Virchows Arch (1999) 435:116–124

ORIGINAL ARTICLE

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Elevated levels of p27, p21 and cyclin D1 correlate with positive oestrogen and progesterone receptor status in node-negative breast carcinoma patients

Received: 26 March 1998 / Accepted: 29 April 1999

Abstract The search for better prognostic indicators and new treatment modalities in node-negative breast carcinoma patients is important. The aim of this study was to determine the immunohistochemical expression of central cell regulator proteins in relation to hormone receptor status, tumour-cell differentiation and prognosis. We investigated the immunoreactivity of p27, p21, cdk4, cyclin D1 and p53 in 77 node-negative breast carcinomas, with long-term follow-up (mean 163 months; range 20–227). Nuclear staining for p27 was seen in 87% of the carcinomas, for cdk4 in 92%, for p21 in 68%, for cyclin D1 in 58% and for p53 in 18%. Oestrogen receptor (ER) and progesterone receptor (PgR) nuclear staining was seen in 69% and 65% of the tumours, respectively. No correlation between the levels of p21 and p53 was observed. P21 overexpression was, however, associated with positive ER status. Elevated levels of p27 and cyclin D1 correlated with positive hormone status (both ER and PgR). We did find a significant correlation between p27 and cyclin D1 and histological grade of the tumours, with extensive positive immunostaining of p27 and cyclin D1 in well-differentiated carcinomas. The only significant prognostic factor in our series was histological grading. Ten-year relapse-free survival was significantly prolonged in patients with histological grade I tumours versus histological grade II and III tumours. Our results suggest that the expression of p27 and cyclin D1 is closely linked to hormone receptor status in breast carcinomas and to tumour differentiation, a finding that may be of importance in the treatment of hormone-dependent tumours.

Key words Breast carcinoma · Cell cycle proteins · p27 · Cyclin D1 · Hormone receptor

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Introduction

The cell cycle is controlled by a family of cyclin-dependent kinases (cdks) whose activity is regulated by cyclin binding, by phosphorylation / dephosphorylation of the cdk subunit, and by association with inhibitory molecules, p15 and p16 of the INK4 family and p21 and p27 of the KIP family being the best characterized of these [42]. Passage through G1- and into S-phase is regulated by the activity of cdk4(6) and cdk2 together with cyclin D, E and A, respectively [43]. Overexpression of cyclin D1 and cyclin E accelerates cell entry into S-phase [31]. The cdk-inhibitor p21 can bind to several different cyclin–CDK complexes and act as an universal inhibitor of these kinases [21]. p21 expression is found to be induced by DNA damage as a direct downstream effector of p53 [13]. However, p21 can also be induced by p53-independent pathways [27, 40] and is possibly involved in cell differentiation, senescence and apoptosis, suggesting the *p21* gene itself is a tumour suppressor gene [10]. The reports on the prognostic value of *p21* are conflicting. Both high expression of *p21* [2, 8] and low expression of *p21* [22, 48] have been related to an unfavourable prognosis.

p27 protein (KIP1) has a significant homology to p21. It binds to cyclin E–cdk2 complexes [34, 47] and cyclin D1–cdk4 [35]. As in the case of *p21*, mutations in the *p27* gene have only rarely been reported [15, 23, 44, 45]. Overexpression of *p27* induces G1 arrest in mammalian cells. Furthermore, the development of pituitary tumours in *p27* knockout mice indicates that *p27* plays an important part in repressing tumour development [24, 32]. Loss of *p27* expression has been reported to be a predictor of poor outcome [9, 36, 46]. Decreased levels of p27

protein may result in altered differentiation and intercellular adhesion.

Cyclin D1 can form a complex with either cdk4 or cdk6, and these complexes have the important task of phosphorylating the retinoblastoma protein (pRB) [5]. It has been observed that cyclin D1 accumulates in differentiated and senescent cells [5]. In a mouse mammary epithelial cell line, some clones with high expression of cyclin D1 showed a marked increase in cell differentiation and apoptosis. Overexpression of cyclin D1 deregulates cell proliferation and can induce tumorigenic changes, suggesting that cyclin D1 has an oncogenic role in breast cancer [49]. High levels of p27 and cyclin D1 have been associated with low-grade malignancy in human breast cancer cells [17, 20].

It is a major challenge in breast cancer oncology to improve patient treatment in node-negative carcinomas. In the present study we have investigated the expression of p21, p27, cyclin D1, cdk4 and p53 in a series of lymph-node-negative breast carcinomas, with a mean follow-up of 163 months. The aim was to determine, by immunohistochemistry, the protein expression of these markers in relation to hormone receptor status, tumour-cell differentiation and the patient's prognosis. We found that positive p21, p27 and cyclin D1 expressions were closely linked to oestrogen receptor expression and to well-differentiated tumours. Only histological grading was of significant prognostic value, with a prolonged 10-year relapse-free survival for patients with well-differentiated tumours.

Materials and methods

Clinical material

The present series was selected from a randomized trial of 448 T0N0-T2N1M0 patients [33]. The first 100 patients with T1-T2N0 were reviewed, leaving 77 patients with complete marker set. These 77 patients were compared with the original study population of 228 node-negative carcinomas. We did not find any significant difference in tumour size, histological grade distribution, age, relapse- free survival or overall survival between our series and the original study population. Thus, the 77 patients were regarded as a representative selection.

Tumour tissue from these 77 T1-T2N0M0 breast carcinomas, treated at The Norwegian Radium Hospital during the period from 1977 to 1979, was retrieved from archival paraffin blocks. Median age of the patients at diagnosis was 57 years (range 36–70 years). Radical (Halsted), modified radical (Patey) or simple mastectomy had been performed. Lymph node dissection was performed in all patients and the median number of axillary lymph nodes removed was 10 (range 1–20). One patient had only one lymph node removed and two patients only three; the others all had more than three lymph nodes examined. None of the patients received adjuvant tamoxifen or radiotherapy at the time of primary treatment. The follow-up time for all patients ranged from 20 to 227 months (mean 163 months) and for patients alive at the last observation it ranged from 175 to 227 months (mean 198 months). No patients were lost to follow-up. Ten-year overall survival was 79% (95% CI 69–89%) and 10-year relapse-free survival, 72% (95% CI 62–82%).

The histopathology slides were reviewed by two of the authors (W.R. and J.M.N.). The following characteristics of the primary tumour were studied: tumour size, histological type according to WHO recommendations, histological grade using Elston's modification [14] of the Bloom and Richardson method [6] on a scale from I to III, with I being well-differentiated and III poorly differentiated tumour. All specimens were evaluated without knowledge of the clinical data.

Immunohistochemistry

Sections for immunohistochemistry were stained using the biotin–streptavidin peroxidase method (Supersensitive Immunodetection System, LP000-UL, Biogenex, Calif.) and OptiMax Plus Automated Cell Staining System (Biogenex). Deparaffinized sections were microwaved in 10 mM citrate buffer pH 6.0 to unmask the epitopes and treated with 1% hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase. The sections were incubated for 30 min at room temperature with the primary antibodies listed in Table 1. The sections were then incubated with biotin-labelled secondary antibody (1:40) and streptavidin peroxidase (1:40) for 20 min each. Tissue was stained for 5 min with 0.05% 3'3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05 M tris(hydroxymethyl)-aminomethane (Tris) buffer at pH 7.6, containing 0.024% H_2O_2, and then counterstained with haematoxylin, dehydrated, and mounted in Diatex. All the dilutions of primary antibodies, biotin-labelled secondary antibody and streptavidin peroxidase were made with phosphate-buffered saline (PBS) pH 7.4, containing 5% bovine serum albumin.

All series included tumour tissue as positive controls. In addition the MCF-7 breast carcinoma cell line was used as positive control for p21, p27, cyclin D1, cdk4 and ER, whereas the T47-D breast carcinoma cell line was used as positive control for p53 and PgR. Negative controls included substitution of polyclonal primary antiserum with normal rabbit serum, whereas negative controls for the monoclonal antibody were performed using mouse myeloma protein of the same subclass and concentration as the primary monoclonal antibody.

The number of immunoreactive cells, when stained with cyclin D1, p27, p21, CDK4 and protein p53, was semiquantitatively estimated: – no positive cells, + <25%, ++ 25–75%, +++ >75% positive cells. Immunoreactivity for ER and PgR was estimated as – no positive cells, + <10%, ++ 10–50%, +++ >50% positive cells [1]. Only distinct nuclear staining was considered positive. The immunostaining intensity was not graded.

Table 1 List of primary antibodies

Antibody[a]	Dilution (immunochemistry)	Dilution source (western blot)	Catalogue
p53	1:6000	1:250	Novocastra Lab. NCL-CMI
p21(WAF-1)	1:60	1:100	Oncog.Res.Prod. OP64
p27	1:200	1:250	Transduction Lab. K25020
Cyclin D1	1:60	1:100|	Oncog.Res.Prod. CC12
cdk4[b]	1:200	1:200	Santa Cruz Biotech. C-22
ER	1:50	1:50	Novocastra Lab. NCL-6F11
PgR	1:50	1:50	Novocastra Lab. NCL-PGR

[a] Unless otherwise specified primary antibodies were monoclonal

[b] Polyclonal antiserum produced in rabbit

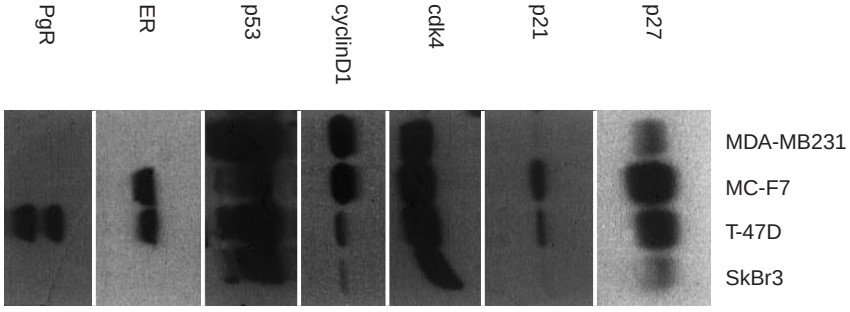

Fig. 1 Representative Western blotting showing the expression of p27, p21, cdk4, cyclin D1, p53, oestrogen receptors (*ER*) and progesterone receptors (*PgR*) in mammary cell lines (MDA-MB231, MCF7, 47D, SKBr3) embedded in paraffin. ×40

Table 2 Immunohistochemical expression in 77 breast carcinomas, in the invasive tumour cells

Antibody	Immunoreactivity *n (%)*			
	–	+	++	+++
p53	63 (82)	7 (9)	4 (5)	3 (4)
p21	25 (32)	49 (64)	3 (4)	
p27	10 (13)	24 (31)	33 (43)	10 (13)
Cyclin D1	32 (42)	35 (45)	9 (12)	1 (1)
cdk4	6 (8)	19 (25)	25 (32)	27 (35)
ER	24 (31)	3 (4)	14 (18)	36 (47)
PgR	27 (35)	16 (21)	15 (19)	19 (25)

In Vitro Cell Growth

MCF-7, T47-D,MDA-MB231, SKBr3 breast carcinoma cell lines were obtained from ATCC (Rockville, Mass.) and grown as monolayer cultures as recommended by the supplier. Cells from monolayer cultures were harvested by scraping, washed in PBS and subsequently harvested for Western blot analysis or fixed in 4% formalin prior to paraffin embedding.

Immunoblotting

Cells from monolayer cultures were lysed in ice-cold lysis buffer (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 0.02 mg/ml each of aprotinin, leupepsin and pepstatin). Subsequently, the lysates were sonicated and clarified by centrifugation. Protein quantitation was done by Bradford analysis (BioRad Laboratories, Hercules, Calif.). From each sample, 25 µg of total protein was separated by SDS-polyacrylamide gel electrophoresis and transferred onto Immobilin-P membranes (Millipore, Bedford, Mass.). As a loading and transfer control the membranes were stained with 0.1% Naphthol blue-black (Sigma Chemical Co., St. Louis, Mo.). The membranes were blocked in TBST (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.25% Tween 20) containing 10% dry milk . Thereafter, the membranes were incubated for 1 h at room temperature with the same antibodies diluted in TBST containing 5% dry milk as used for immunohistochemical analysis (Table 1). After washing in TBST, the immunoreactive proteins were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega, Madison, Wis.) diluted 1:5000 and the ECL Western blotting detection system (Amersham-Pharmacia Biotech.).

Statistics

The relation between the different variables was assessed by the Spearman's rank correlation coefficient. The comparison between lobular and ductal carcinomas and their immunohistochemical staining was done with a nonparametric analysis using the Mann-Whitney U-Wilcoxon rank sum test. Survival was estimated with the Kaplan-Meier procedure and differences between the survival curves compared with the logrank test. P-values ≤0.05 were regarded as statistically significant. The statistical package of SPSS (version 6.1.2) was used.

Results

Of the 77 infiltrating carcinomas, 62 were classified as ductal, 9 as lobular and 6 as belonging to other subgroups (1 mucinous, 1 medullary, 1 mixed lobular and ductal, 1 with neuroendocrine differentiation and 2 secretory). In the group of infiltrating ductal carcinomas, 19 (30%) were histological grade I, 34 (55%) grade II and 9 (14%) grade III. Most, i.e. 57, of the tumours were ≤20 mm in diameter and 20, between 21 and 50 mm.

Comparison of the results from western blot analysis and immunohistochemistry on the same cell lines revealed a very good correlation (see Figs. 1, 2).

Cytoplasmic staining of both nonneoplastic and neoplastic epithelium was seen in cases immunostained with anti-cdk4 and anti-cyclin D1. With anti-p21 and anti-p27, low levels (–/+) of nuclear staining was present in myoepithelial cells and normal ductal epithelium. No positive nuclear staining was identified for p53 and cyclin D1 in normal breast tissue.

The percentage of nuclear staining for all the immunohistochemical markers was estimated in the invasive part of the tumour (Table 2), as the extent of carcinoma in situ was widely variable. Eighty-seven percent of the carcinomas were reactive for p27, 92% for cdk4, 68% for p21, 58% for cyclin D1 and 18% for p53. None of the tumours expressed extensive (>75%) p21 immunostaining, and 25 tumours (32%) were completely negative. For p27, 10 tumours (13%) were negative and 43 tumours (56%) had immunostaining of 25% or more. Only 14 carcinomas (18%) were p53 positive, while 53

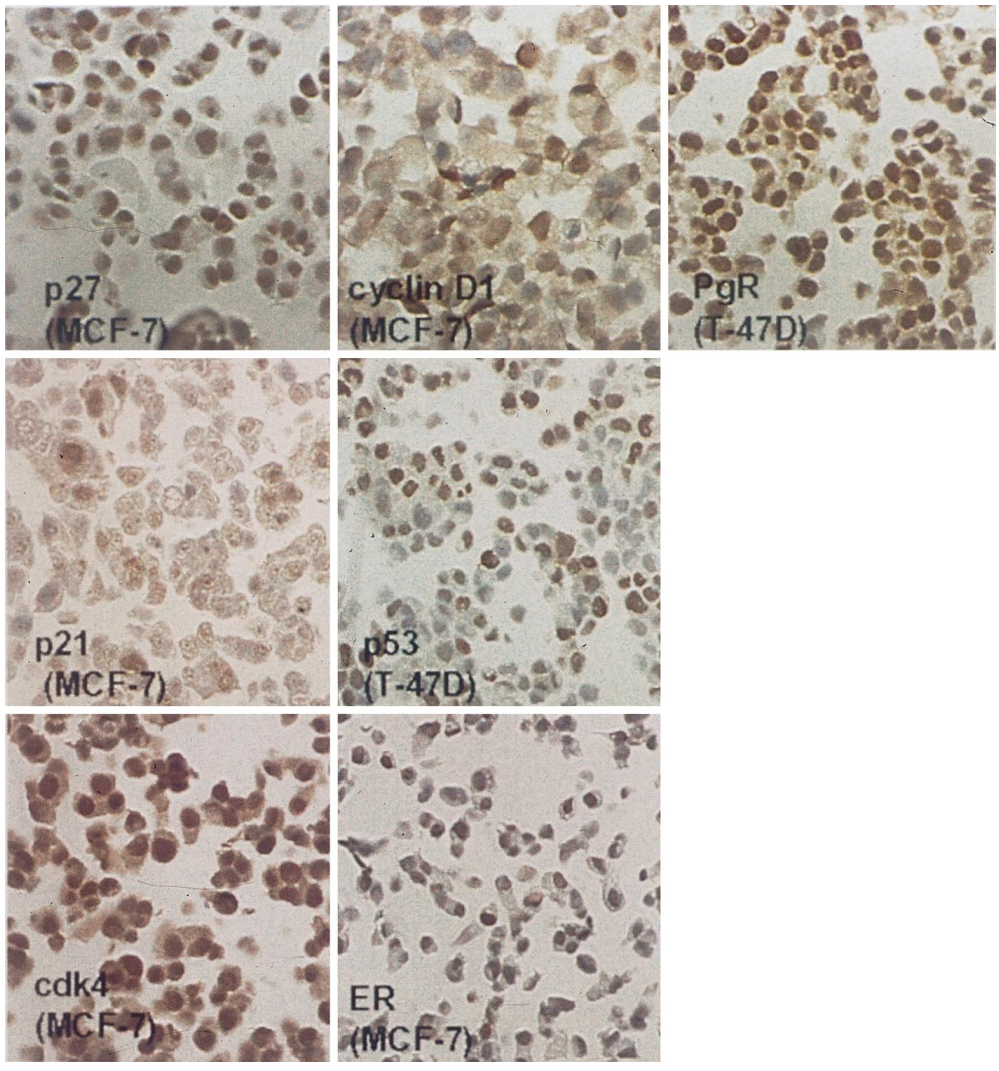

Fig. 2 Immunohistochemical staining of p27, p21 cdk4, cyclin D1, p53, ER and PgR in mammary cell lines (MDA-MB231, MCF7, 47D, SKBr3) embedded in paraffin. ×40

carcinomas (65%) were clinically positive for ER and 34 (44%) for PgR (≥10%).

The pattern of immunostaining was subgrouped for lobular and ductal carcinomas (Table 3). There was a tendency for more extensive p27 immunostaining in the lobular than in the ductal carcinomas (78% versus 55%), with staining in 25% or more of the tumour cells, but the difference was not significant (P=0.056). The only significant difference found in the immunostaining of the cell cycle proteins was for cdk4. Seventy-five percent of the lobular carcinomas (7 cases) were highly positive for cdk4 staining, against 31% (31cases) of the ductal carcinomas (P=0.026). All the lobular carcinomas were negative for p53 staining, but the difference was not significant, as 79% of the ductal carcinomas were also negative (P=0.13). ER and PgR expression were the same in the two groups. We also compared the results of the immunostaining of the ductal carcinomas with the staining results of the whole series and did not find any significant difference.

The histological grade and immunoreactivity for cell-cycle associated proteins were intercorrelated using Spearman's rank correlation coefficient (Table 4). p27 correlated positively with p21 (P=0.05), cyclin D1 and cdk4 (P<0.001). The hormone receptors ER and PgR were positively interrelated with p27 and cyclin D1 (P<0.01). p21 was positively associated with ER (P=0.01), but there was no association with PgR. p53 protein had an inverse correlation with p27 (P<0.001), but no significant correlation was seen between p53 and p21. Higher tumour grade correlated negatively with ER, PgR, cyclin D1 and p27 and positively with p53 protein (P<0.05). Figure 3 illustrates strong positivity for cyclin D1, p27 and ER in a well-differentiated carcinoma.

Relapse-free survival was significantly longer for patients with histological grade I versus grade II and III tumours: 191 months [95% CI 171–223) versus 143 months (95% CI 117–168); P=0.04, Fig. 4]. Median relapse-free survival time was shorter for patients with tumours 21–50 mm in size than for those with smaller tumours: 144 months (95% CI 108–179) versus 179 months (95% CI 159–200), but the difference was not statistically significant. For the survival analyses of the immunohistochemical markers, we analysed each stain-

Table 3 Immunohistochemical expression in lobular versus ductal carcinomas

Antibody	Immunoreactivity	Frequency (%)		*P*-value
		Lobular	Ductal	
p53	–	9 (100)	49 (79)	
	+		6 (10)	
	++		4 (6)	
	+++		3 (5)	0.133
p21	–	4 (44)	17 (28)	
	+	4 (45)	43 (69)	
	++	1 (11)	2 (3)	0.53
p27	–	1 (11)	7 (11)	
	+	1 (11)	21 (34)	
	++	3 (33)	28 (45)	
	+++	4 (45)	6 (10)	0.056
Cyclin D1	–	4 (45)	22 (36)	
	+	3 (33)	32 (52)	
	++	1 (11)	8 (13)	
	+++	1 (11)		0.98
cdk4	–	1 (11)	3 (5)	
	+		17 (27)	
	++	1 (11)	23 (37)	
	+++	7 (78)	19 (31)	**0.026**
ER	–	1 (11)	17 (27)	
	+	1 (11)	2 (3)	
	++	2 (22)	12 (20)	
	+++	5 (56)	31 (50)	0.57
PgR	–		21 (33)	
	+	4 (45)	12 (19)	
	++	2 (22)	13 (21)	
	+++	3 (33)	16 (26)	0.21

Table 4 Spearman's rank correlation coefficients between the investigated parameters in 77 node-negative breast carcinomas. Significant values given in *bold type*

	cdk4	Cyclin D1	ER	Histological grade[a]	p21	p27	p53
PgR	0.05	**0.26**	**0.43**	**–0.40**	–0.04	**0.31**	**–0.33**
p53	–0.22	–0.17	**–0.19**	**0.50**	–0.09	**–0.27**	
p27	**0.50**	**0.43**	**0.40**	**–0.41**	**0.22**		
p21	0.05	**0.37**	**0.26**	–0.20			
Histological grade	–0.10	**–0.29**	**–0.29**				
ER	0.09	**0.52**					
Cyclin D1	**0.25**						

[a] Of the ductal carcinomas

Table 5 Ten-year relapse-free survival according to different prognostic variables (95% confidence intervals in round brackets)

Antibody	–	+	++	+++
p53	65[a] (35–77)	86 (60–100)	75 (31–100)	100[b]
p21	74 (56–92)	67 (53–83)	100[b]	
p27	68 (38–98)	66 (46–86)	72 (56–88)	80 (54–100)
Cyclin D1	71 (53–89)	71 (56–86)	63 (30–96)	100[b]
cdk4	50 (10–90)	72 (51–93)	70 (52–83)	73 (57–89)
ER	67 (44–100)	100[b]	76 (52–100)	71 (58–84)
PgR	63 (45–81)	87 (69–100)	71 (47–95)	72 (51–93)

[a] Proportion of relapse-free patients in each category
[b] All observations are censored after 10 year

ing category individually. We then grouped the staining patterns ++/+++ as positive and +/– as negative. The staining categories +/++/+++ were also evaluated as one positive group. The results did not differ for either subgroup. Survival analyses estimated with the Kaplan-Meier procedure showed no statistically significant association between the immunohistochemical markers and time to first relapse or overall survival (data not

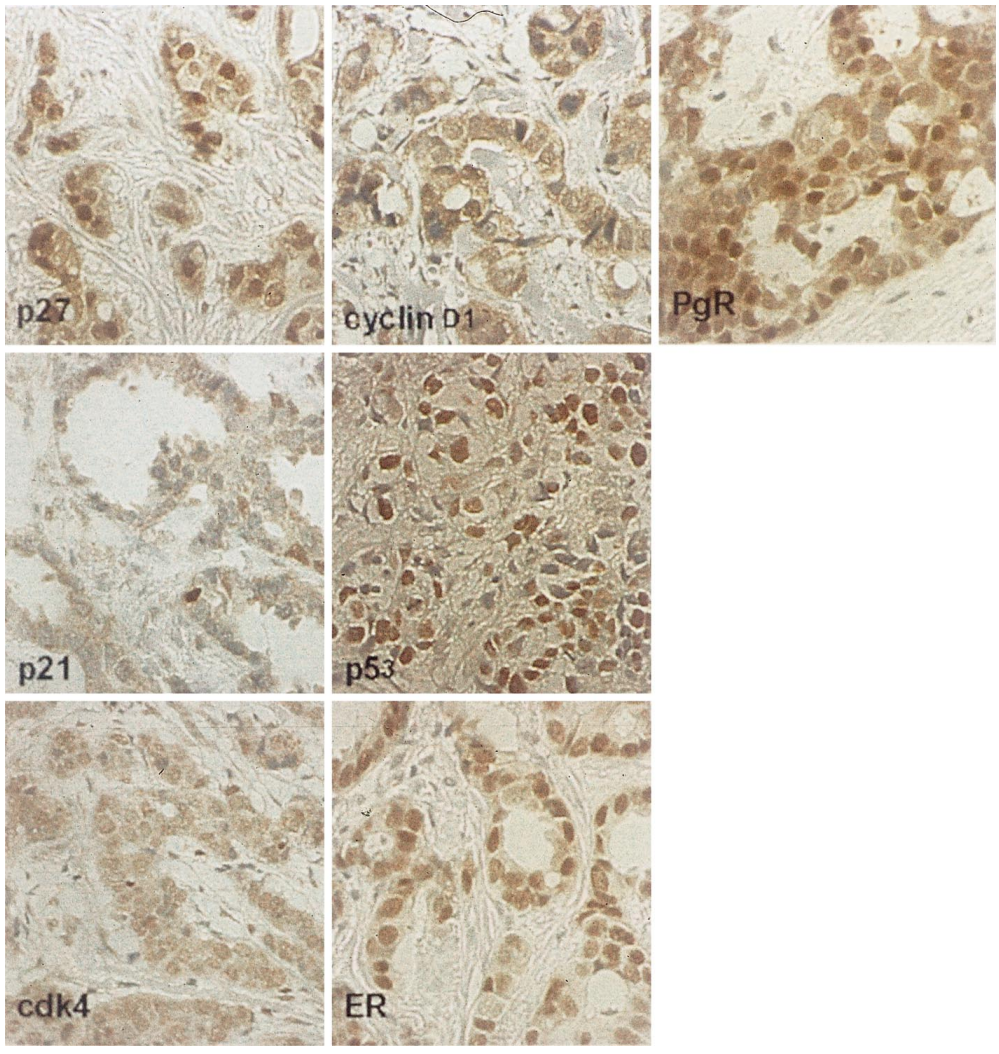

Fig. 3 Immunohistochemical expression of p27, p21, cdk4, cyclin D1, p53, ER and PgR in mammary carcinoma. ×40

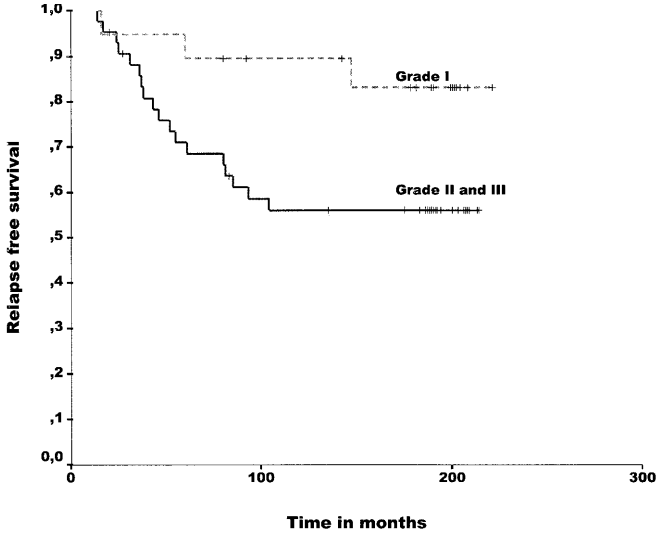

Fig. 4 Kaplan-Meier plot of relapse-free survival for histological grade I versus II and III (P=0.04; + censored observations)

shown). Table 5 illustrates 10-year relapse-free survival rates in each grade of immunostaining for the different immunomarkers. The numbers represent the proportion of relapse-free patients. The confidence intervals are large and there is some degree of overlapping in the four staining categories for all the markers. For p27, there is a tendency towards a better prognosis for tumours with more than 25% positive cells, but the trend is not significant. For the other proteins, there is no clear tendency.

Discussion

In our study we found p21 and p27 expression to be common in node-negative breast carcinomas. This was unexpected, as one major function of p21 and p27 is to bind cyclin–CDK complexes and thereby inhibit cell proliferation and suppress cell growth [21, 47]. Elevated p21 and p27 may reflect a feedback mechanism by which increased levels of cdk4 and cyclin D1 offset the effect of p21 and p27 inhibitor proteins. This hypothesis is strengthened by the fact that we found higher levels of

cdk4 and cyclin D1 in cases that also shared elevated levels of p21 and p27. Another possibility is that *p21* and *p27* genes in tumour cells may be mutated and nonfunctional. This, however, is unlikely, as mutations of *p21* and *p27* genes are reported to be rare events in human cancers [15, 44, 45].

In our series, p21 was expressed in 68% of the tumours. Unlike Bukholm et al. [7], we did not find an association between p53 and p21 expression. However, p53-independent pathways are well documented [27, 38, 41]. In contrast to some studies [2, 8], but in agreement with others [48], we did observe that high levels of p21 expression correlated with positive ER status. ER-positive tumours had a significant positive correlation with well-differentiated tumours, and thus high levels of p21 were indirectly associated with well-differentiated tumours, but this was not significant. Jiang et al. reported no relationship between p21 and ER status.

Reports on the prognostic value of p21 are also conflicting. Both high expression of p21 [2, 8] and low expression of p21 [22, 48] have been related to an unfavourable prognosis. p21 did not have any prognostic significance in our study. Some of the discrepancies may be caused by variations in the number of patients included in the different studies, the length of follow-up and the heterogeneity of p21 immunoexpression in tumours. The definition of positive staining also varies, as some studies define overexpression as >10% positive cells [8, 48], while others include all positive tumours [2]. In a study of malignant melanoma [28] high levels of p21 correlated with thicker lesions, whereas low levels of p21 were observed in the metastases. One explanation for this apparent contradiction may be that cyclin–cdk complexes have reached a threshold level in the metastases, allowing tumour progression, in parallel with a decrease in the p21 level. It is possible that this contradiction also is valid in the primary tumour.

p27 immunoreactivity was present in 87% of the tumours, with a high expression (>25% of tumour cells immunoreactive) in 56% of the cases. This is in agreement with the levels of p27 protein reported in several human breast cancer cell lines and primary breast tumours [9, 39]. Loss of p27 expression has been reported to be a predictor of poor outcome [9, 36]. Reduced p27 levels were not found to be a predictor of poor clinical outcome in our study. We did, however, find that loss of p27 expression correlated with high grade tumours [17] and that high expression of p27 was positively associated with ER and PgR [9]. These findings suggest that p27 may affect differentiation pathways and that decreased p27 protein levels could play a role in tumour progression.

Amplification of the cyclin *D1* gene mapped to chromosome 11q13 has been observed in 10–20% of breast carcinomas [11, 12]. However, the frequency of overexpression of the gene product far exceeds the frequency of DNA amplification and varies in breast carcinomas from 34% to 81% [4, 18, 29, 51]. This suggests that mechanisms other than DNA amplification might have a significant bearing on the aberrant expression of cyclin D1 [18, 26]. We found elevated levels of cyclin D1 in 58% of cases. In agreement with Michalides et al. [29], we did not find any prognostic significance of cyclin D1 expression, but high cyclin D1 levels were positively associated with ER- and PgR-positive tumours, as was also reported by Barnes [3]. It might be that ER-receptor-positive breast carcinomas overexpress cyclin D1 as a result of oestrogen induction, as reported for T47D, an oestrogen-dependent breast tumour cell line [30]. In addition, increased cyclin D1 expression has been observed in oestrogen-induced activation of CDK4 in MCF-7 cells [37]. Gillett et al. [19] found a highly significant association between cyclin D1 and ER and response to tamoxifen for metastatic disease. In vivo, antioestrogens block entry of cells into S-phase and inhibit cell proliferation as a consequence of an early decline in pRB phosphorylation, contributed to by a reduction in cyclin D1–CDK4 activity [16, 50]. If the reduction of cyclin D1/CDK4 activity is critical to antioestrogen action, it could explain the lack of response in ER-negative cyclin D1-negative breast carcinomas. In our series, cyclin D1 had a positive correlation with well-differentiated tumours. It is a paradox that an elevated level of cyclin D1, which is assumed to provide a proliferative advantage to the tumour cell, is associated with well-differentiated tumours. One can speculate that a cyclin-negative phenotype can indicate mutations in the *RB1* gene and that this has more impact on prognosis than cyclin D1 levels. It has also been observed that cyclin D1 accumulates in differentiated and senescent cells [5].

We compared immunohistochemical staining results in lobular carcinomas and ductal carcinomas. We found more extensive staining for cdk4 in the lobular carcinomas. All lobular carcinomas were negative for p53, but this was not statistically significant. In our series we had only 9 lobular carcinomas, so any conclusions must await a larger series.

All these regulatory proteins are localized in the nucleus of the tumour cells and were visualized by nuclear immunostaining. We did, however, find extensive cytoplasmic staining for cdk4 and cyclin D1 in normal epithelial cells and in tumour cells. LaBaer et al. [25] found in mammary epithelial cells that in the presence of p21 a significant fraction of cells displayed an intense and exclusively nuclear staining for both cdk4 and cyclin D1, whereas in the absence of p21 both proteins localized diffusely both to the cytoplasm and the nucleus in the majority of cells. The clinical importance of this has yet to be elucidated.

The only significant prognostic factor in our study was histological grading. Ten-year relapse-free survival was significantly higher for tumours with histological grade I versus histological grades II and III. No significant prognostic value was observed for any of the cell cycle regulators, possibly as a result of the limited number of patients included in our series.

In conclusion, our results indicate that the expression of p21, p27 and cyclin D1 is closely linked to ER in

node-negative breast carcinomas and that this may be of importance in the treatment of hormone-dependent tumours.

Acknowledgements We thank Ellen Hellesylt, Mette Ingrud and Liv Inger Håseth for excellent technical assistance. We also thank Professor Eva Skovlund for her statistical advice. This work was supported by the Norwegian Cancer Society.

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