ORIGINAL ARTICLE

Expression of the cell cycle regulatory proteins $p34^{cdc2}$, $p21^{waf1}$, and p53 in node negative invasive ductal breast carcinoma

H P Kourea, A K Koutras, C D Scopa, M N Marangos, E Tzoracoeleftherakis, D Koukouras, H P Kalofonos

J Clin Pathol: Mol Pathol 2003;**56**:328–335

See end of article for authors' affiliations

Correspondence to: Dr H P Kourea, Department of Pathology, ''Agios Andreas'' Peripheral General Hospital of Patras, Patras, 26335 Greece; hkourea@yahoo.com

Accepted for publication 25 June 2003

Aims: To look for correlations between expression of cell cycle regulatory proteins $p34^{cdc2}$, $p21^{WAF1}$, and p53 in node negative invasive ductal breast carcinoma, or between these proteins and clinicopathological parameters, and to assess their prognostic value.
Methods: Immunohistochemistry using formalin fixed, paraffin wax embedded sections from 94 breast carcinomas. Adjacent benign epithelial breast tissue was available in 74 cases. Median follow up was 72 months.
Results: Nuclear and cytoplasmic $p34^{cdc2}$ expression was seen in 80 and 62 tumours, respectively; nuclear expression was seen in adjacent benign epithelium in 12 cases. $p21^{WAF1}$ and p53 were positive in 48 and 21 tumours, respectively. High expression of $p34^{cdc2}$ in neoplastic nuclei was associated with higher histological grade and p53 expression, but not with tumour size, steroid receptor status, patient age, menopausal status, recurrence, metastasis, disease free survival (DFS), or overall survival (OS). $p34^{cdc2}$ in tumour cytoplasm was associated with $p34^{cdc2}$ nuclear positivity, high tumour grade, and DFS in univariate but not multivariate analysis. In contrast, $p34^{cdc2}$ expression in benign tissue independently predicted DFS and OS in univariate and multivariate analysis. Expression of p53 was associated with high tumour grade and negative steroid receptors, but not with recurrence, metastasis, DFS, or OS. $p21^{WAF1}$ expression was not associated with the examined parameters.
Conclusions: $p34^{cdc2}$, $p21^{WAF1}$, and p53 expression does not predict outcome in node negative breast carcinoma, although $p34^{cdc2}$ expression in benign tissue is related to prognosis. The association between $p34^{cdc2}$ and p53 implicates p53 in G2–M cell cycle checkpoint control, possibly via mediators unrelated to $p21^{WAF1}$.

Breast carcinoma is a heterogeneous disease with variable clinical behaviour. Assessing prognosis is very important for both the prediction of clinical outcome and patient management. Although the lymph node status is a major prognostic parameter,[1] 30% of those patients with node negative breast carcinoma are estimated to die of their disease without adjuvant treatment.[2] Despite the application of valuable prognostic parameters such as tumour size,[1 2] grade,[3–5] and histological type,[6] it is not always feasible to predict the outcome of the disease. Therefore, additional prognostic parameters are needed to identify those patients with node negative breast carcinoma who are more likely to relapse and who might benefit from adjuvant treatment.

Cell cycle deregulation is frequently seen in cancer.[7–9] The cell cycle is directly controlled by a series of cyclin dependent kinases (CDKs), cyclins—the CDK positive regulatory subunits—and CDK inhibitors.[10 11] Progression of the cell cycle from the G2 to the M phase is controlled by a protein kinase complex, called mitosis or maturation promoting factor (MPF).[12 13] MPF consists of two major proteins, the catalytic subunit, $p34^{cdc2}$,[14] and cyclin B1.[15] MPF plays an important role in mitotic induction,[16] regulating a wide range of mitotic events.[17] The complex $p34^{cdc2}$–cyclin B1 is controlled by the $p21^{WAF1}$ protein, which is induced by the wild-type p53 protein.[18] Neoplastic tissues produce high amounts of $p34^{cdc2}$, whereas quiescent cells have low or undetectable amounts.[19 20] A limited number of studies have investigated the role of $p34^{cdc2}$ in the prognosis of breast carcinoma.

> ''Additional prognostic parameters are needed to identify those patients with node negative breast carcinoma who are more likely to relapse and who might benefit from adjuvant treatment''

The p53 tumour suppressor gene is the ''cellular gatekeeper for growth and division''.[21] p53 not only controls the G1–S transition,[10 11 21] but also appears to participate in G2–M cell cycle checkpoint control after DNA damage.[18 22–26] p53 inactivation is the most frequent event in human cancer.[21 27] The prognostic relevance of p53 abnormalities, detected by immunohistochemistry in node negative breast carcinoma, has been highlighted by certain investigators,[28–31] but is still controversial.[32–34]

The purpose of our study was to investigate the potential correlation between p53 and $p34^{cdc2}$, the principal participants in the G1–S and the G2–M checkpoints respectively, their relation with $p21^{WAF1}$, the clinicopathological parameters, and outcome, in addition to the prognostic value of these proteins in node negative invasive ductal breast carcinoma.

Abbreviations: bn, benign; CDK, cyclin dependent kinase; DFS, disease free survival; ER, oestrogen receptor; MPF, mitosis or maturation promoting factor; OS, overall survival; PR, progesterone receptor; TBS, Tris buffered saline; TC, tumour cytoplasm; TN, tumour nuclei

METHODS

Patients and tissue samples

Our study comprised 94 patients with T1–T3, N0, M0 invasive ductal breast carcinoma, for whom paraffin wax embedded tissue blocks and clinical information were available. The administration of neoadjuvant chemotherapy was an exclusion criterion. Information regarding the type of surgery, the greatest tumour diameter, the status of surgical margins, and the number of retrieved axillary lymph nodes was collected from the pathology reports. The clinical records were reviewed for data regarding adjuvant treatment (chemotherapy, hormonal, or radiotherapy) and outcome parameters—that is, the occurrence of recurrence, metastasis or death, disease free survival (DFS), and overall survival (OS).

For each case, representative haematoxylin and eosin stained slides were reviewed to assess tumour grade, according to the Nottingham modification of the Bloom–Richardson grading system,[3] histological type, and presence/extent of an in situ component. Information regarding the status of oestrogen and progesterone receptors was obtained from the patients' records. When such information was not available, the receptor status was examined by immunohistochemistry on paraffin wax embedded tissue.

Immunohistochemical analysis

Formalin fixed, paraffin wax embedded, 5 μm thick sections were dewaxed, rehydrated in graded alcohols, and processed using the streptavidin–biotin–immunoperoxidase method. Briefly, sections were submitted to antigen retrieval by microwave oven treatment for 10 minutes in 0.01 mol/litre citric acid at pH 6.0. This procedure was followed for all antibodies studied. The sections were incubated with 1% hydrogen peroxide for 15 minutes, to block endogenous peroxidase activity, and subsequently with 1% bovine serum albumin diluted in Tris buffered saline (TBS) at pH 7.6 for 20 minutes, to block non-specific binding. The slides were wiped and incubated overnight at 4°C in a humid chamber with appropriately diluted primary antibody. The antibodies used were anti-p53 protein (DO-7) mouse monoclonal antibody (NCL-p53-DO7; Novocastra Laboratories Ltd, Newcastle, Newcastle upon Tyne, UK; 1/50 dilution), anti-cdc2 p34 mouse monoclonal antibody (sc-54; Santa Cruz Biotechnology, Santa Cruz, California, USA; 1/150 dilution), anti-$p21^{WAF1}$ mouse monoclonal antibody (WAF 1 (Ab-1); Oncogene Research Products/Calbiochem, Cambridge, Massachusetts, USA; 1/20 dilution), anti-oestrogen receptor mouse monoclonal antibody (ER1D5; Immunotech, Marseille, France; 1/50 dilution), and anti-progesterone receptor mouse monoclonal antibody (1A6; Immunotech; 1/30 dilution). The slides were then rinsed three times in TBS and incubated with the reagents of the StrAvigen Multilink HRP concentrated detection kit (Biogenex Laboratories, San Ramon, California, USA; 1/80 dilution), according to the manufacturer's instructions. After three washes with TBS, the peroxidase reaction was developed in freshly prepared 0.025% diaminobenzidine/0.1% hydrogen peroxide in TBS. Finally, the sections were counterstained with haematoxylin. Tissues previously known to be positive for $p34^{cdc2}$, $p21^{WAF1}$, and p53 were used as positive controls. Sections prepared with substitution of the primary antibody by TBS were used as negative controls.

Immunohistochemical evaluation and scoring

Two pathologists (HPK and CDS), blinded to the clinical, pathological, and other immunohistochemical results, independently evaluated the immunohistochemically stained slides. Along with the carcinoma, benign breast tissue was available for immunohistochemical examination, on the same or on a separate histological section, in 74 cases. The non-neoplastic tissue consisted of either terminal duct lobular units present in the periphery of the tumour or normal/ectatic ductal structures entrapped within the tumour. Immunohistochemical expression of the proteins was not evaluated in hyperplastic elements. Each histological section was screened and assessed for the percentage of benign and neoplastic nuclei displaying immunostaining. For $p34^{cdc2}$, the tumour cytoplasmic positivity was also recorded separately. $p34^{cdc2}$ immunoreactivity was classified as 1+, 2+, 3+, or 4+ if 0–9%, 10–25%, 26–50%, and 51–100% of the cells, respectively, displayed nuclear or cytoplasmic staining. The $p34^{cdc2}$ positivity was set at $\geqslant$ 10% nuclear or cytoplasmic $p34^{cdc2}$ expression. Immunoreactivity for p53 was classified as 0, 1+, 2+, 3+, or 4+ if 0–9%, 10–25%, 26–50%, 51–75%, and 76–100% of the tumour cell nuclei, respectively, were positive. A carcinoma was classified as p53 positive when at least 10% of the nuclei were immunoreactive. Immunoreactivity for $p21^{WAF1}$ was classified as 1+, 2+, 3+, or 4+ if $<$ 1%, 1–5%, 6–20%, or $>$ 20% of the tumour nuclei, respectively, were positive. A carcinoma was considered $p21^{WAF1}$ positive when $\geqslant$ 6% of the nuclei were immunoreactive. Oestrogen and progesterone receptor expression was considered positive if seen in $\geqslant$ 10% of the neoplastic nuclei. When evaluation between the observers differed by $\geqslant$ 10% or led to a different stratum of immunoreactivity, the case was re-evaluated until a consensus was achieved.

Statistical analysis

The associations between the proteins studied immunohistochemically and the clinicopathological parameters were examined by Pearson's χ^2 test. The effect of these factors on clinical outcome was determined in univariate analysis by the log rank test using the Kaplan–Meier method. Multivariate analysis was performed using the Cox proportional hazard model. Survival was measured in months starting from the date of the first pathological diagnosis. Significance was set at $p \leqslant 0.05$.

RESULTS

Table 1 summarises the clinical and histopathological data of the patients studied.

Table 2 shows the immunohistochemical results for the $p34^{cdc2}$ protein. Figure 1 shows the expression of $p34^{cdc2}$ in tumour cells. Expression of $p34^{cdc2}$ in tumour nuclei ($p34^{cdc2}$TN) compared with adjacent benign tissue ($p34^{cdc2}$bn) was higher in 55, lower in four, and equal in 15 patients. Tumour nuclei showed significantly higher immunoreactivity for $p34^{cdc2}$ (median value, 2+) compared with benign breast epithelium (median value, 1+) ($p < 0.0001$). Tables 3, 4, and 5 show the statistical analysis data for $p34^{cdc2}$TN, $p34^{cdc2}$ in tumour cytoplasm ($p34^{cdc2}$TC), and $p34^{cdc2}$bn, respectively. Tables 6 and 7 and fig 2 show the effect of the examined factors on DFS and OS.

$p34^{cdc2}$TN was associated with histological grade ($p < 0.001$), $p34^{cdc2}$TC ($p < 0.001$), and p53 expression ($p = 0.005$), but it did not correlate with patient age, menopausal status, tumour size, steroid receptor status, recurrence, metastasis, DFS, or OS. $p34^{cdc2}$TC was also associated with grade ($p < 0.001$) and in univariate analysis with DFS ($p = 0.0158$). Although not associated with the examined clinicopathological parameters or the proteins studied, $p34^{cdc2}$bn was associated with longer DFS ($p = 0.0030$) and OS ($p = 0.0046$) in univariate analysis, whereas in multivariate analysis $p34^{cdc2}$bn was the only independent predictor of DFS ($p = 0.001$).

Table 8 shows the immunohistochemical results for p53 and the statistical analysis data for p53 are shown in table 9. Expression of p53 in the tumour is depicted in fig 3. In all cases, benign breast epithelial cells were negative for p53. The

Table 1 Clinical and pathological data of patients (n = 94)

Parameter		No. of patients (%)
Age (years) (median, 55, range, 24–80)		
⩽50		40 (43)
>50		54 (57)
Menopausal status		
Premenopausal		35 (37)
Postmenopausal		57 (61)
Unknown		2 (2)
Surgical treatment		
Partial mastectomy		22 (23)
Total mastectomy		72 (77)
Tumour size		
⩽2 cm		45 (48)
>2 and ⩽5 cm		48 (51)
>5 cm		1 (1)
Tumour grade		
I		21 (22)
II		38 (41)
III		35 (37)
Histological type		
Invasive ductal NOS		80 (85)
Mucinous		5 (5)
Papillary		3 (3)
Medullary		2 (2)
Apocrine		2 (2)
Metaplastic		1 (1)
Tubulolobular		1 (1)
Carcinoma in situ		
Absent		31 (33)
Present (⩽25%)		34 (36)
Extensive (>25%)		27 (29)
Unknown		2 (2)
Oestrogen receptor status		
Positive		54 (57)
Negative		37 (39)
Unknown		3 (3)
Progesterone receptor status		
Positive		47 (50)
Negative		46 (49)
Unknown		1 (1)
Adjuvant treatment		
Chemotherapy		55 (59)
Hormonal treatment		87 (93)
Radiotherapy		41 (44)
Outcome		
No evidence of disease		74 (79)
Relapse		12 (13)
Death		8 (9)
DFS (months)	Median, 69 Range, 12–188	
OS (months)	Median, 72 Range 22–88	

DFS, disease free survival; NOS, not otherwise specified; OS, overall survival.

Table 2 Immunoreactivity for $p34^{cdc2}$ in neoplastic and benign breast tissue

	Score			
	1+	2+	3+	4+
Tumour nuclei (n = 94)	14 (15%)	39 (42%)	34 (36%)	7 (7%)
Tumour cytoplasm (n = 91)	29 (32%)	18 (20%)	17 (18%)	27 (30%)
Benign breast nuclei (n = 74)	62 (84%)	6 (8%)	3 (4%)	3 (4%)

expression of p53 was significantly associated with high tumour grade ($p < 0.001$) and negative oestrogen ($p < 0.001$) and progesterone ($p = 0.005$) receptor status, but there was no correlation with the remaining clinicopathological or outcome parameters.

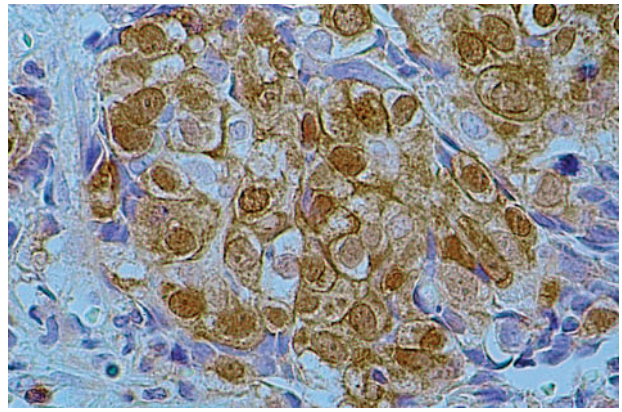

Figure 1 Immunohistochemical reaction for $p34^{cdc2}$ showing 4+ staining (> 50% of tumour nuclei) of invasive ductal breast carcinoma (original magnification, ×400).

Table 8 shows the immunohistochemical results for the $p21^{WAF1}$ protein and the statistical analysis data for $p21^{WAF1}$ are shown in table 10. Figure 4 depicts the expression of $p21^{WAF1}$ in the tumour. No cytoplasmic staining was seen for $p21^{WAF1}$. The expression of this protein did not correlate with the examined clinicopathological or outcome parameters, or the proteins studied.

Table 3 Clinicopathological parameters in relation to $p34^{cdc2}$ expression in tumour nuclei ($p34^{cdc2}$TN)

	$p34^{cdc2}$TN expression				
	1+	2+	3+	4+	p Value
Age (years)					
<50	6	17	12	5	
⩾50	8	22	22	2	0.702
Menopausal					
Pre	4	16	11	4	
Post	9	22	23	3	0.663
T size					
T1	8	16	19	1	
T2	6	23	15	6	0.47
Grade					
1	5	12	4	–	
2	6	19	13	–	
3	3	8	17	7	<0.001
$p34^{cdc2}$TC					
<10%	8	17	4	–	
⩾10%	5	21	29	7	<0.001
$p34^{cdc2}$bn					
<10%	11	26	21	4	
⩾10%	1	3	7	1	0.154
p53					
<10%	13	30	23	3	
⩾10%	1	6	10	4	0.005
$p21^{WAF1}$					
<6%	8	20	14	6	
⩾6%	6	19	20	1	0.875
ER					
Negative	6	13	13	5	
Positive	8	24	20	2	0.364
PR					
Negative	6	18	17	5	
Positive	8	21	16	2	0.244
Relapse					
No	13	32	31	6	
Yes	1	7	3	1	0.900
Death					
No	13	36	31	6	
Yes	1	3	3	1	0.624

ER, oestrogen receptor; $p34^{cdc2}$bn, $p34^{cdc2}$ expression in benign breast tissue; $p34^{cdc2}$TC, $p34^{cdc2}$ expression in tumour cytoplasm; PR, progesterone receptor.

Table 4 Clinicopathological parameters in relation to $p34^{cdc2}$ expression in tumour cytoplasm ($p34^{cdc2}$TC)

	$p34^{cdc2}$ TC expression		
	<10%	⩾10%	p Value
Age (years)			
<50	12	27	
⩾50	17	35	0.848
Menopausal			
Pre	10	23	
Post	17	39	0.996
T size			
T1	15	27	
T2	14	35	0.472
Grade			
1	12	8	
2	14	23	
3	3	31	<0.001
$p34^{cdc2}$TN			
<10%	8	5	
⩾10%	21	57	0.022
p53			
<10%	24	44	
⩾10%	3	16	0.107
$p21^{WAF1}$			
<6%	13	34	
⩾6%	16	28	0.379
ER			
Negative	10	26	
Positive	19	33	0.396
PR			
Negative	14	31	
Positive	15	30	0.824
Relapse			
No	24	55	
Yes	5	7	0.440
Death			
No	26	57	
Yes	3	5	0.724

ER, oestrogen receptor; $p34^{cdc2}$TN, $p34^{cdc2}$ expression in tumour nuclei; PR, progesterone receptor.

Table 5 Clinicopathological parameters in relation to $p34^{cdc2}$ expression in nuclei of benign breast tissue ($p34^{cdc2}$bn)

	$p34^{cdc2}$bn expression		
	<10%	⩾10%	p Value
Age (years)			
<50	26	5	
⩾50	36	7	0.986
Menopausal			
Pre	23	5	
Post	38	6	0.633
T size			
T1	30	7	
T2	32	5	0.535
Grade			
1	16	1	
2	25	6	
3	21	5	0.293
$p34^{cdc2}$TN			
<10%	11	1	
⩾10%	51	11	0.425
$p34^{cdc2}$TC			
<10%	24	2	
⩾10%	36	10	0.128
p53			
<10%	46	10	
⩾10%	13	2	0.683
$p21^{WAF1}$			
<6%	30	6	
⩾6%	32	6	0.920
ER			
Negative	25	5	
Positive	37	6	0.754
PR			
Negative	32	4	
Positive	30	8	0.252
Relapse			
No	54	11	
Yes	8	1	0.663
Death			
No	57	11	
Yes	5	1	0.976

ER, oestrogen receptor; $p34^{cdc2}$TC, $p34^{cdc2}$ expression in tumour cytoplasm; $p34^{cdc2}$TN, $p34^{cdc2}$ expression in tumour nuclei; PR, progesterone receptor.

DISCUSSION

We found significantly higher expression of $p34^{cdc2}$ in carcinoma than in adjacent benign breast tissue. $p34^{cdc2}$ is necessary for the induction of mitosis because it participates in the condensation of chromosomes, the formation of the mitotic spindle, and the breakdown of the nuclear membrane.[35] $p34^{cdc2}$ overexpression in proliferating cells has been reported by several investigators in breast carcinoma[19 36 37] and other tumours.[20 38–40] Because of its participation in the induction of the M phase of the cell cycle, an excess of $p34^{cdc2}$ in the neoplastic tissue provides a proliferative advantage and probably facilitates the neoplastic process.

We examined $p34^{cdc2}$ expression in both tumour nuclei ($p34^{cdc2}$TN) and cytoplasm ($p34^{cdc2}$TC), in addition to the adjacent benign breast tissue ($p34^{cdc2}$bn). An association between $p34^{cdc2}$TN and $p34^{cdc2}$TC expression was noted. Both of these parameters were correlated with higher histopathological grade, unlike the results of previous studies,[35 37] which did not identify an association between $p34^{cdc2}$TN and tumour grade. Our results are in partial agreement with those of Winters and co-workers,[41] who noted a positive association of grade only with $p34^{cdc2}$TC. These findings are probably analogous to the association of proliferative index with grade,[35 42 43] because $p34^{cdc2}$ is thought to be an accurate measure of proliferative cellular activity.[20] Whether these factors are biologically or even aetiologically associated to produce a certain biological tumour profile remains to be elucidated.

''Benign breast epithelium may express $p34^{cdc2}$ as a reactive phenomenon, although the protein may be in an inactive state''

No association was seen between the expression of $p34^{cdc2}$TN or $p34^{cdc2}$TC and patient's age, menopausal status, tumour size, $p34^{cdc2}$bn, or $p21^{WAF1}$. Wiesener and colleagues[35] similarly did not identify an association between $p34^{cdc2}$ and menopausal status, but noted a correlation of $p34^{cdc2}$ expression with oestrogen receptor/progesterone receptor negativity, contrary to our results. $p34^{cdc2}$TN was not associated with DFS or OS, results consistent with recent studies on breast carcinoma[19 35 37] and other types of cancer.[44 45] In contrast, other studies of breast carcinomas[46 47] found $p34^{cdc2}$ expression to be of independent prognostic significance for disease relapse in multivariate analysis. Although $p34^{cdc2}$TC was associated with DFS in univariate analysis, in multivariate analysis it failed to remain significant, and did not appear to affect OS. Therefore, $p34^{cdc2}$TC retention may represent an ineffective mechanism of $p34^{cdc2}$ inactivation. Contrary to these results, the correlation of $p34^{cdc2}$ immunoreactivity with Gleason grade, pathological stage, ploidy abnormalities, presence of metastases,[48] and disease recurrence[49] has been noted in prostatic adenocarcinoma. However in melanoma, although $p34^{cdc2}$ overexpression has been correlated with mitotic activity, tumour thickness, and Clark's level, it was not identified as an independent predictor of prognosis.[40]

Interestingly, in our present study, $p34^{cdc2}$bn was associated with longer DFS in both univariate and multivariate analyses, whereas $p34^{cdc2}$bn was the only parameter that

Table 6 Univariate analysis of examined parameters for disease free survival (DFS)

	Total (n)	Relapse (n)	Median DFS (CI)	p Value
Age (years)				
<50	40	3	73 (64–82)	0.1042
⩾50	54	9		
Menopausal				
Pre	35	2	73 (63–83)	0.1341
Post	57	9	68 (58–78)	
T size				
T1	44	4	67 (65–69)	0.5192
T2	50	8	75 (57–93	
Grade				
1	21	2	67 (56–78)	0.9964
2	38	6	75 (54–96)	0.7221
3	35	4	69 (64–74)	0.4791
$p34^{cdc2}TN$				
<10%	14	1	58 (49–67)	0.1321
⩾10%	80	11	72 (64–80)	
$p34^{cdc2}TC$				
<10%	29	5	63 (56–70)	0.0158
⩾10%	62	7	73 (55–91)	
$p34^{cdc2}$ bn				
<10%	62	8	68 (61–75)	0.0030
⩾10%	12	1	102 (26–178)	
p53				
<10%	69	11	68 (61–75)	0.5671
⩾10%	21	0	69 (63–75)	
$p21^{WAF1}$				
<6%	48	8	68 (64–72)	0.5019
⩾6%	46	4	72 (53–91)	
ER				
Negative	37	4	68 (61–75)	0.7945
Positive	54	7	70 (60–80)	
PR				
Negative	46	6	67 (61–73)	0.4512
Positive	47	6	73 (61–85)	

Multivariate analysis for DFS: $p34^{cdc2}$bn, p=0.001; $p34^{cdc2}$TC, p=0.074.
CI, confidence interval; ER, oestrogen receptor; $p34^{cdc2}$bn, $p34^{cdc2}$ expression in benign breast tissue; $p34^{cdc2}$TC, $p34^{cdc2}$ expression in tumour cytoplasm; $p34^{cdc2}$TN, $p34^{cdc2}$ expression in tumour nuclei; PR, progesterone receptor.

Table 7 Univariate analysis of examined parameters for overall survival (OS)

	Total (n)	DOD (n)	Median OS (CI)	p Value
Age (years)				
<50	40	3	73 (65–81)	0.9560
⩾50	54	5	68 (60–76)	
Menopausal				
Pre	35	2	73 (63–83)	0.1785
Post	57	4	70 (53–87)	
T size				
T1	44	2	68 (62–74)	0.5203
T2	50	6	75 (57–93)	
Grade				
1	21	1	67 (36–98)	0.9815
2	38	3	82 (68–96)	0.9164
3	35	4	69 (64–74)	0.6925
$p34^{cdc2}TN$				
<10%	14	1	62 (53–71)	0.1065
⩾10%	80	7	73 (59–87)	
$p34^{cdc2}TC$				
<10%	29	3	67 (62–72)	0.0715
⩾10%	62	5	75 (58–92)	
$p34^{cdc2}bn$				
<10%	62	5	69 (63–75)	0.0046
⩾10%	12	1	102 (26–178)	
p53				
<10%	69	8	72 (60–84)	0.7237
⩾10%	21	0	69 (63–75)	
$p21^{WAF1}$				
<6%	48	5	69 (63–75)	0.5611
⩾6%	46	3	77 (59–95)	
ER				
Negative	37	4	69 (62–76)	0.9610
Positive	54	4	72 (62–82)	
PR				
Negative	46	5	68 (62–74)	0.5110
Positive	47	3	77 (64–90)	

CI, confidence interval; DOD, died of disease; ER, oestrogen receptor; $p34^{cdc2}$bn, $p34^{cdc2}$ expression in benign breast tissue; $p34^{cdc2}$TC, $p34^{cdc2}$ expression in tumour cytoplasm; $p34^{cdc2}$TN, $p34^{cdc2}$ expression in tumour nuclei; PR, progesterone receptor.

affected OS. The reasons for this unexpected finding are unclear, even more so given that $p34^{cdc2}$bn was not associated with the examined clinicopathological parameters or the examined proteins. Localisation of $p34^{cdc2}$ in the nucleus may either be associated with inactive $p34^{cdc2}$ state or may represent a reactive secondary phenomenon to injurious stimuli. This last hypothesis is supported by the observation that G2 arrest after exposure of human cells to ionising radiation may be accompanied by nuclear translocation of $p34^{cdc2}$.[18] Thus, benign breast epithelium may express $p34^{cdc2}$ as a reactive phenomenon, although the protein may be in an inactive state. The association of $p34^{cdc2}$ expression in benign epithelium with better survival may be explained by a combination of both hypotheses. Namely, injurious stimuli may result in secondary nuclear translocation of $p34^{cdc2}$, although additional protective cellular mechanisms (such as phosphorylation or protein binding) inactivate the kinase in both benign and neoplastic cells, thus preventing cellular proliferation. The evaluation of this finding merits further investigation using biochemical methods in larger series of patients.

In general, positive immunohistochemical staining for p53 has been associated with mutant p53 gene status, resulting in a protein product with a longer half life that allows its visualisation using immunohistochemistry. Our study confirmed previous reports[50] connecting p53 overexpression with higher grade and negative oestrogen and progesterone receptor status. No association of p53 expression with patient's age, menopausal status, tumour size, DFS, or OS were identified. Similar results have been reported by others.[32 34 50 51] Recently, a consensus statement of the College of American Pathologists included p53 in category II of prognostic factors, indicating ''its import needs to be validated further in statistically robust studies''.[33] However, it should be noted that the detection of p53 gene mutations has been shown to be of prognostic importance.[52–54]

$p34^{cdc2}$TN expression paralleled that of p53, contrary to previous observations.[41] Although most carcinomas displayed a p53−/$p34^{cdc2}$+ phenotype, most p53 negative tumours, assumed to possess wild-type p53, expressed lower amounts of $p34^{cdc2}$. This probably reflects the fact that intact p53 can cause G2 arrest by reduction of the expression of $p34^{cdc2}$.[26] Our findings associate p53 with the amount of nuclear $p34^{cdc2}$, a factor crucial for the induction of mitosis, thus associating p53 with G2–M cell cycle checkpoint control. Previous reports have also implicated p53 in G2–M cell cycle checkpoint control.[18 22–26]

Additional links between $p34^{cdc2}$ and p53 are proteins that are transcriptionally activated by p53, such as $p21^{WAF1}$, 14-3-3σ, and GADD45.[26] $p21^{WAF1}$ directly inhibits $p34^{cdc2}$, and it has been shown that the cyclin B–cdc2 kinase complex is negatively regulated by wild-type p53 mediated transcriptional induction of $p21^{WAF1}$.[18 22 24] In our study, we detected the presence $p21^{WAF1}$ in the nuclei only. The absence of cytoplasmic staining is probably related to the use of an acidic citrate buffer (pH 6.0) for antigen retrieval.[41] $p21^{WAF1}$ was not associated with the examined clinicopathological parameters, the proteins analysed, or outcome, similar to previous observations.[41] Contrary to these findings, the association of high $p21^{WAF1}$ with high grade and shorter relapse free

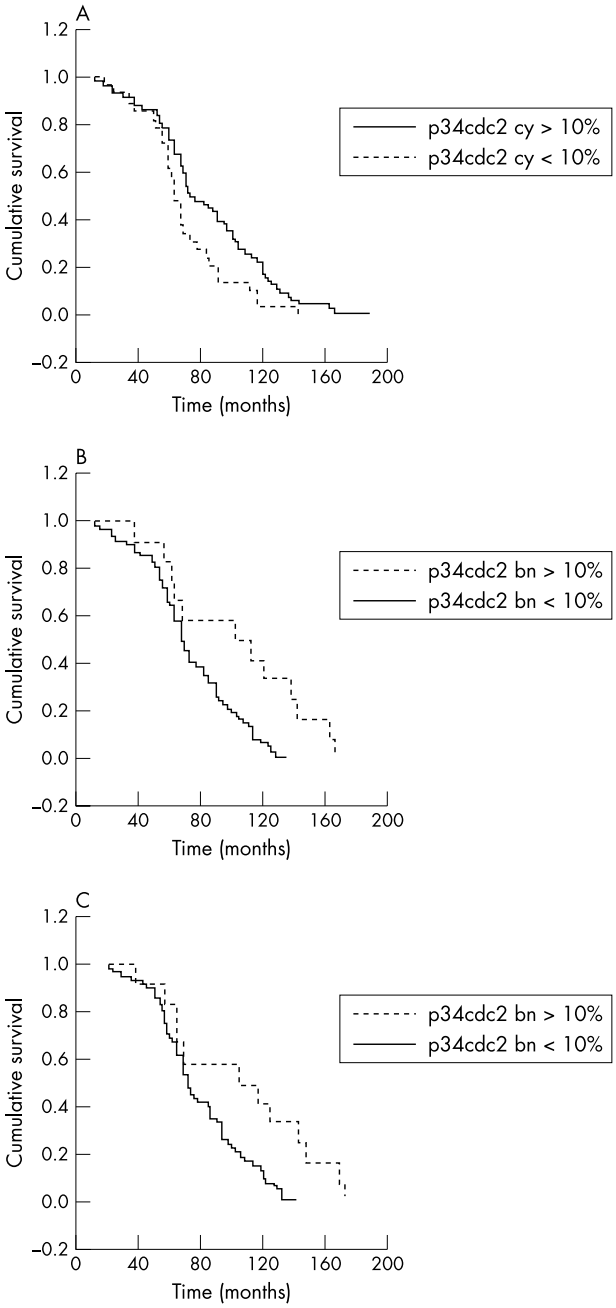

Figure 2 (A) Kaplan–Meier curve for disease free survival stratified according to $p34^{cdc2}$ cytoplasmic expression; low, < 10% of positive tumour cells; high, ⩾ 10% of positive tumour cells. (B) Kaplan–Meier curve for disease free survival stratified according to $p34^{cdc2}$ nuclear expression in benign breast epithelial elements; low, < 10% of positive benign cells; high, ⩾ 10% of positive benign cells. (C) Kaplan–Meier curve for overall survival stratified according to $p34^{cdc2}$ nuclear expression in benign breast epithelial elements; low, < 10% of positive benign cells; high, ⩾ 10% of positive benign cells.

Table 8 Immunoreactivity for p53 and $p21^{WAF1}$ in neoplastic breast tissue

	Score				
	0	1+	2+	3+	4+
p53 (n=90)	69 (77%)	1 (1%)	3 (3%)	7 (8%)	10 (11%)
$p21^{WAF1}$ (n=94)		27 (29%)	21 (22%)	35 (37%)	11 (12%)

Table 9 Clinicopathological parameters in relation to p53 expression in the tumour

	p53 expression		
	<10%	⩾10%	p Value
Age (years)			
<50	28	11	
⩾50	41	10	0.345
Menopausal			
Pre	24	10	
Post	43	11	0.338
T size			
T1	34	8	
T2	35	13	0.374
Grade			
1	16	1	
2	36	2	
3	17	18	0.001
$p34^{cdc2}TN$			
<10%	13	1	
⩾10%	56	20	0.122
$p34^{cdc2}TC$			
<10%	24	3	
⩾10%	44	16	0.107
$p34^{cdc2}bn$			
<10%	46	13	
⩾10%	10	2	0.683
$p21^{WAF1}$			
<6%	32	13	
⩾6%	37	8	0.217
ER			
Negative	18	17	
Positive	48	4	0.001
PR			
Negative	28	16	
Positive	40	5	0.005
Relapse			
No	58	21	
Yes	11	–	0.052
Death			
No	61	21	
Yes	8	–	0.104

ER, oestrogen receptor; $p34^{cdc2}bn$, $p34^{cdc2}$ expression in benign breast tissue; $p34^{cdc2}TC$, $p34^{cdc2}$ expression in tumour cytoplasm; $p34^{cdc2}TN$, $p34^{cdc2}$ expression in tumour nuclei; PR, progesterone receptor.

survival has been previously noted.[55] Because of the complex interactions of $p21^{WAF1}$, in addition to its differing functions according to its stoichiometry (induction of cyclin–cyclin dependent kinase complex formation at low concentration and inhibition of the complex at higher concentrations[56]), direct or possibly simplistic conclusions regarding the

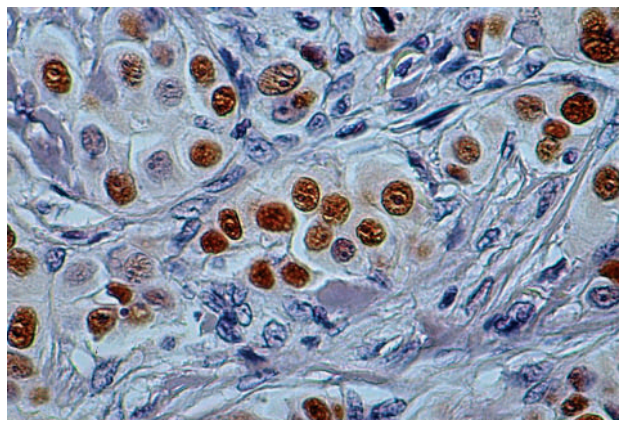

Figure 3 Immunohistochemical reaction for p53 showing 4+ staining (> 75% of tumour nuclei) of invasive ductal breast carcinoma (original magnification, ×400).

Table 10 Clinicopathological parameters in relation to $p21^{WAF1}$ expression in the tumour

	$p21^{WAF1}$ expression		
	<6%	⩾6%	p Value
Age (years)			
<50	21	19	
⩾50	27	27	0.813
Menopausal			
Pre	18	17	
Post	29	28	0.960
T size			
T1	20	24	
T2	28	22	0.313
Grade			
1	10	11	
2	17	21	
3	21	14	0.299
$p34^{cdc2}$TN			
<10%	8	6	
⩾10%	40	40	0.626
$p34^{cdc2}$TC			
<10%	13	16	
⩾10%	34	20	0.379
$p34^{cdc2}$bn			
<10%	30	32	
⩾10%	6	6	0.920
p53			
<10%	32	37	
⩾10%	13	8	0.217
ER			
Negative	23	14	
Positive	25	29	0.140
PR			
Negative	27	19	
Positive	21	26	0.180
Relapse			
No	40	42	
Yes	8	4	0.252
Death			
No	43	43	
Yes	5	3	0.504

ER, oestrogen receptor; $p34^{cdc2}$bn, $p34^{cdc2}$ expression in benign breast tissue; $p34^{cdc2}$TC, $p34^{cdc2}$ expression in tumour cytoplasm; $p34^{cdc2}$TN, $p34^{cdc2}$ expression in tumour nuclei; PR, progesterone receptor.

prognostic role of $p21^{WAF1}$ cannot be made with the use of immunohistochemistry alone.

Two parameters may have adversely affected our results. These are the relatively small number of patients and the administration of adjuvant treatment to all patients studied. This last factor imposes additional difficulties in the identification of those patients who would have relapsed without such treatment. Furthermore, although the median length of the follow up period (72 months) is considered adequate, re-evaluation of the data after extension of the follow up period might provide us with additional information. In the meantime, results concerning DFS and OS should be considered with caution.

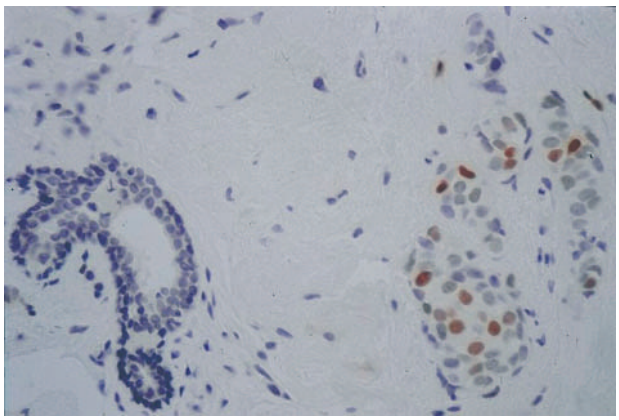

Figure 4 Immunohistochemical reaction for $p21^{WAF1}$ showing 3+ staining (6–20% of tumour nuclei) of invasive ductal breast carcinoma in the right side of the figure (original magnification, ×200).

Take home messages

- Tumour expression of the cell cycle regulators $p34^{cdc2}$, $p21^{WAF1}$, and p53 does not predict outcome in node negative breast carcinoma
- $p34^{cdc2}$ expression in benign tissue adjacent to the tumour is related to prognosis
- Additional studies in patients with node negative breast carcinoma are needed before any final conclusions can be drawn on the prognostic role of these proteins
- The association between $p34^{cdc2}$ and p53 implicates p53 in G2–M cell cycle checkpoint control, possibly involving mediators unrelated to $p21^{WAF1}$

> ''Our findings associate p53 with the amount of nuclear $p34^{cdc2}$, a factor crucial for the induction of mitosis, thus associating p53 with the G2–M cell cycle checkpoint control''

In conclusion, in our study we found that $p34^{cdc2}$ was overexpressed in node negative invasive ductal breast carcinoma compared with benign breast tissue, and detected a strong correlation between nuclear and cytoplasmic $p34^{cdc2}$ overexpression by the tumour and histopathological grade. However, $p34^{cdc2}$ tumour expression did not affect the patients' outcome, tumour size, or steroid receptor status. $p34^{cdc2}$ expression by the benign tissue adjacent to the tumour independently correlated with prognosis. Furthermore, although there was an association of p53 with histopathological grade and negative steroid receptor status, there was no effect of p53 on patient outcome. Similarly, $p21^{WAF1}$ was not associated with the examined clinicopathological parameters, the proteins analysed, or the clinical outcome. In view of the contradictory results regarding the effect of $p34^{cdc2}$ and p53 expression on clinical outcome in the literature, it is apparent that additional studies in patients with node negative breast carcinoma are necessary, before drawing any final conclusions on the prognostic role of these proteins. Finally, the relation of $p34^{cdc2}$ to p53 supports the theories implicating the p53 protein in G2–M cell cycle checkpoint control, thus expanding the complexity of the cellular events involved in cellular homeostasis and neoplastic proliferation. Further studies in patients with breast carcinoma and other neoplasms are needed for a better understanding of the complex cellular mechanisms of cell cycle control.

ACKNOWLEDGEMENTS

This work was supported in part by a grant provided by the Greek National Ministry of Health and the Greek Anticancer Organisation.

Authors' affiliations

H P Kourea, C D Scopa, Department of Pathology, University Hospital of Patras, Patras Medical School, Patras, Rion 26500, Greece
A K Koutras, M N Marangos, H P Kalofonos, Department of Internal Medicine, University Hospital of Patras
E Tzoracoeleftherakis, D Koukouras, Department of Surgery, University Hospital of Patras

REFERENCES

1 **Carter CL**, Allen C, Henson DE. Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases. *Cancer* 1989;**63**:181–7.
2 **McGuire EG**, Tandon AK, Allred DC, *et al.* How to use prognostic factors in axillary node-negative breast cancer patients. *J Natl Cancer Inst* 1990;**82**:1006–15.
3 **Elston CW**, Ellis IO. Pathological prognostic factors in breast cancer I. The value of histological grade in breast cancer: experience from a larger study with long-term follow-up, *Histopathology* 1991;**19**:403–10.
4 **Page DL**. Prognosis and breast cancer. Recognition of lethal and favorable prognostic types. *Am J Surg Pathol* 1991;**15**:334–49.
5 **Pinder SE**, Murray S, Ellis IO, *et al.* The importance of histologic grade of invasive breast carcinoma and response to chemotherapy. *Cancer* 1998;**83**:1529–39.
6 **Dixon JM**, Page DL, Anderson TJ, *et al.* Long term survivors after breast cancer. *Br J Surg* 1985;**72**:445–8.
7 **Hartwell LH**, Kastan MB. Cell cycle control and cancer. *Science* 1994;**266**:1821–8.
8 **Bartek J**, Lukas J, Bartkova J. Perspective: defects in cell cycle control and cancer. *J Pathol* 1999;**187**:95–9.
9 **Dictor M**, Ehinger M, Mertens F, *et al.* Abnormal cell cycle regulation in malignancy. *Am J Clin Pathol* 1999;**112**:40–52.
10 **Cordon-Cardo C**. Mutation of cell cycle regulators. *Am J Pathol* 1995;**147**:545–60.
11 **Hirama T**, Koeffler P. Role of the cyclin-dependent kinase inhibitors in the development of cancer. *Blood* 1995;**86**:841–54.
12 **Masui Y**, Markert CL. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J Exp Zool* 1971;**177**:129–46.
13 **Lohka ML**, Hayes MK, Maller J. Purification of maturation promoting factor, an intracellular regulator of early mitotic events. *Proc Natl Acad Sci U S A* 1988;**85**:3009–13.
14 **Dunphy WG**, Brizuela L, Beach D, *et al.* The xenopus cdc-2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell* 1988;**54**:423–31.
15 **Gautier J**, Minshull J, Lohka M, *et al.* Cyclin is a component of maturation-promoting factor from Xenopus. *Cell* 1990;**60**:487–94.
16 **Hoffmann I**, Clarke PR, Marcote MJ, *et al.* Phosphorylation and activation of human cdc25-C and cdc2-cyclin B and its involvement in the self-amplification of MPF at mitosis. *EMBO J* 1993;**12**:53–63.
17 **Gerhart J**, Wu M, Kirschner M. Cell cycle dynamics of an M-phase-specific cytoplasmic factor in Xenopus laevis oocytes and eggs. *J Cell Biol* 1984;**98**:1247–55.
18 **Winters ZE**, Ongkeko WM, Harris AL, *et al.* p53 regulates Cdc2 independently of inhibitory phosphorylation to reinforce radiation-induced G2 arrest in human cells. *Oncogene* 1998;**17**:673–84.
19 **Kawamoto H**, Koizumi H, Uchikoshi T. Expression of the G2–M checkpoint regulators cyclin B1 and cdc2 in nonmalignant and malignant human breast lesions: immunocytochemical and quantitative image analyses. *Am J Pathol* 1997;**150**:15–23.
20 **Gannon JV**, Nebreda A, Goodger NM, *et al.* A measure of the mitotic index: studies of the abundance and half-life of p34cdc2 in cultured cells and normal and neoplastic tissues. *Genes Cells* 1998;**3**:17–27.
21 **Levine AJ**. P53, the cellular gatekeeper for growth and division. *Cell* 1997;**88**:323–31.
22 **Agarwall ML**, Agarwall A, Taylor WR, *et al.* p53 controls both the G2–M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci U S A* 1995;**92**:8493–97.
23 **Cross SM**, Sanchez CA, Morgan CA, *et al.* A p53 dependent mouse spindle checkpoint. *Science* 1995;**267**:1353–56.
24 **Bunz F**, Dutriaux A, Lengauer C, *et al.* Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 1998;**282**:1497–1501.
25 **Innocente SA**, Abrahamson JLA, Cogswell JP, *et al.* p53 regulates a G2 checkpoint through cyclin B1. *Proc Natl Acad Sci U S A* 1999;**96**:2147–52.
26 **Taylor WR**, Stark GR. Regulation of G2/M transition by p53. *Oncogene* 2001;**20**:1803–15.
27 **Levine AJ**, Momand J, Finland CA. The p53 tumor suppressor gene. *Nature* 1991;**351**:453–5.
28 **Thor AD**, Moore DH II, Edgerton SM, *et al.* Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. *J Natl Cancer Inst* 1992;**84**:845–55.
29 **Allred DC**, Clark GM, Elledge R, *et al.* Association of p53 protein expression with tumour cell proliferation rate and clinical outcome in node negative breast cancer. *J Natl Cancer Inst* 1993;**85**:200–6.
30 **Barnes DM**, Dublin EA, Fisher CJ, *et al.* Immunohistochemical detection of p53 protein in mammary carcinoma: an important new independent indicator of prognosis? *Hum Pathol* 1993;**24**:469–76.
31 **Isola J**, Visakorpi T, Holli K, *et al.* Association of overexpression of tumor suppressor protein p53 with rapid cell proliferation and poor prognosis in node negative breast cancer patients. *J Natl Cancer Inst* 1993;**84**:1109–14.
32 **Rosen PP**, Lesser ML, Arroyo CD, *et al.* p53 in node-negative breast carcinoma: an immunohistochemical study of epidemiologic risk factors, histologic features, and prognosis. *J Clin Oncol* 1995;**13**:821–30.
33 **Fitzgibbons PL**, Page DL, Weaver D, *et al.* Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999. *Arch Pathol Lab Med* 2000;**124**:966–78.
34 **Reed W**, Hannisdal E, Boehler PJ, *et al.* The prognostic value of p53 and c-erb B2 immunostaining is overrated for patients with lymph node negative breast carcinoma. *Cancer* 2000;**88**:804–13.
35 **Wiesener B**, Hauser-Kronberger CE, Zipperer E, *et al.* $p34^{cdc2}$ in invasive breast cancer: relationship to DNA content, Ki67 index and c-erbB-2 expression. *Histopathology* 1998;**33**:522–30.
36 **Megha T**, Lazzi S, Ferrari F, *et al.* Expression of the G2–M checkpoint regulators cyclin B1 and $p34^{cdc2}$ in breast cancer: a correlation with cellular kinetics. *Anticancer Res* 1999;**19**:163–70.
37 **Depowski PL**, Brien TP, Sheehan CE, *et al.* Prognostic significance of $p34^{cdc2}$ cyclin-dependent kinase and MIB1 overexpression, and Her-2/neu gene amplification detected by fluorescence in situ hybridization in breast cancer. *Am J Clin Pathol* 1999;**112**:459–69.
38 **Yasui W**, Ayhan A, Kitadai Y, *et al.* Increased expression of p34cdc2 and its kinase activity in human gastric and colonic carcinomas. *Int J Cancer* 1993;**53**:36–41.
39 **Goodger NM**, Gannon J, Hunt T, *et al.* The localization of $p34^{cdc2}$ in the cells of normal, hyperplastic, and malignant epithelial and lymphoid tissues of the oral cavity. *J Pathol* 1996;**178**:422–8.
40 **Tran T**, Ross JS, Carlson A, *et al.* Mitotic cyclins and cyclin-dependent kinases in melanocytic lesions. *Hum Pathol* 1998;**29**:1085–90.
41 **Winters ZE**, Hunt NC, Bradburn MJ, *et al.* Subcellular localization of cyclin B, Cdc2 and $p21^{WAF1/CIP1}$ in breast cancer: association with prognosis. *Eur J Cancer* 2001;**37**:2405–12.
42 **Wintzer HO**, Zipfel I, Schulte-Mönting J, *et al.* Ki-67 immunostaining in human breast tumours and its relationship to prognosis. *Cancer* 1991;**67**:421–8.
43 **Pinder SE**, Wencyk P, Sibbering DM, *et al.* Assessment of the new proliferative marker MIB-1 in breast carcinoma using image analysis: associations with other prognostic factors and survival. *Br J Cancer* 1995;**71**:146–9.
44 **Yamamoto H**, Monden T, Ikeda K, *et al.* Coexpression of cdk2/cdc2 and retinoblastoma gene products in colorectal cancer. *Br J Cancer* 1995;**71**:1231–6.
45 **Brien TP**, Depowski PL, Sheehan CE, *et al.* Prognostic factors in gastric cancer. *Mod Pathol* 1998;**11**:870–7.
46 **Ohta T**, Fukuda M, Arima K, *et al.* Analysis of Cdc2 and cyclin D1 expression in breast cancer by immunoblotting. *Breast Cancer* 1997;**4**:17–24.
47 **Umemura S**, Komaki K, Noguchi S, *et al.* Prognostic factors for node-negative breast cancers: results of a study program by the Japanese Breast Cancer Society. *Breast Cancer* 1998;**5**:243–9.
48 **Kallakury BV**, Sheehan CE, Ambros RA, *et al.* The prognostic significance of $p34^{cdc2}$ and cyclin D1 protein expression in prostate adenocarcinoma. *Cancer* 1997;**80**:753–63.
49 **Kallakury BV**, Sheehan CE, Rhee SJ, *et al.* The prognostic significance of proliferation-associated nucleolar protein p120 expression in prostate adenocarcinoma: a comparison with cyclins A and B1, Ki-67, proliferating cell nuclear antigen, and p34cdc2. *Cancer* 1999;**85**:1569–76.
50 **Michalides R**, Hageman P, van Tinteren H, *et al.* A clinicopathological study on overexpression of cyclin D1 and of p53 in a series of 248 patients with operable breast cancer. *Br J Cancer* 1996;**73**:728–34.
51 **Zolota V**, Gerokosta A, Melachrinou M, *et al.* Microvessel density, proliferating activity, p53 and bcl-2 expression in in situ ductal carcinoma of the breast. *Anticancer Res* 1999;**19**:3269–74.
52 **Elledge RM**, Fuqua SA, Clark GM, *et al.* Prognostic significance of p53 gene alterations in node negative breast cancer. *Breast Cancer Res Treat* 1993;**26**:225–35.
53 **Iacopetta B**, Grieu F, Powell B, *et al.* Analysis of p53 gene mutation by polymerase chain reaction-single strand conformation polymorphism provides independent prognostic information in node-negative breast cancer. *Clin Cancer Res* 1998;**4**:1597–602.
54 **Pharoah PDP**, Day NE, Caldas C, *et al.* Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis. *Br J Cancer* 1999;**80**:1968–73.
55 **Barbareshi M**, Caffo O, Doglioni C, *et al.* $p21^{WAF1}$ immunohistochemical expression in breast carcinoma: correlations with clinicopathological data, oestrogen receptor status, MIB1 expression, p53 gene and protein alterations and relapse-free survival. *Br J Cancer* 1996;**74**:208–15.
56 **LaBaer J**, Garrett MD, Stevenson LF, *et al.* New functional activities for the p21 family of CDK inhibitors. *Genes Dev* 1997;**11**:847–62.