

타목시펜 치료를 받은 에스트로젠 수용체 양성 유방암 환자의 Cyclin D1 단백질 발현에 따른 치료 효과의 비교

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Cyclin D1 Expression and Patient Outcome after Tamoxifen Therapy in Estrogen ReceptorPositive Breast Cancer

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Background and Objectives: Cyclin D1 expression is closely related with ER in breast cancer. We conducted this study to evaluate whether therapeutic response to tamoxifen is varied with levels of cyclin D1 expression in ER positive breast cancer patients. **Methods:** Immunohistochemical assay for cyclin D1 protein was performed in 66 patients treated with tamoxifen for more than 2 years. Patient survival and correlation between cyclin D1 expression and biologic data of the patients were analyzed. **Results:** Cyclin D1 expression was detected in 46 (69.7%) and significantly reduced in poorly differentiated cancer ($p=0.023$). Cyclin D1 expression was high in the tumors expressing Myc (15/15 vs. 31/51; $p=0.002$), and was markedly increased in the tumors in which p27Kip1 expression was repressed (30/38, 78.9%). However, the difference was not statistically significant ($p=0.051$). There was no significant relationship between cyclin D1 expression and S-phase. Patients with tumors expressing cyclinD1 showed better disease free survival and overall survival but the difference was not statistically significant. **Conclusions:** Cyclin D1 expression was associated with cell differentiation but not useful in discriminating high risk group with tamoxifen treatment. Cyclin D1 may have a role other than cell cycle regulator in ER positive breast cancer, such as differentiation signal. (*Journal of Korean Breast Cancer Society* 2000;1:000~000)

Key Words: Differentiation, Myc, p27Kip1, Prognosis, Treatment

Introduction

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Cyclin D1 protein is a key regulator of the G1
phase progression during the cell cycle. The cyclin
D1 gene, CCND1, is amplified in approximately

20% of human breast cancers¹⁾, and the protein is over-expressed in around 50% of the cases²⁾. A number of studies have been conducted whether cyclin D1 could be a useful biological marker in the management of breast cancer patients³⁻⁵⁾. The results are equivocal according to patient selection, tissue preparation, and detection methods of the conducted studies. However, one of the most consistent trends among clinical studies is intimate correlation of cyclin D1 overexpression and tumor estrogen receptor (ER) positivity. Most studies in breast cancer have found that high levels of cyclin D1 expression are associated with positive ER expression and with well differentiated cancers⁶⁻⁸⁾.

Cyclin D1 expression is induced by estrogen and thought to act as a cellular sensor for their presence in breast cancer cells⁹⁾. Cyclin D1 was reported to bind liganded and unliganded ER, and tamoxifen could inhibit cyclin D1 activation of ER¹⁰⁾. Therefore, cyclin D1 is considered to serve non-cell cycle function in breast cancer. A recent study demonstrated that constitutive overexpression of cyclin D1 does not prevent inhibition of hormone-responsive human breast cancer cell growth by antiestrogens such as tamoxifen or ICI 182,780¹¹⁾.

ER positivity is associated with a good response to endocrine therapy, but the treatment is not effective in all patients with ER positive tumors. The biological and clinical diversity of breast cancer is responsible for various response to hormonal therapy. Major clinical utility of measuring ER in breast cancer is to predict response to hormonal therapy in the adjuvant setting. If expression of cyclin D1 is related to ER expression of breast cancer, measurement of cyclin D1 expression could be a useful adjunct to ER status in determining hormonal therapy in the management of breast cancer patients.

We conducted this study to investigate the correlation between cyclin D1 expression and response to hormonal therapy in postmenopausal women with

breast cancer. Correlations between cyclin D1 expression and status of Myc and p27Kip1 expression, representative regulators of cell cycle, are also described.

Materials and Methods

A total of 66 post-menopausal patients with ER positive breast cancer who underwent modified radical mastectomy or breast conservation surgery at Inje University Sanggye Paik Hospital between January 1992 and December 1993 were evaluated. Important selection criteria for entry to the study were feasible freshness of cancer tissues for immunohistochemical assay of cyclin D1 protein and treatment with tamoxifen only after surgical management. Mean age of studied patients was 54 years ranging from 47 to 79 years, and all patients were confirmed as post-menopausal women. ER status of studied patients was assessed with dextran coated charcoal assay, regarding receptor levels >3 fmol/mg of cytosolic protein as positive. The stage of each patient was determined according to the TNM classification of the American Joint Committee on Cancer; 16 with stage I (24.2%), 28 with stage IIa (42.4%), 16 with stage IIb (24.2%), 6 with stage IIIa (9.2%). All patients did not received any form of postoperative adjuvant chemotherapy but were treated with tamoxifen 20 mg/day for minimum 2 years (range: 2.3 - 6.4 years).

1. Cell cycle analysis

Flow cytometry analysis was performed on cell suspensions obtained from breast cancer tissues. After centrifugation, supernatants were discarded, and the cell pellets were resuspended in 250 μ l of buffer solution (10mM Citrate, pH 7.5, 20mM NaCl, 20mM MgC¹²⁾. After adding 10 μ l/ml of trypsin, trypsin inhibitor and DNase-free RNase, nuclei were incubated at room temperature for 30 minutes. DNA

staining was obtained with 500 μ l of propidium iodide solution (PI; Molecular Probes, Eugene, OR) in PBS (100 μ l/ml PI, 0.1% Triton X-100, 1% FCS) for 1 hour at 4°C in the dark, followed by flow analysis. The DNA fluorescence was analyzed using a FACScan (Becton-Dickinson, Bedford, MA). Data acquisition was performed using the Cell Fit software (Becton-Dickinson) and data analysis using the Phoenix Flow System Multicycle AV software.

2. Immunohistochemical assay

The same paraffin blocks as for flow cytometry from breast cancer patients were retrieved and neoplastic tissues of these breast cancers were examined for expression of cyclin D1 protein using the avidin-biotin complex (ABC) immunoperoxidase method. We used commercially available monoclonal antibody; NCL-CYCLIN D1-GM (1:50 dilution) for cyclin D1 protein assay (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). Immune staining was performed as described previously¹²⁾. Counterstaining with hematoxylin was done after ABC immune staining and two pathologists evaluated immunohistochemical staining separately without information of patients' outcome data. Two pathologists reviewed the slides if interpretation of the immunohis-

tochemical analysis was different. Three separate blocks containing malignant cells were stained and scored by calculating the stained cells from 500 observed cancer cells in percentage. Sections of breast cancer observed to express homogenous and/or intense nuclear immunohistochemical staining for cyclin D1 protein in more than 5% of the observed field were considered to be positive for expression (Fig. 1).

Data for the immunohistochemical assay were merged with clinicopathologic characteristics of patients to determine the frequency of cyclin D1 protein expression, and to evaluate the role of cyclin D1 as cell cycle regulator in human breast cancer by correlating with proliferative activity of the cancer cells. We also analyzed the correlation between cyclin D1 expression and expression status of p27Kip1 and Myc protein which had been performed in the tumor tissues of same patient population. Statistical analyses were performed using SPSS package program (7.5 version) of personal computer statistics software. Correlation between clinical/biological parameters and expression of cyclin D1 protein was estimated by chi-square test. Statistical analysis of patients' survival according to cyclin D1 expression status was carried out by log-rank test.

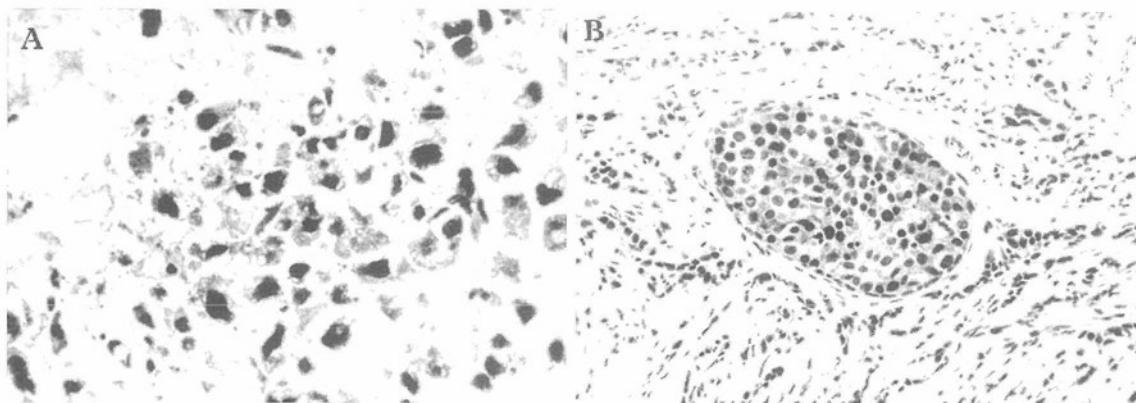


Fig. 1. A, Cyclin D1 expression in invasive ductal cancer ($\times 400$). Intense nuclear staining is observed in more than 80% of cells. B, Cyclin D1 expression is evident in in situ cancer component as well as in invasive breast cancer ($\times 200$).

Results

Of 66 studied samples of breast cancer with ER positivity, 46 (69.7%) showed cyclin D1 expression. In 18 tumors which are less than 2 cm in their size, cyclin D1 expression was observed in 13 (72.2%)

while its expression rate was 68.8%(33/48) in the tumors larger than 2 cm in their size. There was no difference of cyclin D1 expression according to size of the tumors (Table 1). When we analyzed expression pattern of cyclin D1 according to lymph node status, cyclin D1 expression was observed in the tumors with negative axillary lymph node (24/32) as

Table 1. Correlation between Cyclin D1 Expression and Clinical/Biological Parameters

	Cyclin D1 expression (%)			p-value
	<5%	≥5%	Total	
Progesterone receptor				0.394
Negative	7(35.0%)	13(65.0%)	20	
Positive	13(28.3%)	33(71.7%)	46	
Nuclear grade				0.023
I	5(38.5%)	19(61.5%)	24	
II	6(24.0%)	19(76.0%)	25	
III	9(56.3%)	7(43.8%)	16	0.158
Tumor size				
<2cm	5(27.8%)	13(72.2%)	18	
>2cm	15(31.3%)	33(68.8%)	48	
Lymph node metastasis				0.409
0	8(25.0%)	24(75.0%)	32	
1-3	5(35.7%)	9(64.3%)	14	
> 3	7(35.0%)	13(65.0%)	20	0.385
ploidy				
diploid	11(34.4%)	21(65.6%)	32	
non-diploid	8(27.6%)	21(72.4%)	29	
S-phase				0.579
<10%	8(30.8%)	18(69.2%)	26	
≥10%	12(30.0%)	28(70.0%)	40	

Table 2. Change of Cyclin D1 Expression according to Expression of Myc and p27Kipl

	Cyclin D1 expression (%)			p-value
	< 5%	≥ 5%	Total	
myc expression				0.002
<5%	20 (39.2%)	31 (60.8%)	51	
≥5%	0 (0%)	15 (100%)	15	
P27Kipl expression				0.051
<20%	8 (21.1%)	30 (78.9%)	38	
≥20%	12 (42.9%)	16 (57.1%)	28	

Table 3. Correlation between Nuclear Grade of the Tumors and Studied Molecules

	Nuclear Grade (%)			p-value
	I	II	III	
Cyclin D1				0.020
<5%	5 (20.0)	6 (24.0)	9 (56.3)	
>5%	20 (80.0)	19 (76.0)	7 (43.7)	
P27Kip1				0.004
<20%	21 (84.0)	11 (44.0)	6 (37.5)	
>20%	4 (16.0)	14 (56.0)	10 (62.5)	
myc				NS
<5%	19 (73.1)	19 (76.0)	13 (81.3)	
>5%	7 (26.9)	6 (24.0)	3 (18.7)	

well as in the tumors with involved axillary lymph nodes (22/34). Expression pattern of cyclin D1 was not different with axillary lymph node status.

There was a significant difference of cyclin D1 expression according to nuclear grade of the tumors. Frequency of cyclin D1 was more than 70% in low or intermediate grade tumors, but the frequency decreased to 43.7% (7/16) in high grade tumors ($p=0.023$). Cyclin D1 expression was analyzed according to progesterone receptor (PR) status of the patients. PR was expressed in 46 (69.7%) out of total 66 patients. However, there was no significant correlation between cyclin D1 expression and PR expression of the tumors (Table 1).

Results of cell cycle analyses using flow cytometry showed that 32 patients (48.4%) had diploid tumors while 29 patients (43.9%) had non-diploid tumors. We could not find a significant association between ploidy of the tumors and cyclin D1 expression. The finding was same when we analyzed cyclin D1 expression according to the S-phase of the tumors. Eighteen tumor samples (69.2%) out of 26, in which S-phase fraction was less than 10%, showed high expression of cyclin D1 and 28 tumors (70.0%) out of 40, which showed high S-phase frac-

tion, had increased level of cyclin D1 expression. Change of cyclin D1 expression was not associated with S-phase of the tumors. We correlated cyclin D1 expression with expression of p27Kip1, an inhibitor of cyclin-dependent kinases (cdk), and Myc protein which have been performed with the same tumor tissues. Cyclin D1 was expressed in 31 (60.8%) out of 51 tumors which did not show Myc expression whereas cyclin D1 expression was evident in all tumors which showed high expression of Myc protein (15/15). Cyclin D1 expression was significantly increased in the tumors expressing Myc protein ($p=0.002$). In the tumors with persistent expression of p27Kip1, cyclin D1 was expressed in 57.1% (16/28). Cyclin D1 expression was markedly increased in the tumors in which p27Kip1 expression was repressed (30/38, 78.9%). However, the difference was not statistically significant ($p=0.051$). Change of cyclin D1 expression according to expression of p27Kip1 and Myc are summarized in Table 2.

With median follow-up period of 46 months (35-75 months), 21 patients (31.8%) had recurrent disease and 16 patients (24.2%) had expired due to recurrence of the disease. In survival analysis, there was a trend for decreased recurrence in the patients with cyclin D1 overexpression. However, the difference was not statistically significant ($p=0.154$ by log-rank test). Fourteen patients (30.4%) out of 46, who had increased level of cyclin D1 expression at the time of surgery, had recurrent disease whereas 7 patients (35%) out of 20 patients, who did not showed cyclin D1 expression, had recurrent disease (Fig. 2). Of 20 patients who did not showed cyclin D1 expression, 5 patients (25%) had died due to recurrence of breast cancer, while 11 patients (23.9%) out of 46 patients, who showed increased level of cyclin D1 expression, had died because of recurrent disease. Overall survival of the patients was not associated with cyclin D1 expression ($p=0.243$).

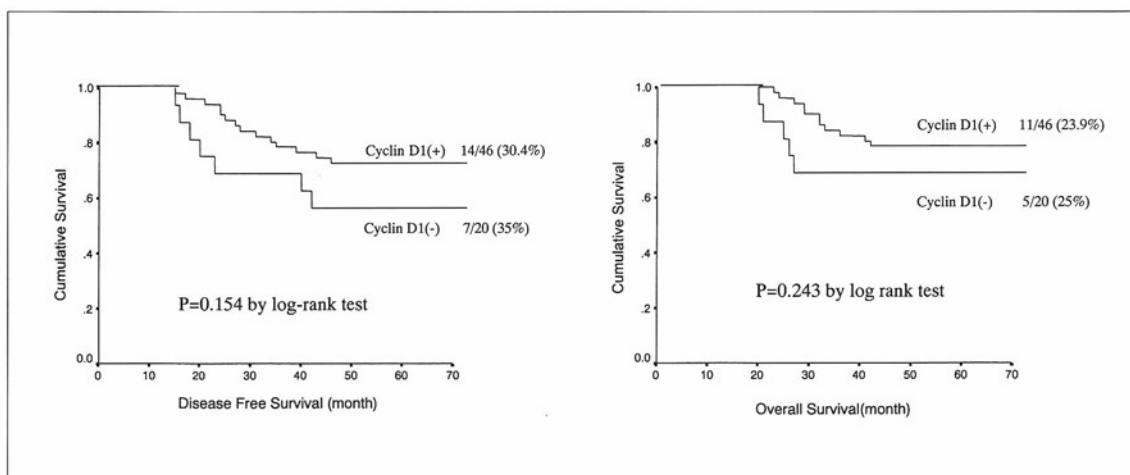


Fig. 2. Patients with cyclin D1 expression showed better survival than patients with cyclin D1 negative tumors, but the difference was not statistically significant. A, Disease free survival. B, Overall survival of the patients.

by log-rank test).

Discussion

In breast cancer cell lines, expression of either Myc or cyclin D1 precedes estrogen-induced progression through G1 phase of the cell cycle¹³. A number of reports have examined cyclin D1 expression among panels of human breast carcinoma cell lines, and found those ER positive cell lines to quantitatively overexpress cyclin D1¹⁴⁻¹⁸. If cyclin D1 expression is closely linked to ER expression in breast tissues, response to tamoxifen treatment could be varied with cyclin D1 expression. However, we could not observe any difference in recurrence free survival or overall survival of the patients according to the status of cyclin D1 expression after adjuvant treatment with tamoxifen. This result contrasts with the good response to tamoxifen therapy for metastatic disease in the patients showing high levels of cyclin D1 expression².

Wilcken et al reported the in vitro finding that ER positive cells which over-express cyclin D1 can continue to proliferate in the presence of anti-

estrogens¹⁹. Diversity of clinical response to hormonal therapy may be due to the alteration of genes other than CCND1 or ER that accumulates in individual tumor. Recently, Collecchi et al reported that cyclin D1 expression showed different pattern according to axillary lymph node status²⁰ and the finding suggests that cyclin D1 may be differently regulated during tumor progression. Progesterone receptor (PR) expression is also known to be regulated by ER, but there was no significant correlation between PR and cyclin D1 expression in the result of present study. It seems likely that cyclin D1 expression is regulated by signals other than ER in human breast cancer. Therapeutic effects of tamoxifen in human breast cancer may be quite complex and may include activity which is independent of the ER or cyclin D1, since in vitro studies cannot simulate the complex environment that tumor cells normally experience in vivo.

Most studies in breast cancer have found that high levels of cyclin D1 expression are associated with well differentiated cancers⁶⁻⁸. We could observe a similar finding from the result of present study. Cyclin D1 expression was markedly decreased in high grade tumor compared to the tumors showing

low or intermediate nuclear grade (Table 3). Result of the present study suggests that cyclin D1 expression in primary breast cancer may have a differentiation signal.

Cyclin D1 and its kinase partners are involved in cell-cycle regulation and its over-expression accelerates and shortens the G1 phase²¹⁻²³. However, we could not find a significant correlation between cyclin D1 expression and S-phase of primary breast cancer tissues. Lack of significant association between cyclin D1 expression and S-phase coincides with the findings of other studies^{7,20}. We also analyzed the change of cyclin D1 expression along with p27Kip1 expression. We could observe that frequency of cyclin D1 expression decreased in the tumors in which p27Kip1 expression was well preserved. However, the difference was not statistically significant. p27Kip1 acts during G0 and the early G1 phase of the cell cycle to inhibit cyclin D1-cdk4 and cyclin E-cdk2 complexes²⁴. Recent studies suggest that p27Kip1 also functions in cellular differentiation and development²⁵⁻²⁷. We have reported that p27Kip1 expression is linked to cell differentiation and proliferation in another population of breast cancer patients²⁸. Expression of cyclin D1 and p27Kip1 may be different according to tumor cell clones, or cell proliferation may be influenced by molecules other than cyclin D1 or p27Kip1. It is also possible that cyclin D1 plays a role in processes other than cell proliferation of breast cancer, such as cellular differentiation or genetic instabilities.

Overexpression of Myc has been shown to overcome p27Kip1 mediated growth arrest, allowing cyclin-cdk activity in the presence of physiological and even elevated levels of p27Kip1²⁹. In the result of present study, we could find that cyclin D1 expression was significantly increased in the tumors expressing Myc. Prall et al suggested that inducible expression of either c-Myc or cyclin D1 was sufficient for S-phase entry in cells previously

arrested in G1 phase by pretreatment with ICI118780, a potent estrogen antagonist¹³. Growth inhibition of ER positive breast cancer cells by antiestrogens is also accompanied by a decrease in c-Myc expression, both in vivo³⁰ and in vitro³¹. There are conflicting reports about the effects of Myc on cyclin D1 expression, with some suggesting repression^{32,33} and others indicating upregulation of cyclin D1 by Myc³⁴.

Myc may also be involved in the regulation of cdk activity through increased cyclin expression. Seth et al suggested that Map kinase pathway cooperates with Myc to regulate cdk expression by direct phosphorylation of the Myc transactivation domain³⁵. In the results of our study, cyclin D1 expression was strongly associated with Myc expression and there was a tendency that p27Kip1 expression was inversely correlated with cyclin D1 expression. Expression of cyclin D1 in human breast cancer may be interrelated with expression status of Myc and/or p27Kip1. However, it should be determined by further study whether the proteins function in complementary or linear pathways.

In summary, cyclin D1 expression was closely related with cell differentiation and expression of Myc protein in ER positive breast cancer. However, it is not clear whether response to tamoxifen treatment could be predicted by cyclin D1 expression status.

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