Cyclin D1 Expression in Invasive Breast Cancer

Correlations and Prognostic Value

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Cyclin D1 overexpression, detected by standard immunohistochemistry, was correlated with other prognostic variables and its prognostic value was evaluated in a group of 148 invasive breast cancers with long-term follow-up. Overexpression of cyclin D1 (59% of cases) was negatively correlated (χ^2 test) with histological grade (P = 0.0001), mean nuclear area (P = 0.004), mean nuclear volume (P = 0.02), and mitotic activity (P = 0.03) and positively correlated with estrogen receptor (P = 0.0001). There was a strong correlation between cyclin D1 overexpression and histological type (P = 0.0001). Positive cyclin D1 staining was seen in 11 of 13 tubular carcinomas, 3 of 3 mucinous carcinomas, 4 of 4 invasive cribriform carcinomas, and 17 of 20 lobular carcinomas. Of 102 ductal cancers, 52 were positive, and all 6 medullary carcinomas were negative. There were no significant correlations with lymph node status, tumor size, or DNA ploidy. In survival analysis, cyclin D1 overexpression did not provide significant univariate or multivariate prognostic value. In conclusion, cyclin D1 is mainly overexpressed in the well differentiated and lobular types of invasive breast cancer and is strongly associated with estrogen receptor positivity. It is negatively correlated with the proliferation marker mitoses count and with the differentiation markers nuclear area and nuclear volume. However, cyclin D1 overexpression does not seem to have prognostic value in invasive breast cancer when no adjuvant treatment is given.   (Am J Pathol 1997, 150:705–711)

In breast cancer, high tumor cell proliferation as assessed by mitotic counts,[1–6] DNA flow cytometric S-phase fraction,[7,8] Ki-67 expression,[9,10] or tritiated thymidine uptake[11,12] has been shown to indicate poor prognosis. Little is known, however, of the molecular and cell biological background of increased proliferation in breast cancer.

Recently, more insight has been gained in the role of the cyclin protein family in the complex regulation of different phases of the cell cycle[13–19]. Of the cyclin proteins, the D cyclins are preferentially expressed in the G1 phase. Cyclin D1 is especially interesting as it has been shown that induction of cyclin D1 is sufficient for cells arrested in the early G1 phase to complete the cell cycle.[17] Amplification of cyclin D1 is frequently found in a number of tumors, including squamous cell carcinoma of the head and neck[15] and invasive breast carcinomas[19,20] and has been correlated with poor prognosis in these tumors. Overexpression of the cyclin D1 protein may be present due to amplification[15,16] or chromosomal translocation as has been found in parathyroid adenomas[21] and centrocytic lymphomas.[22] Cyclin D1 overexpression has been the subject of several studies,[14–16,23–27] but only few clinical studies on cyclin D1 overexpression have been described.[15,16,25,26] In a limited series, cyclin D1 overexpression was positively correlated with high tumor grade in sarcomas.[27] In breast cancer, Zhang et al[25] found overexpression of cyclin D1 in over 80% of cases using a polyclonal antibody. Most of these cases, however, showed no amplification. Micha-

lides et al[16] also found cyclin D1 overexpression in the absence of amplification in invasive breast cancer, correlated to estrogen receptor positivity.

The aim of this study was to correlate cyclin D1 overexpression using a well characterized antibody with other qualitative and quantitative prognostic variables and to evaluate its prognostic value in a group of 148 invasive breast cancer patients with long-term follow-up.

Materials and Methods

Patients

The patients from this group were selected from a previously described group of 189 cases with invasive breast cancer,[28] diagnosed between 1971 and 1981 in the Free University Hospital or the Netherlands Cancer Institute. All patients were treated with radical or modified radical mastectomy. Postoperative locoregional radiotherapy was given in all lymph node positive cases, and none of the patients received any form of adjuvant systemic therapy. Follow-up time was 83 months on average (range, 4 to 138 months). For 41 cases, no tumor material was left in the original blocks, leaving 148 cases. None of these patients were included in our previous study.[16]

Specimen Preparation

The fresh operation specimens were cut in slices of approximately 0.5 cm, tumor size was measured, and the material was fixed in neutral 4% buffered formaldehyde. Representative tumor samples were taken, taking special care that the periphery of the tumor was sampled, and embedded in paraffin. The 4-μm-thick sections were cut for cyclin D1 immunohistochemistry and routine staining with hematoxylin and eosin (H&E) for diagnosis and histological typing according to the World Health Organization criteria,[29] mitoses counting, and morphometry.

For DNA cytometry, cell suspensions were prepared from 50-μm-thick slices of the representative paraffin block of the primary tumor according to standard procedures[30] and stained with diamidino-phenyl-indole-dihydrochloride, taking 4-μm sandwich slides before and after the thick slices to check them for the presence of invasive tumor parts.

Estrogen receptor content was assessed with the dextran-coated charcoal assay on tumor cytosols, regarding receptor levels $\geq$10 fmol/mg of cytosolic protein as positive.

Immunohistochemistry

The primary antibody used for cyclin D1 staining of sections was an affinity-purified rabbit anti-cyclin D1 antibody that was generated by injection of a β-galactosidase-cyclin D1 fusion protein, using the carboxyl-terminal part of cyclin D1 (amino acids 217 to 296, corresponding to the *NcoI-DdeI* fragment of cyclin D1) into rabbits. Antibodies directed against the cyclin D1 part of the fusion protein were affinity purified on a glutathione *S*-transferase-cyclin-D1 fusion protein, using the whole-size cyclin D1 protein (corresponding to the *NcoI-HindIII* fragment of cyclin D1), coupled covalently to a CH-activated Sepharose-4B (Pharmacia, Uppsala, Sweden). With this procedure, antibodies reactive with the β-galactosidase and (contaminating) bacterial proteins were removed. In immunoprecipitation experiments, the antibody was shown to be specific for cyclin D1[15] and did not cross-react with either cyclin D2 or cyclin D3, as no immunostaining was found with H9 cells that express these members of the cyclin D family, but not cyclin D1.[15] The B31S antibody detected a level of cyclin D1 that corresponds at least with protein levels present in tumors with a threefold amplification of the cyclin D1 gene.[16] We used the antibody B31S in a 1:80 dilution using phosphate-buffered saline/1% bovine serum albumin.

After antigen retrieval (15 minutes of microwave cooking (setting at 450 W) in citrate buffer (10 mmol/L, pH 6)), the 4-μm sections were incubated with the primary antibody for 16 hours at 4°C and with the peroxidase-labeled conjugate for 30 minutes at room temperature. A two-stage streptavidin-biotin-peroxidase technique was used (Dako Duet Kit, DAKO, Glostrup, Denmark). Negative controls consisted of omission of the antiserum from the first incubation, and as positive control a head and neck squamous cell carcinoma with known cyclin D1 amplification and overexpression[15] was used.

This staining procedure detects a level of cyclin D1 protein expression that is also found in breast cancers with a greater than threefold amplification of the cyclin D1 gene.[16] Staining was found in the nucleus, and tumor cells showed a range of intensities of staining (both in the breast cancers as well as in the positive control). This variation in staining may well be due to the cell-cycle-related expression pattern of cyclin D1 protein with a peak in G1, which is maintained in tumor cells with an overexpression of cyclin D1.[14,16,23] Despite this variation, a straightforward scoring of the staining based on intensity and percentage of nuclei staining was possible, which was done by two observers in 100 cells. In agree-
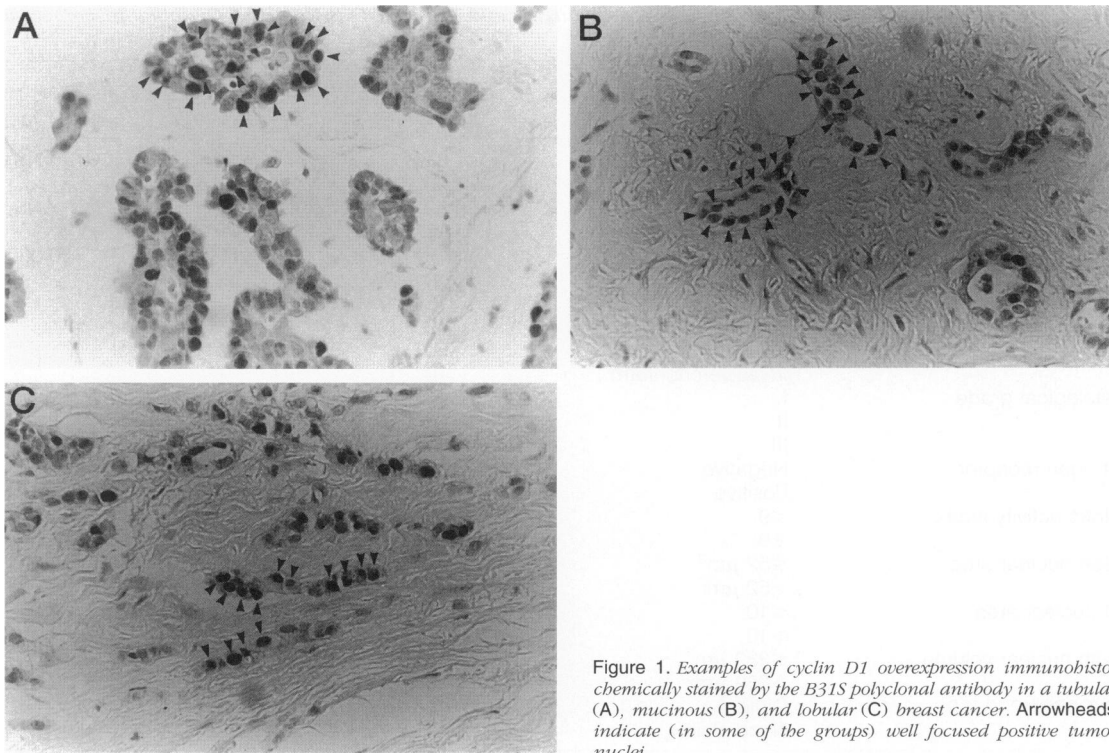
Figure 1. *Examples of cyclin D1 overexpression immunohisto-chemically stained by the B31S polyclonal antibody in a tubular* (A), *mucinous* (B), *and lobular* (C) *breast cancer.* Arrowheads *indicate (in some of the groups) well focused positive tumor nuclei.*

ment with previous studies,[16] cases were regarded as negative when <5% of nuclei showed staining and as positive when ≥5% of nuclei stained.

Very rarely, staining of normal mammary epithelium was observed in our specimens, but stromal tissue was always negative. Cytoplasmic tumor cell staining that was observed in some cases was ignored.

Morphometry

In the H&E-stained slides, the most cellular area containing the highest density of mitotic figures (usually at the periphery) was selected, rejecting areas with *in situ* carcinoma, necrosis, and many nonmalignant cells. A measurement field of approximately 0.5 × 0.5 cm in size was marked for counting mitotic figures and nuclear morphometric measurements.

Mitotic figures were counted in 10 consecutive high-power fields at ×400 magnification using a 40× objective (numerical aperture, 0.75; field diameter, 450 μm), starting at the spot within the measurement field with the highest density of mitotic figures. The total number of mitotic figures counted in these 10 fields was taken as the mitotic activity index.[2]

Nuclear morphometry was performed using an interactive digitizing video overlay system (QPRODIT, Leica, Cambridge, UK) at a final magni-

fication of approximately ×3000. For assessment of mean nuclear area, 100 nuclei were selected according to a systematic random sampling method,[31] and their contours were traced. For each case, mean and SD of the nuclear area were calculated. Volume weighted mean nuclear volume was assessed by measuring the intercepts along parallel test lines through 100 point sampled nuclei,[32] taking the cube of each intercept, and multiplying the mean cubed intercept length by $\pi/3$.

DNA Flow Cytometry

DNA flow cytometry was performed with a mercury-lamp-based PAS II flow cytometer (Partec, Münster, Germany) within 3 hours after diamidino-phenyl-indole-dihydrochloride staining. The first peak in the DNA histograms was assumed to represent DNA-diploid cells. DNA diploidy was defined by the presence of only one cell cycle in the DNA histogram, and DNA nondiploidy by the presence of more than one cell cycle.

Statistics

For statistical analysis, grouping was performed using logical classes for the discrete variables, and for

Table 1. *Correlations (χ^2 Test) between Expression of Cyclin D1 and Clinical, Classical, and Quantitative Pathological Variables*

Variable	Grouping	Cyclin D1 negative	Cyclin D1 positive	*P* value
Lymph node status	Negative	31	40	NS
	Positive	30	38	
Tumor size	≤2.5 cm	32	46	NS
	>2.5 cm	29	41	
Histological type	Tubular	2	11	0.0001
	Mucinous	0	3	
	Medullary	6	0	
	Lobular	3	17	
	Ductal	50	52	
	Invasive cribriform	0	4	
Histological grade	I	16	47	0.0001
	II	22	31	
	III	23	9	
Estrogen receptor	Negative	25	11	0.0001
	Positive	8	28	
Mitotic activity index	<9	25	51	0.03
	≥9	36	36	
Mean nuclear area	≤52 μm^2	23	54	0.004
	>52 μm^2	38	33	
SD nuclear area	≤10	21	44	0.05
	>10	40	43	
Mean nuclear volume	≤239 μm^3	24	51	0.02
	>239 μm^3	37	36	
DNA ploidy	Diploid	19	35	NS
	Nondiploid	42	52	

Unknown cases are not listed. NS, not significant.

the continuous variables the median values were used.

To assess correlations, confusion matrices were computed and tested for significance with the χ^2 test. For survival analysis, overall survival time (defined as the time between date of operation and death from recurrent disease) was used as the follow-up parameter. Patients dying from causes unrelated to breast cancer were censored at the time of death. Kaplan-Meier curves were plotted, and differences between the curves were analyzed using the Mantel-Cox test. Multivariate analysis was performed with the Cox regression model (enter and remove limits of 0.1) to evaluate additional prognostic value of cyclin D1 overexpression to the other prognostic variables. All of these tests were carried out with the biomedical package from Statistical Solutions (Cork, Ireland). *P* values of <0.05 were regarded as significant.

Results

Overexpression of cyclin D1 was found in 87 of 148 cases (59%). Figure 1 shows examples of cyclin D1 overexpression in different types of invasive breast cancer. Cyclin D1 overexpression was significantly negatively correlated (Table 1) with histological

grade (P = 0.0001), mean nuclear area (P = 0.004), mean nuclear volume (P = 0.02), mitotic activity index (P = 0.03), and SD of nuclear area (P = 0.05). Estrogen receptor-positive cases showed significantly more often cyclin D1 overexpression (P = 0.0001).

There was a strong correlation between cyclin D1 overexpression and histological type (P = 0.0001). Positive cyclin D1 staining was seen in 11 of 13 tubular carcinomas, 3 of 3 mucinous carcinomas, and 4 of 4 invasive cribriform carcinomas. Of the lobular carcinomas, 17 of 20 were positive. Of 102 ductal cancers, 52 were positive, and all 6 medullary carcinomas were completely negative. The highest percentage of cyclin-D1-positive cells seen in these different tumor types was 90% for lobular cancers, 80% in tubular, mucinous, and ductal types, and 55% in invasive cribriform cancers. There were no significant correlations between cyclin D1 overexpression and lymph node status, tumor size, or DNA ploidy.

In univariate survival analysis (Table 2), there was a trend for better prognosis of cases showing cyclin D1 overexpression using the 5% cutoff value, which was, however, not significant (Figure 2). Other thresholds did not provide essentially different results. Histological grade, tumor size, lymph node

Table 2.   *Univariate Survival Analysis Results*

Variable	Cutoff	n	% dead	% survival	P value	Mantel-Cox
Cyclin D1	Negative	61	23	62	0.34	0.9
	Positive	87	26	70		
Lymph node status	Negative	68	13	81	0.0015	10.1
	Positive	71	31	56		
Tumor size	≤2.5 cm	78	12	85	0.0001	27.5
	>2.5 cm	70	37	47		
Histological grade	I	63	13	79	0.0008	14.2
	II	53	18	66		
	III	32	18	44		
Estrogen receptor	Positive	36	12	67	0.84	0.04
	Negative	36	12	67		
Mitotic activity index	<9	76	14	82	0.0001	16.0
	≥9	72	35	51		
Mean nuclear area	≤52 μm^2	77	16	79	0.0015	10.1
	>52 μm^2	71	33	54		
SD nuclear area	≤10	65	14	78	0.0082	7.0
	>10	83	35	58		
Mean nuclear volume	≤239 μm^3	74	16	78	0.0004	12.5
	>239 μm^3	74	34	55		
DNA ploidy	Diploid	54	15	72	0.23	1.4
	Nondiploid	94	34	64		

status, mitotic activity index, mean and SD of the nuclear area, and mean nuclear volume showed their usual prognostic value. DNA ploidy and the estrogen receptor status were not significant.

In multivariate survival analysis, cyclin D1 overexpression did not have additional prognostic value to the mitotic activity index, tumor size, and lymph node status.

Discussion

The aim of this study was to correlate cyclin D1 overexpression with other qualitative and quantitative prognostic variables and to evaluate its prognostic value in a well documented group of 148 invasive breast cancer patients with long-term follow-up.

Cyclin D1 was overexpressed in 59% of cases, in accordance with the results from Bartkova et al[23]

Figure 2. *Survival curves of cases with and without cyclin D1 overexpression.*

and Zhang et al[25] but is somewhat higher than in the study by Michalides et al.[16] It is also higher than the amplification found in 19% of cases in a previous study.[19] This implies that there may be overexpression of cyclin D1 in the absence of amplification, as has been found by others.[16,19,22,24] This could be due to a mutation-induced longer half-life of the protein or a translocation or as a result of increased hormone sensitivity. As to the latter, cyclin D1 overexpression was indeed strongly correlated to estrogen receptor positivity, as in a previous study.[16]

There were no significant correlations with lymph node status, tumor size, or DNA ploidy. Interestingly, cyclin D1 overexpression was especially found in tubular, mucinous, and invasive cribriform cancers. These are well differentiated tumor types with relatively low nuclear atypicality, low proliferation, positive steroid receptor status, and favorable prognosis.[33] Overexpression was also frequently found in lobular cancer, which also usually show low nuclear atypicality, low proliferation, and positive steroid receptor status and are associated with an intermediate prognosis.[33] All medullary cancers that in general show high nuclear atypicality, high proliferation, and poor prognosis[33] were cyclin D1 negative. The ductal cancers with their broad spectrum of nuclear atypicality and proliferation did not show such clear trends. Indeed, overexpression of cyclin D1 was strongly negatively correlated with mitotic activity index (as marker of proliferation), mean and SD of nuclear area, and mean nuclear volume (as markers of nuclear differentiation).

In the well differentiated tumor types, overexpression therefore does not seem to result in a higher proliferation as reflected by mitotic activity. Although cyclin D1 overexpression results in a shortening of the G1 phase,[17,34,35] this may be compensated by a lengthening of other parts of the cell cycle. Besides cyclin D1, also other regulators such as the p53 and retinoblastoma (Rb) proteins affect transition through the cell cycle that may result in a relative G1 arrest despite cyclin D1 overexpression. In actual invasive breast cancer, cell cycle regulation seems to be more complex than in some cell lines, as previous studies showed that overexpression of cyclin D1 triggers autonomous proliferation[17] (R.M.L. Zwijssen, R. Klompmaker, E.B.H.G.M. Wientjens, P.M.P. Kristel, B. van der Burg, R.J.A.M. Michalides, submitted for publication). As to the poorly differentiated ductal and medullary cancers, many of them may have a cyclin-D1-independent proliferation pathway considering their complete cyclin D1 negativity. This could be related to defects in the Rb gene as it has been shown that these may lead to proliferation despite low levels of cyclin D1 mRNA and protein.[36]

Considering the correlations between cyclin D1 overexpression and the well established prognostic variables mean and SD of nuclear area[2,4-6] and nuclear volume,[32] mitotic activity index,[2,4-6] and histological tumor type,[33] we expected to also find prognostic value of cyclin D1 overexpression. In contrast with a previous study in which amplification was correlated to prognosis, no significance of cyclin D1 overexpression was found in univariate and multivariate survival analysis as in our previous study on overexpression in an independent group of patients.[16] Although some selection of cases could not be avoided as some blocks ran out of tumor, the study group can still be considered to be representative as all established prognosticators (tumor size, lymph node status, mitotic index, histological grade, and nuclear area) showed their usual prognostic value. However, the results of this study on patients that did not receive adjuvant treatment cannot be generalized just like that. It requires additional investigation to evaluate whether the same results apply to patients receiving adjuvant treatment, especially those receiving chemotherapy.

Apparently, one has to distinguish overexpression of cyclin D1 due to genetic alterations such as amplification and translocation from overexpression as a result of, for example, increased hormone sensitivity. The prognostic value of these two forms of cyclin D1 overexpression may well be contradictory and, therefore, confound the prognostic value of overexpression of cyclin D1 in general.

In conclusion, cyclin D1 shows mainly overexpression in the well differentiated and lobular types of invasive breast cancer, and overexpression is strongly correlated to estrogen receptor positivity. It is negatively correlated with the proliferation marker mitoses count and with the differentiation markers mean and SD of nuclear area and mean nuclear volume. However, cyclin D1 overexpression does not seem to have prognostic value when no adjuvant treatment is given. These results provide motivation for additional studies on the role of cyclin D1 in oncogenesis, proliferation, differentiation, and clinical behavior of invasive breast cancer.

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