303	52	
Age				
<35 y	46 (13.0)	37	9	0.312
≥35 y	309 (87.0)	266	43	
Size				0.955
≤2 cm	83 (23.4)	70	13	
2-5 cm	231 (65.1)	198	33	
>5 cm	41 (11.5)	35	6	
Grade				0.052
I	26 (7.3)	18	8	
II	218 (61.4)	188	30	
III	111 (31.3)	97	14	
Lymph node				<0.001
0	169 (47.6)	150	19	
1	186 (52.4)	153	33	
Subtypes				0.004
Luminal A	91 (25.6)	76	15	
Luminal B	149 (42.0)	120	29	
Her-2	81 (22.8)	74	7	
Basal like	34 (9.6)	33	1	
ER				<0.001
Negative	123 (34.6)	118	5	
Positive	232 (65.4)	185	47	
PR				<0.001
Negative	152 (42.8)	140	12	
Positive	203 (57.2)	163	40	
HER-2				0.028
Negative	232 (65.4)	191	41	
Positive	123 (34.6)	112	11	
Ki67				0.756
Low	129 (36.3)	109	20	
High	226 (63.7)	194	32	
P53				0.001
Negative	286 (80.6)	235	51	
Positive	69 (19.4)	68	1	

pre-invasive and invasive lesions for the same patient and found similar CyclinD1 levels in most cases (345 out of 355, 97.2%). CyclinD1 expression was seen to be significantly correlated with tumor increased grade, Ki67, postitive ER and PR, negative HER-2 and p53 (P<0.05, **Table 1**). Higher CyclinD1 expression was found in luminal B tumors, whereas, loss of expression or very weak expression of CyclinD1 was observed in basal-like tumors.

Relationship between CCND1 amplification and clinicopathological variables

We analyzed CCND1 gene copy numbers in 355 cases by FISH. CCND1 amplification was detected in 52 (14.65%) tumors, including 38 with high-level gene amplification (clustered) and 14 with low-level increase of gene copy number (<10 copies, dot-like) (**Figure 2**). As seen for CyclinD1, comparing CCND1 status in DCIS and invasive carcinoma, there is no significant difference (12% vs. 15%, P = 1). These findings revealed CCND1/CyclinD1 changes occur in early-stage of breast cancer. The associations between CCND1 amplification and clinicopathological variables were summarized in **Table 2**. CCND1 gene amplification was positively correlated with ER, PR and lymph node status (P<0.001) while negatively correlated with HER-2 amplification, p53 status (P<0.05). Furthermore, CCND1 amplification tended to be correlated with tumor grade (P = 0.052). Similar as high CyclinD1 expression tumors, CCND1+ tumors were more often of luminal type (44 out of 52, 84.6%) while only one CCND1+ tumor (1.9%) which showed low level gene copy number increase was basal-like.

Correlation between CCND1 amplification and CyclinD1 expression

Data of CCND1 amplification and CyclinD1 expression were available in all 355 cases. All of 52 cases with CCND1 gene amplification were found to have moderate or strong CyclinD1 expression, including 38 tumors with high level amplification showing diffuse strong staining (>50%, +++), and 14 cases with low level amplification exhibiting diffuse moderate or moderate to strong staining. Unsurprisingly there was a significant correlation between CCND1 amplification and CyclinD1 high expression (P<0.001). Furthermore, the immunostaining scores of CyclinD1 protein expression was divided into two subgroups according to CCND1 gene amplification or not. Box-and-whisker graph demonstrated that immunohistochemical scores of CyclinD1 in CCND1 amplified group were significantly higher than those in non-amplified group (P<0.001, **Figure 3**).

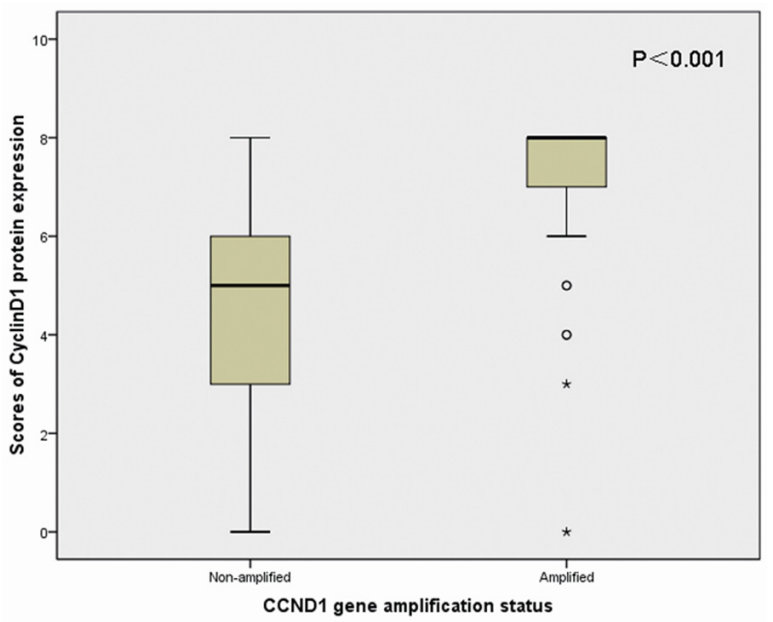

Figure 3. Box-and-whisker graph demonstrated that immunohistochemical scores of CyclinD1 in CCND1 amplified group were significantly higher than those of CCND1 non-amplified group (P<0.001).

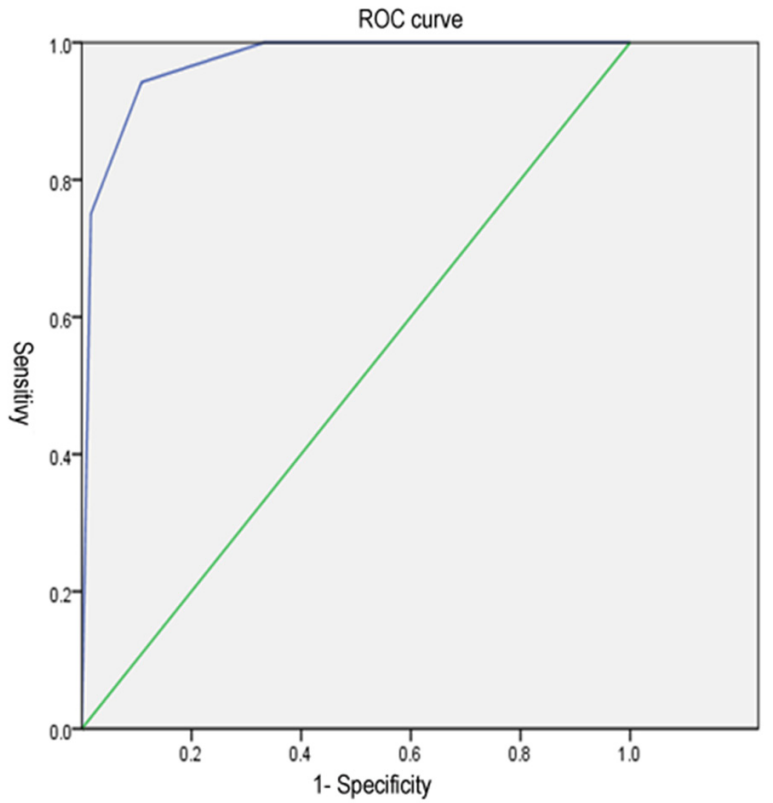

Figure 4. ROC curve analysis of immunostaining scores of CyclinD1 for predicting the CCND1 gene amplification. AUC = area under curve.

ROC curve to confirm a cut-off point for predicting gene amplification

Through ROC curve analysis, it was found that the immunostaining score of CyclinD1 at 6.5 had the optimal performance ability for predicting the CCND1 gene amplification with a sensitivity of 94.2% and specificity of 87.8%. The area under the curve was 96.7% (P<0.001, **Figure 4**). In other words, patients with the immunostaining score 7 or 8 had an approximately 88% chance of CCND1 gene amplification while patients with the immunostaining score <6.5 had only an approximately 6% chance of CCND1 amplification. Then we evaluated the relationship between CyclinD1 expression and clinicopathological characteristics based on the cut-off point. Similar results as CCND1 amplification was obtained.

CCND1 amplification/ CyclinD1 overexpression in ER/PR-positive group

47 out of 52 (90.4%) tumors harboring CCND1 amplification and 173/233 (74.2%) cases with CyclinD1 moderate to strong expression were ER positive. We explored the relationships between CCND1/CyclinD1 status and clinicopathological variables in ER positive group respectively. There is no significant correlation between CCND1 amplification/CyclinD1 overexpression and clinicopathological characteristics in ER positive group.

Discussion

It has been widely accepted that CyclinD1 is linked to

breast carcinogenesis, which is due to promoting cell proliferation and differentiation by shortening the G1/S transition and the interaction with ER. Our present study showed that CyclinD1 expression was up-regulated in breast cancer tissues. The frequency of CCND1 amplification (14.6%) defined by FISH is concordance with that reported in the previous studies [5, 24-26]. As expected there is a strong significant correlation between CCND1 gene amplification and CyclinD1 expression. All 52 CCND1+ tumors showed rather high CyclinD1 level, while most of CCND1- tumors were stained with CyclinD1 mildly to moderately. We also compared the CCND1/CyclinD1 status in early lesions and the invasive settings. The data explored that there were identical alteration in both lesions, further confirming it should be an early event in the development of breast cancer.

This study demonstrated that a significant relationship was noted between CCND1 and increased grade, lymph node metastasis, negative HER-2 and p53 as well as ER and PR positivity. As to CyclinD1 expression similar data was obtained. Though seldom seen in basal-like breast cancer, a high grade type of breast cancer, CCND1/CyclinD1 was found to tend to be higher histological grade, suggesting that CCND1/CyclinD1 is linked to more aggressive phenotype except for basal-like tumors. Beyond expectation, there was no significant correlation of CCND1/CyclinD1 with other pathological features in ER positive tumors, which was not in concordance with some of previous studies. It might be owing to low patient numbers, more HER-2 positive tumors in this cohort, and different cut-off value for ER positive. As the cases in this study were from recent years, the following-up data were not available to further analyze the effects of CyclinD1/CCND1 on prognosis. Hence, further relevant studies and in-depth assessments are needed to carry out in order to ensure the accuracy and specificity.

Further evidence was present that the majority of the CCND1 amplification/high CyclinD1 breast cancers were ER positive and PR positive, and classified as luminal B, which is easy to understand in consideration of CyclinD1 acting as a co-factor of ER independently of the ligand. On the contrary, among basal like or p53-mutated tumors loss of CyclinD1 expression were more frequent, which was in line with other studies [13]. So it was thought CyclinD1 was unlikely to play a role in these tumors, while we considered down-regulation or loss expression of CyclinD1 might be an important event in the initiation and/or progression of tumors in such subtypes. Lehn et al. [27] found increased migration and proliferation in breast cancer cells by silencing of CyclinD1 and down-regulation of CyclinD1 is linked to unfavorable prognostic features. They supposed that CyclinD1 has distinct functions in different cell cycle phases, moreover, the mechanism is complex and not mediated by a single pathway or gene product. Basal-like or p53-mutated tumors are highly malignant and more invasive. In this regard, CyclinD1 might play an important role in defining malignant behaviors rather than it is not involved in their tumorigenesis. Different from ER positive tumors, basal-like breast cancers frequently bear p53 mutations and other genes mutations such as BRCA1 and BRCA2. Therefore we supposed that a genetic background of abnormality influences how CyclinD1 affects cell proliferation and malignant behavior. It deserves further attention to better under stand biological importance and its mechanism in basal-like breast cancer.

Whilst endocrine therapy represents a major treatment for patients with ER positive breast cancer, up to 40% of the patients are resistant to the therapy. However, the mechanism of resistance remains a major issue and there is yet no biomarker to assist clinician in selecting patients who are resistant to endocrine therapy. As mentioned above, studies showed CCND1 gene amplification as a possible marker to predict prognosis and tamoxifen resistance. A poor response to tamoxifen has been observed in ER positive tumors with CCND1 amplification [15]. In vitro study, our data confirmed that the breast cancer cells were more sensitive to tamoxifen and toremifene bysilencing of CCND1 (to be published). But as to the predictive impact of CyclinD1, despite intensive studies, conflicting results have been reported [28, 29]. Various factors could contribute to conflicting roles as well as the wide range of overexpression rate of CyclinD1 (from 40% to 90%). According to our experience and also discussed by other authors [17, 25], the most reasonable possibility for the discordant results is different cut-off point or criteria to evaluate

CyclinD1 "overexpression". Some researchers consider CyclinD1 expression higher than that of normal breast tissue as overexpression, some take expression of this protein in more than 10% tumor cells as overexpression, while others define it quite strictly. It is obvious and the influence of CyclinD1 with diffuse strong staining on patient survival and response to therapy would be significantly different from that of 10% moderate positive. Tumors bearing CCND1 amplification all exhibit rather diffuse and strong CyclinD1 expression. Considering that normal breast tissue shows CyclinD1 expression to some extent and benign epithelial hyperplasia also could have high CyclinD1 levels, it is reasonable to hold that the CyclinD1 levels comparable to those with gene amplification could be counted as true "high expression" as reported for other genes such as HER-2. Jirström et al. [25] showed a positive link between resistance to tamoxifen and intensity of CyclinD1 staining other than the nuclear fraction. Lundgron [19] obtained contrast results when comparing nuclear fraction of CyclinD1 and CCND1 amplification with patient's prognosis. Data from the study of Jirström et al also revealed that the intensity of the nuclear staining by immunohistochemistry rather than the nuclear fraction was indicative of treatment response [25]. The reason for this discrepancy remains to be elucidated, but the nuclear intensity of CyclinD1 might be linked well to the degree of amplification of the CCND1 gene. It was noted the treatment-predictive value of these two variables differed: there was an adverse tamoxifen effect in amplified tumors, but when defining high CyclinD1 protein content either as strong nuclear intensity or >50% positive cells, there was a significantly positive tamoxifen effect [25], further supporting CCND1+ rather than a high protein expression may be a biomarker of negative predictive value for tamoxifen benefit. But no one gave evaluating criteria. Taking together with other studies we can conclude although CyclinD1 protein expression correlated strongly with CCND1 gene amplification in this study, the latter was by far the most powerful predictor of tamoxifen response. However, compared with FISH analysis, immunohistochemistry is much simpler and easier to use in clinical practice. Herein we hope to get a cut-off point of CyclinD1 overexpressionto predict CCND1 gene amplification in breast cancer precisely. Using ROC we got the cut-off point in which it has the optimal sensitivity and specificity is 6.5 ($P<0.001$, AUC = 0.969). That's to say, breast cancers with CyclinD1 immunostaining scores7 or 8 have the CCND1 amplification with possibility of 97% while tumors with scores 6 or less than 6 have very low possibility. Our current study first reported to predict CCND1 gene amplification using immunostaining scores of CyclinD1 protein.

In recent years, the success of HER2-targeted therapy is bringing us toward a new era of personalized medicine. HER-2 amplification which is found in approximately 20% of the breast cancers is associated with a poor prognosis and a good response to tratuzumab and targeting HER-2 is supposed to be a successful way in breast cancer treatment [30]. However, if the ER+/HER-2- patients are resistant to endocrine therapy, how could we do? The story of HER-2 enlightened us that CCND1 may not only serve as a predictive role for prognosis and response to endocrine therapy, but serve as a marker for target therapy. It is necessary to give anti-CyclinD1 treatmentin breast cancer with CCND1 amplification. As we know, the amplification of CCND1 gene is a frequent event in breast cancer, cut-off point should be used to evaluate CyclinD1 status and give clinician more information when they are making therapy plans.

The present study demonstrated that CyclinD1 overexpression and CCND1 amplification are frequent in breast cancers and there was a strong correlation between them in this series of patients. The CCND1/CyclinD1 changes are identical in early and the invasive lesions. Our data have also shown that there were significant correlations between hormone receptors, negative HER-2 and CyclinD1/CCND1 status. We found majority of basal-like breast cancers showed deregulation or loss expression of CyclinD1, so CyclinD1 might play an important role in defining malignant behaviors. More important, we confirmed an optimal cut-off value of immunostaining scores of CyclinD1 protein which could be used to predict the status of CCND1 gene amplification. It would be useful to identify a subset of patients with ER positive breast cancer who have poor response to endocrine agents. In future it may serve as a target of anticancer drugs.

Materials and methods

Patients

A cohort of breast cancers were collected from patients diagnosed at the Department of Pathology of Qilu Hospital, Shandong University between 2010 and 2013. All the tumors were invasive carcinoma of no specific type with foci of pre-invasive components-DCIS. Patients were excluded if their data were lost or incomplete, or the paraffin-embedded tissue blocks were not available for immunostaining and FISH analysis. At last, 355 eligible patients who underwent potentially curative surgeryand complete axillary clearance were identified for this study. For each tumor, malignancy grade, tumor size (maximum diameter), lymphnode status and ER, progesterone receptor (PR), HER-2, p53, ki67, CK5/6 immunostaining at the time of diagnosis were evaluated. Tumors weregraded accordingto a modified Elston & Ellis scoring system, andsize was categorized in accordance with the TNM staging criteria. In detail, patient and tumor characteristics of 355 cases were listed in **Table 1**. This study was approved by the Ethics Committee and previous informed consent was obtained from all patients for collection of breast cancer specimens in accordance with the guidelines of Qilu Hospital.

TMA sconstruction

The TMAs were arrayed as previously described [31-34]. Briefly, H&E-stained sections from each available tumor block were reviewed to define the most representative areas of tumors. Two tissue cores (diameter 1 mm) were taken from DCIS and invasive area per case for TMA construction. Subsequently contiguous sections were prepared from array blocks for H&E staining, immunohistochemistry and FISH procedure (thickness 4 μm).

Fluorescence in situ hybridization

FISH analysis was performed to detect gene copy number changes ofHER-2 and CCND1 with a commercial FISH probe kit (Abbott Molecular Inc.). The slides were pre-treated in sodium thiocyanate and exposed target DNA by pepsin digestion at 37°C for 30 min. Denaturation was executed by incubation in formamide solution at 72°C. Hybridization with the DNA probe was performed in humidified chamber at 37°C for overnight. After washing in sodium citrate (SSC) and detergent solutions, sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Signal evaluation was carried out using an Olympus BX53 epi-fluorescence microscope equipped with DAPI, Spectrum Green, Spectrum orange filter cubes. Two observers (LL, XJW) carried out all investigations independently. The exact copy number of the signals per nucleus was recorded, and at least 100 non-overlapping nuclei per sample were analyzed. Evaluation of HER-2 amplification was performed according to ASCO/CAP Her2 clinical practice guideline [35]. CCND1 amplification (CCND1+) was defined by the presence of an excess in the number of gene loci over the number of corresponding chromosomes on more than 20% of counted cells [36].

Immunohistochemistry

The sections were deparaffinized with xylene, rehydrated through a graded alcohol series and microwaved at 500 W for 2×5 min in 10 mM citrate buffer (pH 6.0). After rinsing in Tris-buffered saline (TBS, pH 7.6), sections were immersed in 3% hydrogen peroxide to exhaust endogenous peroxidase activity followed by 5% normal horse serumand then incubated overnight at 4°C with the monoclonal primary antibodies for CyclinD1 (SP4, diluted 1:50, Neomarkers). Then appropriate second antibodies and streptavidin-peroxidase conjugate were applied, and antibody-specific binding was visualized with 3,3-diaminobenzidine solution (DAB). Lastly, the sections were counterstained with hematoxylin and mounted with neutral balsam. Positive controls were included in each slide run. Normal fetus serum was used as a negative controlby replacement of the relevant primary antibody.

Only distinct nuclear staining was accepted as a positive reaction for CyclinD1 and staining intensity and nuclear fraction were scoredsemi-quantitatively by two pathologists (LL and XJW) on a multiheaded light microscope using the Allred score method [37]. With this method, the intensity of the immunohistochemical reaction was recorded as 0, negative (no staining of any nuclei); 1, weak; 2, moderate; or 3, strong. The fraction of tumor nuclei was also recorded

as either: 0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3 and 5, >2/3. The final staining score was the sum of staining intensity and percentage of positive cells, which ranged from 0 to 8. Thus it was further graded into four groups as follows: negative (0-2); weak (3-4); intermediate (5-6) and strong (7-8). The immunostained slides of ER, PR, HER-2, p53, ki67 and CK5/6 were reviewed and reevaluated. Tumors were counted asposi-tive for ER and PR if >1% of the nuclei of neoplastic cells showed definitive staining. Ki67 status was scored low if <14% of the nuclei of neoplastic cells were positive, and high if ≥14% of the nuclei of neoplastic cells were positive. p53 was considered positive only when more than 75% of cancer cells with distinct, strong nuclear staining and negative when 75% or less were stained [38]. HER-2 was scored according to ASCO/CAP Her2 clinical practice guideline [35].

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (SPSS Inc. Chicago, IL). The Chi-square and Fisher exact test was used to evaluate the statistical significance between clinicopathological variables and CyclinD1/CCND1. Correlations were studied using the Spearman test. Mann-Whitney test were used to detect statistical significance in Box-and-whisker graph. Receiver-operating characteristic curve (ROC) was used to assess the ability of immunostaining scoring of CyclinD1 to predict CCND1 gene amplification. The optimal cut-off value was calculated by determining immunostaining scores that provided the greatest sum of sensitivity and specificity. Statistical significance was set at 0.05 and all *P* value is two-sided.

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