Altered expression of cyclin E and the retinoblastoma protein influences the effect of adjuvant therapy in breast cancer

MARIE AHNSTRÖM WALTERSSON[1], MARIE STENMARK ASKMALM[1], BO NORDENSKJÖLD[1], TOMMY FORNANDER[2], LAMBERT SKOOG[3] and OLLE STÅL[1]

[1]Linköping University, Faculty of Health Sciences, Department of Clinical and Experimental Medicine, Division of Oncology, SE-581 85 Linköping; [2]Department of Oncology, Karolinska University Hospital, SE-141 86 Stockholm; [3]Department of Cytology, Karolinska University Hospital, SE-104 01 Stockholm, Sweden

Received August 18, 2008; Accepted October 14, 2008

DOI: 10.3892/ijo_00000168

Abstract. Cyclin E and the retinoblastoma protein (Rb) are both important regulators of the G_1 phase in the cell cycle. Overexpression of cyclin E and lost expression of Rb has previously been observed in breast tumours at frequencies of 10-50% and 20-30%, respectively. We explored the prognostic role of cyclin E and Rb in breast cancer patients randomised for tamoxifen (TAM), CMF (cyclophosphamide, metotrexate, 5-fluorouracil) chemotherapy and radiotherapy (RT) and how their expression affects the patients' response to treatment. Protein expression was assessed with immunohistochemistry. We found overexpression of cyclin E in 32.1% (71/221) of the tumours and loss of Rb expression in 25.0% (59/236). Increased expression of cyclin E correlated to dysfunctional p53 (P=0.003) while loss of Rb correlated to normal p53 status (P=0.001). Our results suggest that patients with high cyclin E tumours have less benefit from tamoxifen (ER+, TAM vs. no TAM; RR=0.97; 95% CI, 0.36-2.60) than patients whose tumours show low expression (ER+, TAM vs. no TAM; RR =0.41; 95% CI, 0.24-0.72). Cyclin E also tended to predict the benefit from radiotherapy with a local recurrence rate of 0.31 (RT vs. CMF; 95% CI, 0.12-0.83) for patients with low expression and 0.68 (RT vs. CMF; 95% CI, 0.2-2.32) for patients with high expression of cyclin E. When the p53 status was taken in consideration the results showed that patients with both normal p53 and normal Rb expression had considerably lower locoregional recurrence rate when treated with radiotherapy instead of CMF (RR=0.17; 95% CI, 0.052-0.58) as compared to patients with either altered Rb or p53 or both (RR=0.70; 95% CI, 0.28-1.73).

Correspondence to: Dr Marie Ahnström Waltersson, Linköping University, KEF/Oncology laboratory, University Hospital, SE-581 85 Linköping, Sweden
E-mail: marie.ahnstrom@liu.se

Key words: cell cycle, radiotherapy, chemotherapy, overexpression, Rb, p53

Introduction

The G_1 phase of the cell cycle is a critical step in which the cell decides whether or not to initiate DNA replication. The retinoblastoma protein (Rb) plays an important role in the G_1 phase as it binds and inactivates the transcription factor E2F1 (1,2). E2F1 is essential for replication to start and its release is facilitated by phosphorylation of Rb by cyclin D/CDK4/6 and cyclin E/CDK2 complexes. Unlike cyclin D, cyclin E functions independently of mitogens and is instead activated by the release of E2F1 from Rb. Both the cyclin D/CDK4/6 and the cyclin E/CDK2 complex are also regulated by the cell cycle inhibitor $p21^{CIP1/WAF1}$. The inhibitor moves between the complexes and when the cyclin D1 protein levels are high the inhibitor is titered away from the cyclin E/CDK2 complex. While the cyclin D/CDK4/6 complex is activated by p21 binding the cyclinE/CDK2 complex is inhibited. Upon cellular stress such as DNA damage, p53 is activated and this can lead to increased expression of the cell cycle inhibitor p21 and hence, cell cycle arrest.

Overexpression of cyclin E is common in breast cancer although the reported frequencies vary widely from 10-50% (3-6). In most studies a correlation between cyclin E overexpression and oestrogen receptor (ER) negative cancer has been observed (4,5,7) although some failed to see this connection (3,6). Overexpression of cyclin E also seems to be related to worse prognosis (3-5,7) and an association of cyclin E with increased proliferative activity has been reported (5,7,8).

The retinoblastoma protein is often lost in cancer development and this is also reported in breast cancer. Despite the apparent central role of Rb in cell cycle regulation most studies have failed to see any prognostic importance of dysfunction of the protein. There does not seem to exist any relationship between lost Rb and oestrogen receptor status (9,10), although Ceccarelli *et al* (11) reported that tumours with lost Rb function are predominantly ER negative. The previously reported frequencies of lost Rb in breast cancer are most often 20-30% (9,10,12,13).

We studied the prognostic role of cyclin E and Rb in breast cancer patients randomised to tamoxifen (TAM), CMF (cyclophosphamide, metotrexate, 5-fluorouracil) chemo-

therapy and radiotherapy (RT) and how their expression affects the patient's response to treatment. Their relation to each other and other cell cycle regulators were also investigated.

Materials and methods

Patients. The breast cancer patients included in this study were postmenopausal women who participated in a Swedish trial (14) where they were randomised to either adjuvant chemotherapy or postoperative radiotherapy. The patients included in the trial had either histologically verified lymph node metastases or a tumour diameter exceeding 30 mm. The patients did not receive any preoperative treatment and surgery consisted of modified radical mastectomy. The chemotherapy treatment consisted of 12 courses of CMF given according to the original Milan protocol (cyclophosphamide 100 mg/m^2 orally on days 1-14, metotrexate 40 mg/m^2 i.v. on days 1 and 8, and 5-fluorouracil 600 mg/m^2 i.v. on days 1 and 8). Radiotherapy was given with the high-voltage technique with a total dose of 46 Gy. Using a 2x2 factorial study design, the patients were also randomised to either tamoxifen or no adjuvant endocrine therapy. Tamoxifen treatment was given postoperatively at a dose of 40 mg daily for at least 2 years. The current study included a subset consisting of 255 patients for whom frozen tumour tissue was still available after hormone receptor assays. The median period of follow-up was 10.6 years for recurrence-free patients. Approval for this specific study was obtained by the regional ethics committee at Karolinska Hospital. In accordance with the approval the patients were not asked for a written informed concent.

Immunohistochemical detection of cyclin E and Rb. The immunohistochemical method was previously described elsewhere (15). The frozen tissue sections were fixed in 4% formalin for 30 min and boiled in 10 mM citrate buffer pH 6.0 in a pressure cooker for 10 min. After cooling to room temperature the samples were placed in 3% H_2O_2 in methanol for 5 min and then incubated with serum free protein block (Dako, Denmark) for 10 min. The slides were incubated with a mouse monoclonal cyclin E primary antibody, clone HE12 (Pharmingen, USA) or a mouse monoclonal retinoblastoma antibody, clone Rb1 (1F8) (Dako) at 4˚C overnight. The cyclin E antibody was followed by secondary Multilink swine anti-goat/mouse/rabbit antibody (Dako) conjugated with biotin. Streptavidin-horseradish peroxidase was then applied. The Rb antibody was followed by secondary Dako Evision+ antibody (Dako, CA, USA). Positive cells were visualised with 3.3-diaminobenzidin hydrochloride (DAB) and before counterstaining with haematoxylin the slides were incubated in 0.5% $CuSO_4$ in 0.85% NaCl for 2 min to enhance contrast. For cyclin E, a mouse IgG1 antibody was used as negative control. All washing steps were performed in PBS with 0.2% tween and 0.5% BSA. Antibodies and DAB were diluted in PBS with 0.5% BSA.

Cyclin D1 and p53. Cyclin D1 protein levels was previously analysed immunohistochemically (15). p53 was previously analysed for gene mutations and protein accumulation with single-strand conformation polymorphism followed by direct sequencing and immunohistochemistry respectively (16).

Scoring. For Rb the slides were graded by frequency of positive cells as negative, <25%, 25-50%, 50-75% or >75%. The cyclin E staining was graded according to proportion of positive cells as <1%, 1-10% or >10%. Only the fraction of cyclin E positive cells was graded, as the staining intensity in positive cells was uniform unlike the frequency, which varied significantly between tumours. Although some cytoplasmic staining was observed, only nuclear staining was considered. The slides were evaluated by two independent observers.

Statistical analysis. The relationships between different grouped variables were analysed with the χ^2 test, or the χ^2 test for trend when required. Survival curves were produced according to the life-table method described by Kaplan and Meier. Analysis of recurrence rates was performed with Cox proportional hazard regression. All procedures are comprised in the statistical package STATISTICA 7.0 (StatSoft Scandinavia AB, Sweden). The criterion for statistical significance was $P<0.05$.

Results

The staining of cyclin E was informative in 221 tumours of which 41 (18.6%) had <1% positive cells, 109 (49.3%) had 1-10% positive cells and 71 (32.1%) had >10% positive cells (Fig. 1). Only nuclear staining was considered. Overexpression of cyclin E was defined as tumours having more than 10% positive cells. Increasing expression of cyclin E was correlated to oestrogen receptor negativity (P=0.007), S-phase fraction (P=0.015) and tumour size (P=0.04) (Table I). High expression of cyclin E was also correlated to negative lymph nodes (P=0.009). Analysis of the relationship between cyclin E and cyclin D1 showed that higher expression of cyclin E was associated with higher expression of cyclin D1 (P=0.03) (Table I).

The staining of Rb was informative in 236 tumours. Of those 38 (16.1%) were negative, 21 (8.9%) had <25% positive cells, 29 (12.3%) had 25-50% positive cells, 75 (31.8%) had 50-75% positive cells and 73 (30.9%) had >75% positive cells (Fig. 1). Loss of Rb function was defined as less than 25% positive cells, resulting in 59 (25.0%) patients with loss of Rb function. The expression of Rb was not correlated to oestrogen receptor status or any other prognostic factors except tumour size, where positive Rb staining was associated with larger tumours (P=0.011) (Table I). The expression of cyclin E and loss of Rb function was not correlated to each other.

The relation of p53 to Rb and cyclin E. The p53 status was analysed by combining the results from mutational analysis and immunohistochemical analysis made in previous work by Askmalm *et al* (16). Mutation in p53 and/or accumulated p53 protein was considered as dysfunctional p53. Dysfunctional p53 was inversely correlated to loss of Rb function (P=0.001) (Table I). Dysfunctional p53 was also correlated to increasing expression of cyclin E (P=0.003).

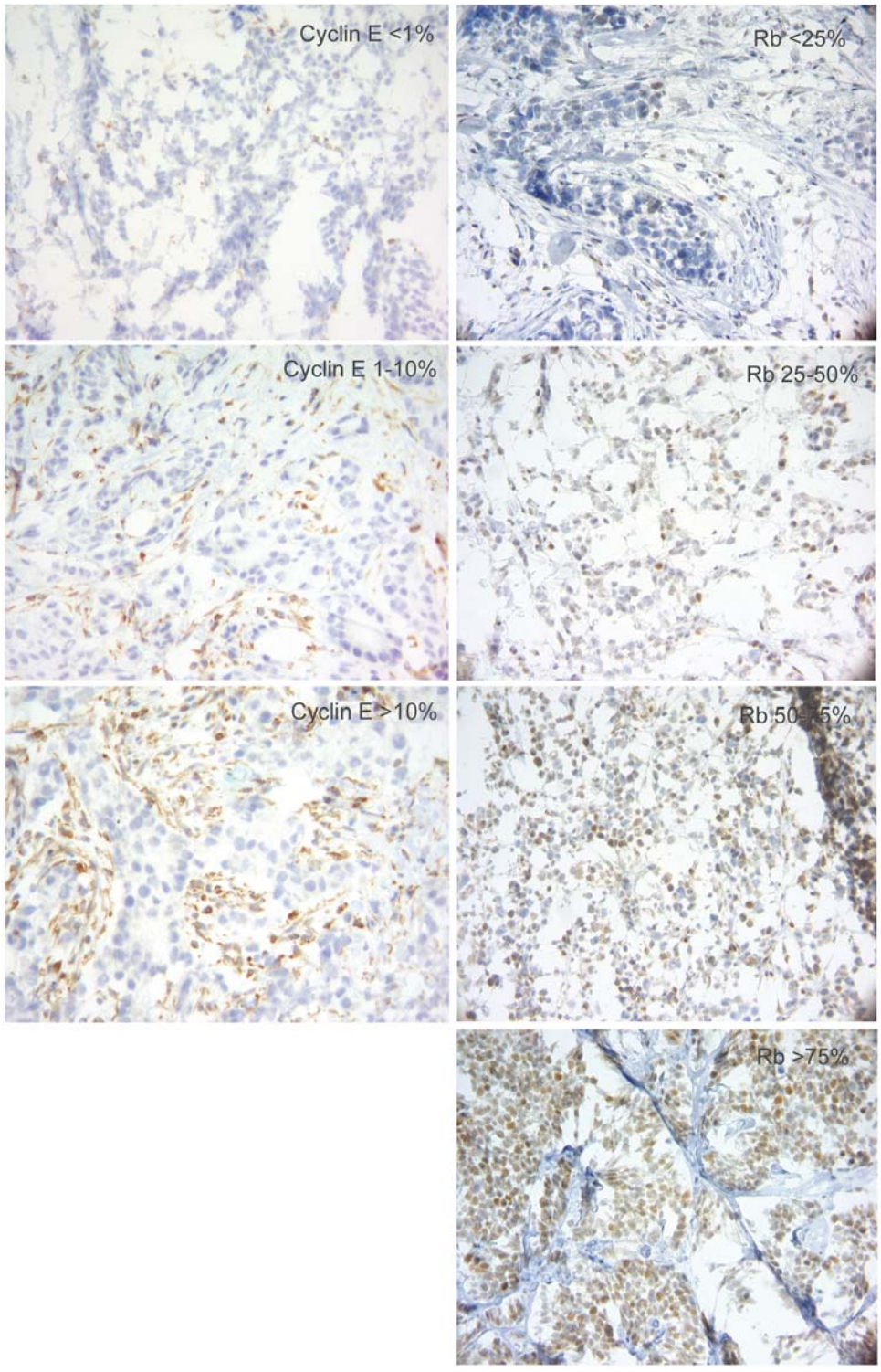

Figure 1. Immunohistochemical staining of cyclin E and Rb in tumours with different expression levels.

The prognostic importance of cyclin E and Rb expression. Recurrence free survival was analysed both in the whole material and depending on ER receptor status. Neither cyclin E overexpression (cyclin E >10% vs. cyclin E <10%; RR=0.74; 95% CI, 0.49-1.13) nor loss of Rb function (Rb+ vs. Rb-; RR=1.50; 95% CI, 0.96-2.32) seemed to affect the recurrence rate. For cyclin E, similar results were found when analysing local recurrence and breast cancer survival. Rb expression on the other hand seemed to influence the risk of local recurrence where lost expression of Rb was associated to reduced risk of local recurrence compared to normal expression (Rb+ vs. Rb-; RR=2.43; 95% CI, 1.03-5.74) (Fig. 2).

Radiotherapy and local recurrence. In the aspect of radiotherapy and local recurrence both the patients with high and low expression of cyclin E had a decreased risk of local recurrence when treated with radiotherapy instead of

Table I. Cyclin E and Rb expression in relation to clinico-pathological factors.

	Cyclin E			Rb	
	<1% n (%)	1%-10% n (%)	>10% n (%)	≤25% n (%)	>25% n (%)
Lymph node status					
0	2 (8)	7 (28)	16 (64)[b]	4 (19)	17 (81)
1-3	26 (20)	70 (53)	35 (27)	39 (28)	99 (72)
>3	13 (20)	32 (49)	20 (31)	16 (21)	61 (79)
Tumour size					
≤20 mm	21(23)	47 (51)	24 (26)[a]	31 (31)	68 (69)[a]
21-30 mm	11 (16)	36 (52)	22 (32)	20 (27)	54 (73)
>30 mm	9 (15)	26 (43)	25 (42)	8 (13)	55 (87)
S-phase fraction					
<5%	16 (26)	34 (55)	12 (19)[a]	18 (26)	51 (74)
5-10%	10 (14)	33 (48)	26 (38)	18 (24)	57 (76)
>10%	10 (15)	32 (47)	26 (38)	18 (27)	48 (73)
Er status					
ER-	10 (16)	23 (36)	31 (48)[b]	16 (22)	58 (78)
ER+	31 (20)	83 (54)	40 (26)	43 (27)	116 (73)
Cyclin D1					
Weak	36 (22)	79 (48)	49 (30)[a]	41 (25)	118 (74)
Strong	4 (9)	23 (49)	20 (43)	12 (25)	36 (75)
p53					
Functional	32 (21)	78 (52)	39 (26)[b]	51 (32)	109 (68)[c]
Dysfunctional	7 (11)	28 (43)	30 (46)	8 (12)	61 (88)
Rb					
>25%	25 (7)	73 (49)	50 (34)		
≤25%	8 (5)	29 (56)	15 (29)		

[a]P<0.05, [b]P<0.01, [c]P=0.001.

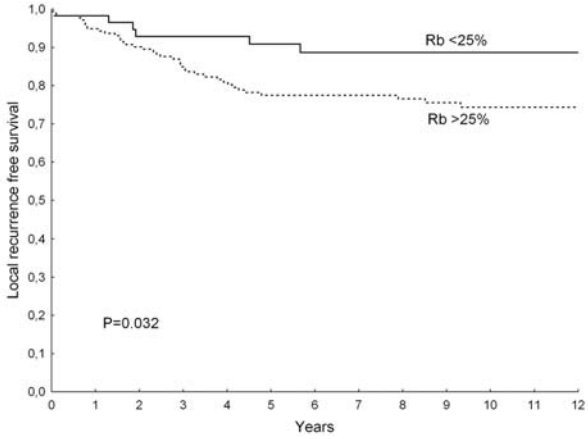

Figure 2. Risk of local recurrence depending on Rb expression.

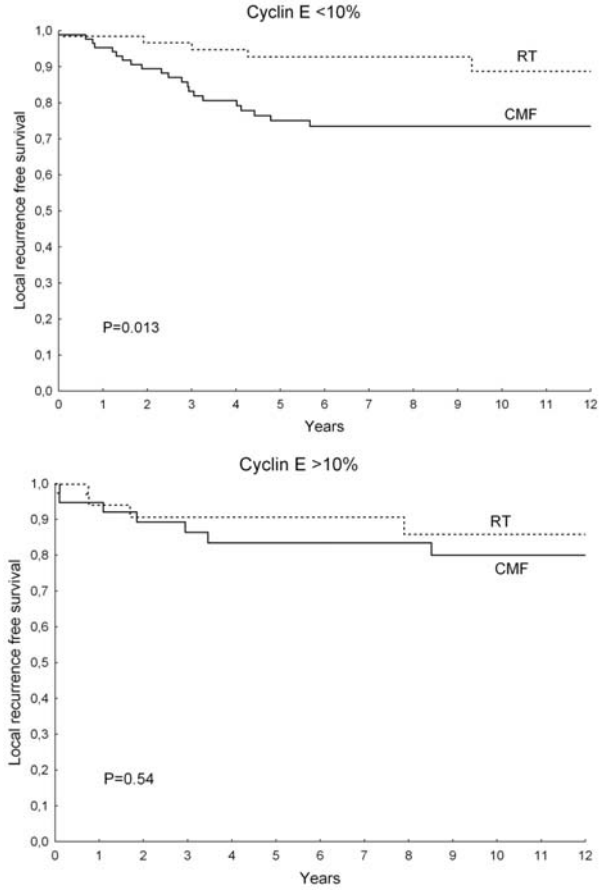

Figure 3. The impact of cyclin E expression on the patients benefit from radiotherapy vs. chemotherapy in relation to local recurrence.

chemotherapy, although only significant in the group with low expression (RT vs. CMF; RR=0.31; 95% CI, 0.12-0.83) (Fig. 3). This result illustrates that radiotherapy is more effective in preventing local recurrence than chemotherapy. The effect of radiotherapy was less marked in the group of patients with high expression of cyclin E (RT vs. CMF; RR=0.68; 95% CI, 0.2-2.32), although the test for interaction was not significant (P=0.34). Patients with lost Rb expression had a decreased risk of local recurrence, compared to patients with normal Rb expression. The group with normal expression of Rb had a significantly lower risk of local recurrence if treated with radiotherapy compared to CMF (Table II). In the group with lost Rb this relation was not obvious. The possibility of p53 status affecting the results was investigated by analysing lost Rb expression in combination with lost p53 function. Patients with both normal p53 and Rb expression had significantly reduced risk of local recurrence when treated with radiotherapy instead of CMF (Table II, Fig. 4). Patients with either lost p53 function or low Rb expression or both did not significantly benefit from radiotherapy.

Cyclin E and response to tamoxifen treatment. When analysing the patients' response to tamoxifen treatment only the oestrogen receptor positive patients were included. As expected for the whole population, the patients with low

Table II. Rb and p53 expression and risk of local recurrence in relation to radiotherapy or CMF treatment.

	No. of patients	Local recurrence rate	95% CI	P-value	P interaction
Rb ≤25%					
CMF	35	1			
RT	24	0.27	0.032-2.33	0.23	
Rb >25%					
CMF	103	1			
RT	74	0.40	0.19-0.84	0.016	0.73
Rb ≤25% and/or lost p53 function					
CMF	73	1			
RT	55	0.70	0.28-1.73	0.44	
Rb >25% and normal p53					
CMF	64	1			
RT	45	0.17	0.052-0.58	0.005	0.070

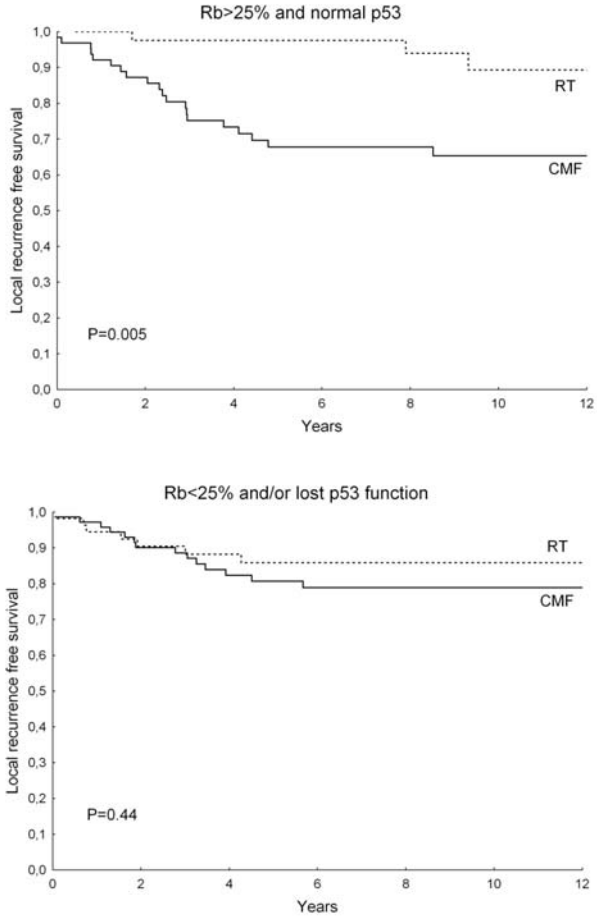

Figure 4. The impact of Rb and p53 on the patients benefit from radiotherapy vs. chemotherapy in relation to local recurrence.

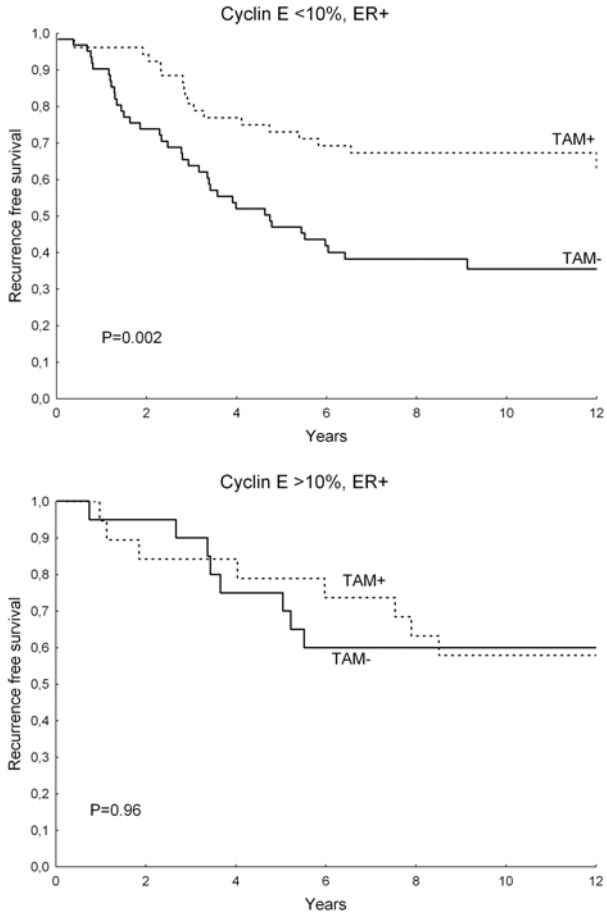

Figure 5. The effect of tamoxifen treatment on recurrence depending on cyclin E expression.

cyclin E expression who received tamoxifen treatment showed decreased risk of recurrence, in contrast to patients with high expression, for whom on the other hand no significant difference in response was seen (Table III, Fig. 5). A test of the difference between the two groups showed borderline significance (P=0.086). Combining the expression of cyclin E and cyclin D1 in one analysis showed the same pattern,

Table III. Cyclin expression and risk of recurrence in relation to tamoxifen treatment for oestrogen receptor positive patients.

	No. of patients	Local recurrence rate	95% CI	P-value	P interaction
Cyclin E ≤10%					
Tamoxifen-	62	1			
Tamoxifen+	52	0.41	0.24-0.72	0.002	
Cyclin E >10%					
Tamoxifen-	20	1			
Tamoxifen+	20	0.97	0.36-2.60	0.96	0.086
Cyclin E ≤10% and cyclin D1 low					
Tamoxifen-	48	1			
Tamoxifen+	42	0.39	0.21-0.73	0.003	
Cyclin E >10% and/or cyclin D1 high					
Tamoxifen-	32	1			
Tamoxifen+	30	0.86	0.40-1.87	0.96	0.12

with approximately the same significance in the test for interaction (Table III). The expression of Rb did not affect the patients benefit from tamoxifen (data not shown).

Discussion

The results of this study show high expression of cyclin E to be associated with oestrogen receptor negativity. This is consistent with previous studies (4,5,7) although some have failed to see this connection (3). In agreement with Lindahl *et al* (5) we also found a correlation to tumour size and lymph node status. Nielsen *et al* (17) found cyclin E expression to be inversely correlated to cyclin D1 expression. Opposing this, our results showed that high expression of cyclin E was associated with high expression of cyclin D1. We found cyclin E expression to be associated with S-phase fraction, a measure of cell proliferation. This has previously been reported by several other groups (5,7,8).

The staining of Rb was graded according to fraction of positive cells and we found loss of Rb in 25.0% of the tumours. The somewhat different frequencies of lost Rb reported so far are probably due to differences in methods and grading systems. Our result is in line with Anderson *et al* (10) and Sawan *et al* (12) who both used immunohistochemistry and a grading system similar to ours. Nielsen *et al* (17) reported only 9% abnormal Rb but then only negative tumours were included. As others (6,9,10) we failed to see any connection between Rb expression and oestrogen receptor status, although Ceccarelli *et al* (11) found a correlation between loss of Rb and oestrogen receptor negativity.

Similar to others, we did not see any correlation between lost Rb and prognosis (9,10,12). Unlike many other authors (3,4,7,18) we did not find cyclin E expression to affect the risk of recurrence. This was still true when the material was divided by oestrogen receptor status. However, the relationship between cyclin E and prognosis seems to be complex and has been reported to be dependent on the growth pattern (19).

Unlike Sawan *et al* (12), who did not find any association between p53 and Rb, we found an inverse correlation between loss of functional Rb and abnomal p53 hence opening up for the speculation that loss of Rb and dysfunctional p53 represent two different pathways in cancer development. We also saw a correlation between high expression of cyclin E and abnormal p53 and this is in line with Lindahl *et al* (5) and Lodén *et al* (20).

Our results showed decreased radiosensitivity among patients with high tumour expression of cyclin E. Upon radiation-induced DNA-damage, p53 is expressed leading to increased p21 levels, followed by G1 arrest (21). In this study high expression of cyclin E was associated with high expression of cyclin D1 and abnormal p53. Radiation will in this case fail to induce p21 and cyclin E inhibition and at the same time, high expression of the two cyclins will continue to move the cells forward in cell cycle. Abnormal p53 has been associated with resistance to radiotherapy (16), probably caused by diminished p21 induction and failure to induce apoptosis. Tumours with normal Rb expression did respond to radiotherapy. Diminished Rb function may undermine the role p53 has in signalling G1-arrest upon DNA-damage since E2F1 is constantly free. In this aspect Rb positive tumours are expected to respond better to radiation than tumours without Rb. However, worth noting is the correlation between normal Rb and lost p53 function which should affect the radiosensitivity. Indeed, in our material, tumours with both normal p53 and Rb expression showed increased response to radiotherapy compared to tumours with altered expression of either p53 or Rb or both.

Our results suggest that patients with high cyclin E tumours have less benefit from tamoxifen than patients whose tumours show low expression. Several experimental studies support our findings. Christov *et al* (22) describe in their report that tamoxifen treatment inhibits tumour growth in rats by decreasing proliferation and inducing apoptosis. The cyclin D1 and cyclin E protein levels were decreased by the treatment. In another study Hui *et al* (23) showed that

cyclin E overexpression mediates some short-term anti-oestrogen resistance in MCF-7 cells, although cyclin D1 overexpression resulted in a more evident resistance. Similarly, Dhillon *et al* (24) reported that anti-oestrogen resistance in cyclin E overexpressing MCF-7 cells was dependent on modification of the Rb/E2F signalling pathway. Several studies show that tamoxifen causes cell cycle arrest through up-regulation of p21 and $p27^{KIP2}$ protein levels and increasing their binding to the cyclin E/CDK2 complex (25,26). Inhibition of either p21 or p27 will lead to anti-oestrogen resistance although Planas-Silva *et al* (26) only found this to be true for p21. In support of this Pérez-Tenorio *et al* (27) found that p21 delocatisation to the cytoplasm was associated with tamoxifen resistance whereas nuclear localisation predicted benefit from the treatment.

Akli *et al* (28) analysed the importance of overexpression of low molecular weight forms (LMW) of cyclin E and found higher kinase activity and a more effective binding between them and CDK2 compared to the full length protein. The LMW forms were also resistant to inhibition by p21 and p27 despite normal binding of the inhibitors to the CDK2/cyclin E complex. The inability of p21 to inhibit the complex caused anti-oestrogen resistance. In a clinical material Akli *et al* (28) could not see any benefit from tamoxifen among patients with tumours overexpressing cyclin E. In previous work we found that overexpression of cyclin D1 indicated decreased benefit from tamoxifen treatment (15). Here we found that high expression of cyclin E was associated to cyclin D1 overexpression, hence, overexpression of the two cyclins might have a combined effect on anti-oestrogen response. However, elevated expression of both cyclins did not show increased anti-oestrogen resistance, as compared to overexpression of cyclin E alone, in our material.

The Rb antibody used in this project binds to both phosphorylated and hypophosphorylated protein. Measurements showing both types separately would be useful in order to present a clearer picture of the impact of Rb in breast cancer. The antibody against cyclin E used in this study reacts with both full length and low molecular weight proteins and as reported by Akli *et al* overexpression of low molecular weight forms of cyclin E might have a greater impact on patient outcome than the full length protein.

In conclusion, our study shows that the cell cycle regulators cyclin E and Rb may be of importance in treatment prediction. Overexpression of cyclin E affected the patients benefit from tamoxifen and radiotherapy. Lost Rb expression in combination with lost p53 function influenced the patients benefit from radiotherapy, hence, taking the two proteins into account may help to predict radioresistance. Continued research in this field is necessary to further explore the relation between cell cycle regulators and response to therapy.

Acknowledgements

This work was funded by the Swedish Cancer Society.

References

1. Sherr CJ: The Pezcoller lecture: cancer cell cycles revisited. Cancer Res 60: 3689-3695, 2000.
2. Steeg PS and Zhou Q: Cyclins and breast cancer. Breast Cancer Res Treat 52: 17-28, 1998.
3. Kim HK, Park IA, Heo DS, Noh DY, Choe KJ, Bang YJ and Kim NK: Cyclin E overexpression as an independent risk factor of visceral relapse in breast cancer. Eur J Surg Oncol 27: 464-471, 2001.
4. Nielsen NH, Arnerlov C, Emdin SO and Landberg G: Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to oestrogen receptor status. Br J Cancer 74: 874-880, 1996.
5. Lindahl T, Landberg G, Ahlgren J, Nordgren H, Norberg T, Klaar S, Holmberg L and Bergh J: Overexpression of cyclin E protein is associated with specific mutation types in the p53 gene and poor survival in human breast cancer. Carcinogenesis 25: 375-380, 2004.
6. Milde-Langosch K, Bamberger AM, Methner C, Rieck G and Loning T: Expression of cell cycle-regulatory proteins rb, p16/MTS1, p27/KIP1, p21/WAF1, cyclin D1 and cyclin E in breast cancer: correlations with expression of activating protein-1 family members. Int J Cancer 87: 468-472, 2000.
7. Kuhling H, Alm P, Olsson H, Ferno M, Baldetorp B, Parwaresch R and Rudolph P: Expression of cyclins E, A, and B, and prognosis in lymph node-negative breast cancer. J Pathol 199: 424-431, 2003.
8. Nielsen NH, Arnerlov C, Cajander S and Landberg G: Cyclin E expression and proliferation in breast cancer. Anal Cell Pathol 17: 177-188, 1998.
9. Pietilainen T, Lipponen P, Aaltomaa S, Eskelinen M, Kosma VM and Syrjanen K: Expression of retinoblastoma gene protein (Rb) in breast cancer as related to established prognostic factors and survival. Eur J Cancer 31A: 329-333, 1995.
10. Anderson JJ, Tiniakos DG, McIntosh GG, Autzen P, Henry JA, Thomas MD, Reed J, Horne GM, Lennard TW, Angus B and Horne CH: Retinoblastoma protein in human breast carcinoma: immunohistochemical study using a new monoclonal antibody effective on routinely processed tissues. J Pathol 180: 65-70, 1996.
11. Ceccarelli C, Santini D, Chieco P, Taffurelli M, Gamberini M, Pileri SA and Marrano D: Retinoblastoma (RB1) gene product expression in breast carcinoma. Correlation with Ki-67 growth fraction and biopathological profile. J Clin Pathol 51: 818-824, 1998.
12. Sawan A, Randall B, Angus B, Wright C, Henry JA, Ostrowski J, Hennessy C, Lennard TW, Corbett I and Horne CH: Retinoblastoma and p53 gene expression related to relapse and survival in human breast cancer: an immunohistochemical study. J Pathol 168: 23-28, 1992.
13. Wakasugi E, Kobayashi T, Tamaki Y, Nakano Y, Ito Y, Miyashiro I, Komoike Y, Miyazaki M, Takeda T, Monden T and Monden M: Analysis of phosphorylation of pRB and its regulatory proteins in breast cancer. J Clin Pathol 50: 407-412, 1997.
14. Rutqvist LE and Johansson H: Long-term follow-up of the Stockholm randomized trials of postoperative radiation therapy versus adjuvant chemotherapy among 'high risk' pre- and postmenopausal breast cancer patients. Acta Oncol 45: 517-527, 2006.
15. Ahnstrom M, Nordenskjold B, Rutqvist LE, Skoog L and Stal O: Role of cyclin D1 in ErbB2-positive breast cancer and tamoxifen resistance. Breast Cancer Res Treat 91: 145-151, 2005.
16. Askmalm MS, Carstensen J, Nordenskjold B, Olsson B, Rutqvist LE, Skoog L and Stal O: Mutation and accumulation of p53 related to results of adjuvant therapy of postmenopausal breast cancer patients. Acta Oncol 43: 235-244, 2004.
17. Nielsen NH, Emdin SO, Cajander J and Landberg G: Deregulation of cyclin E and D1 in breast cancer is associated with inactivation of the retinoblastoma protein. Oncogene 14: 295-304, 1997.
18. Keyomarsi K, Tucker SL, Buchholz TA, Callister M, Ding Y, Hortobagyi GN, Bedrosian I, Knickerbocker C, Toyofuku W, Lowe M, Herliczek TW and Bacus SS: Cyclin E and survival in patients with breast cancer. N Engl J Med 347: 1566-1575, 2002.
19. Berglund P, Stighall M, Jirstrom K, Ryden L, Ferno M, Nordenskjold B and Landberg G: Cyclin E confers a prognostic value in premenopausal breast cancer patients with tumours exhibiting an infiltrative growth pattern. J Clin Pathol 61: 184-191, 2008.
20. Lodén M, Stighall M, Nielsen NH, Roos G, Emdin SO, Ostlund H and Landberg G: The cyclin D1 high and cyclin E high sub-groups of breast cancer: separate pathways in tumorigenesis based on pattern of genetic aberrations and inactivation of the pRb node. Oncogene 21: 4680-4690, 2002.

21. Pawlik TM and Keyomarsi K: Role of cell cycle in mediating sensitivity to radiotherapy. Int J Radiat Oncol Biol Phys 59: 928-942, 2004.
22. Christov K, Ikui A, Shilkaitis A, Green A, Yao R, You M, Grubbs C, Steele V, Lubet R and Weinstein IB: Cell proliferation, apoptosis, and expression of cyclin D1 and cyclin E as potential biomarkers in tamoxifen-treated mammary tumors. Breast Cancer Res Treat 77: 253-264, 2003.
23. Hui R, Finney GL, Carroll JS, Lee CS, Musgrove EA and Sutherland RL: Constitutive overexpression of cyclin D1 but not cyclin E confers acute resistance to antiestrogens in T-47D breast cancer cells. Cancer Res 62: 6916-6923, 2002.
24. Dhillon NK and Mudryj M: Ectopic expression of cyclin E in estrogen responsive cells abrogates antiestrogen mediated growth arrest. Oncogene 21: 4626-4634, 2002.
25. Cariou S, Donovan JC, Flanagan WM, Milic A, Bhattacharya N and Slingerland JM: Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells. Proc Natl Acad Sci USA 97: 9042-9046, 2000.
26. Planas-Silva MD and Weinberg RA: Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. Mol Cell Biol 17: 4059-4069, 1997.
27. Perez-Tenorio G, Berglund F, Esguerra Merca A, Nordenskjold B, Rutqvist LE, Skoog L and Stal O: Cytoplasmic p21WAF1/CIP1 correlates with Akt activation and poor response to tamoxifen in breast cancer. Int J Oncol 28: 1031-1042, 2006.
28. Akli S, Zheng PJ, Multani AS, Wingate HF, Pathak S, Zhang N, Tucker SL, Chang S and Keyomarsi K: Tumor-specific low molecular weight forms of cyclin E induce genomic instability and resistance to p21, p27, and antiestrogens in breast cancer. Cancer Res 64: 3198-3208, 2004.