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Subcellular localisation of cyclin B, Cdc2 and p21$^{WAF1/CIP1}$ in breast cancer: association with prognosis

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Abstract

The heterodimeric cyclin B/Cdc2 protein kinase governs entry into mitosis, and can be negatively regulated through p53-mediated transcriptional induction of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$. Ectopic expression of p21$^{WAF1/CIP1}$ in cultured cells has been shown previously to influence the subcellular distribution of the cyclin-dependent kinases (CDKs) including Cdc2. In this study, we have examined the subcellular localisation of Cdc2, cyclin B and p21$^{WAF1/CIP1}$ by immunohistochemistry in a well characterised series of primary breast cancers. Surprisingly, p21$^{WAF1/CIP1}$ was predominantly cytoplasmic in many of the tumours, where it was associated with high p53 levels; cytoplasmic p21$^{WAF1/CIP1}$ and high cyclin B levels were also significant predictors of poor prognosis. We conclude that breast tumorigenesis may be characterised by abnormalities in pathways determining not only levels of expression of key regulatory molecules, but also their subcellular localisation. Investigation of the subcellular distribution of cell cycle regulatory proteins, particularly p21$^{WAF1/CIP1}$, could provide valuable prognostic markers in breast cancer.

Keywords: CDK; CDK inhibitor; Prognostic markers; Immunohistochemistry

1. Introduction

Cyclins and cyclin-dependent kinases (CDKs) are key regulators of the eukaryotic cell cycle. Entry into mitosis requires the association of cyclin B1 with Cdc2, the prototypical CDK, and activation of Cdc2 protein kinase. The tumour suppressor p53 is essential for arrest in G1 following irradiation of mammalian cells [1]. This requirement for p53 reflects its induction of the p21$^{WAF1/CIP1}$ CDK inhibitor after DNA damage, with consequent inhibition of CDKs required for S phase entry. p53 and p21$^{WAF1/CIP1}$ also regulate the G2/M transition and are essential to sustain G2 arrest after DNA damage [2–4]. In cervical carcinoma cells, cyclin B1/Cdc2 is cytoplasmic during interphase and is transported into the nucleus at the onset of mitosis [5]. In keeping with these findings, normal oral epithelia and breast tissues showed a predominant cytoplasmic localisation of cyclin B1 and Cdc2, with malignancies demonstrating a higher proportion of nuclear staining [6,7].

Alterations in cyclin B1 and Cdc2 are a widespread feature of tumorigenesis. A relationship between increased cyclin B1/Cdc2 levels and tumour invasion and aggressiveness suggests that their immunohistochemical detection could be of prognostic value [6–11]. Proportionately increased expression of cyclin B1 and Cdc2 in breast cancer correlated with mitotic and/or ana-telophase index, and was associated with an increased incidence of lymph node metastasis [12].

Normal breast epithelium has a characteristically low p21$^{WAF1/CIP1}$ reactivity indicative of its low turnover rate [13,14]. High p21$^{WAF1/CIP1}$ expression might be

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expected to be related to normal p53 function, and this is frequently the case in colonic carcinomas [15], but several additional mechanisms may contribute to $p21^{WAF1/CIP1}$ induction. Recent studies suggest that nuclear accumulation of $p21^{WAF1/CIP1}$ promotes cell cycle arrest, while cytoplasmic $p21^{WAF1/CIP1}$ inhibits apoptosis [16,17].

We previously found that p53-null H1299 (non-small cell lung cancer) cells that were arrested in G2 following DNA damage demonstrated a preferential cytoplasmic localisation of cyclin B1 and Cdc2 *in vitro* [4]. By contrast, activation of the G2 checkpoint in association with functional p53 and $p21^{WAF1/CIP1}$ produced a significant nuclear localisation of cyclin B1 and Cdc2 in these cells. Prompted by these *in vitro* findings, here we have investigated the subcellular distribution of cyclin B1, Cdc2 and $p21^{WAF1/CIP1}$ in a series of breast cancers.

2. Patients and methods

2.1. Patients

The study included 73 patients aged from 32 to 80 years (median 55 years) with primary infiltrating carcinoma diagnosed between 1989 and 1992 at the John Radcliffe Hospital (Table 1). Eligibility criteria were: histological diagnosis of breast carcinoma, level one or complete axillary lymph node dissection, no distant metastases and unilateral tumour. 49 (67%) cases were node-negative and 24 (33%) cases were node-positive (N1-mobile ipsilateral or N2-fixed ipsilateral). Adjuvant treatment groups comprised: tamoxifen (16 node-negative, 14 node-positive); CMF (cyclophosphamide, methotrexate, 5-fluorouracil; 6 node-negative, 6 node-positive); both (tamoxifen + CMF; 2 node-negative, 4 node-positive). 25 node-negative women received no adjuvant treatment. The median follow-up duration was 65 months (range 46–92 months).

Table 1
Clinicopathological variables in the patients entered into this study

Characteristic	No. of patients (%)
Total	73 (100)
Tumour size (cm)	
⩽2	22 (30)
>2	51 (70)
Tumour grade	
Ductal grade I	5 (7)
Ductal grade II	24 (33)
Ductal grade III	30 (41)
Non-ductal	14 (19)
ER status (fmol/mg)	
⩽10	38 (52)
>10	35 (48)
p53	
0	33 (45)
1+	19 (26)
2+/3+	21 (29)
Age (years)	
⩽50	43 (59)
>50	30 (41)

ER, oestrogen receptor.

Tumour samples were collected shortly after surgery and were fixed in buffered formalin for 24–48 h at room temperature. Tumours were classified according to Azzopardi [18]. Invasive ductal carcinomas were graded by the modified Bloom's grading system described by Elston and Ellis [19]. Oestrogen receptor (ER) status was determined using an enzyme-lined immunosorbent assay (ELISA) assay (Abbott-ERICA; Abbott Laboratories, UK); tumours were classified as ER-positive if oestradiol binding exceeded 10 fmol/mg cytosolic protein.

2.2. Immunohistochemistry

p53 immunostaining was performed as described elsewhere in Refs. [20,21] using the DO7 monoclonal antibody (Novocastra Laboratories, Newcastle upon Tyne, UK). Nuclear p53 staining was graded from 1 to 3 according to its intensity and extent of immunoreactivity [22]. For intensity staining, 1 represented weakly positive tumour cells and 3 represented strongly positive cells. For the extent of staining, 1 denoted samples in which less than one-third of the tumour cells had positive staining, 2 signified those with positive staining in one-third to two-thirds of the tumour cells, and 3 denoted those with more than two-thirds positive staining. The results obtained with the two scales were multiplied; scores of 1 and 2 were considered to be low staining (1+), 3 and 4 medium (2+), and 6 and 9 high staining (3+). Breast carcinomas known to overexpress p53 and sections of atypical fibroxanthoma with known *TP53* gene mutation and protein accumulation were used as positive controls. Negative controls were obtained by omitting the primary antibodies.

$p21^{WAF1/CIP1}$ immunoreactivity was evaluated using the mouse monoclonal antibody Ab-1 (Calbiochem, Cambridge, MA, USA) at 0.5 μg/ml. Monoclonal antibodies against cyclin B1 (V152) and Cdc2 (A17; kindly provided by Dr Julian Gannon, ICRF, Clare Hall, UK) were used at 6 and 24 μg/ml, respectively. The specificity of each antibody for its respective antigen in immunohistochemistry has been demonstrated previously [7,23]. Horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (for Cdc2 and cyclin B1) and biotinylated goat anti-mouse IgG (for $p21^{WAF1/CIP1}$; DAKO, Glostrup, Denmark) secondary antibodies were used at final concentrations of 10 and 15 μg/ml, respectively. Formalin-fixed paraffin sections of breast cancer tissue were mounted on polylysine-coated glass slides and

were baked for 10 min at 56–60 °C, prior to dewaxing in Citroclear (HD Supplies, Aylesbury, UK). Positive controls were tonsil sections for Cdc2 and cyclin B1, and lung for $p21^{WAF1/CIP1}$. The tissue was rehydrated by sequential immersion in 100 and 50% ethanol. The slides were washed in water, incubated for 30 min in 0.3% (v/v) hydrogen peroxide and washed once in phosphate-buffered saline (PBS)/0.1% (v/v) Tween-20 prior to microwave antigen retrieval. The slides were placed in 100 ml of Tris/ethylene diamine tetraacetic acid (EDTA) buffer (50 mM Tris base, 2 mM EDTA, pH 9.0) and microwaved at 800 W (2×4 min). The slides were then washed in PBS/0.1% (v/v) Tween-20, and were incubated at room temperature for 30–60 min with 200 μl of the appropriate dilution of primary antibody in Roswell Park Memorial Institute (RPMI)-1640/10% (v/v) fetal bovine serum/0.02% (v/v) sodium azide. Slides were washed twice in PBS/0.1% (v/v) Tween-20 and incubated with secondary antibodies for 30–60 min. The StreptABC Duet kit (DAKO, Glostrup, Denmark) was used for detection of $p21^{WAF1/CIP1}$ and involved an additional 30-min incubation with Tris buffered saline/biotinylated streptavidin/HRP. After one PBS/Tween-20 wash, the slides were developed in 3,3 diaminobenzidine and counterstained with haematoxylin before mounting.

Immunostaining was assessed using a Laborlux microscope (Leitz Weitzlar, Germany) at 400× overall magnification with a field diameter of 0.46 mm. In the neoplastic cell population, the degree of staining intensity and the proportion of cells with $p21^{WAF1/CIP1}$, cyclin B1 and Cdc2 immunoreactivity in the nucleus and cytoplasm were graded semi-quantitatively to produce an intensity distribution score (IDS) for each localisation [24]. The most intense areas of tumour staining were selected and evaluated by measuring the percentage of positive cells in a high power field (×25 objective). Calculation of the IDS was performed as follows: IDS (maximum score 300) = 1×% of weakly stained cells + 2×% moderately stained cells + 3×% of strongly stained cells). A similar approach has been used recently by others to evaluate p53 levels [25]. Average IDS values were determined by examination of 10 fields.

2.3. Statistical analysis

Data were analysed using STATA version 5 software (STATA corporation, College Station, Texas, USA). The relationship between p53, $p21^{WAF1/CIP1}$, cyclin B1 and Cdc2 and prognostic factors was assessed using standard logistic regression, ordered logistic regression (proportional odds model) or multinomial logistic regression, with the IDS being the independent variable in each case. Pearson correlation coefficients were used to determine the association between the subcellular localisation of $p21^{WAF1/CIP1}$, cyclin B1 and Cdc2. Wilcoxon's sign test was used to assess differences between the staining levels in the nucleus and cytoplasm for $p21^{WAF1/CIP1}$, cyclin B1 and Cdc2. Analyses of survival data were performed using Cox's regression model or log-rank test.

3. Results

3.1. Localisation of cyclin B1, Cdc2 and $p21^{WAF1/CIP1}$

$p21^{WAF1/CIP1}$ immunoreactivity was evaluable in 66 (90%) cases, Cdc2 in 69 (95%) and cyclin B1 in 72 (99%). Cyclin B1 (Fig. 1a and b), Cdc2 (Fig. 1c and d) and $p21^{WAF1/CIP1}$ (Fig. 1e and h) immunoreactivity was both cytoplasmic and nuclear in the majority of the breast cancers. In general, there was significantly higher expression (IDS) of cyclin B1, Cdc2 and $p21^{WAF1/CIP1}$ in the cytoplasm compared with the nucleus (Table 2) ($P < 0.0001$). Eight cancers (12%) showed no nuclear $p21^{WAF1/CIP1}$ expression, whereas only four (6%) lacked $p21^{WAF1/CIP1}$ staining in the cytoplasm and only 14 (21%) demonstrated predominantly nuclear $p21^{WAF1/CIP1}$ immunoreactivity.

3.2. Association of cyclin B1, Cdc2 and $p21^{WAF1/CIP1}$ with clinicopathological factors

There was no significant association between cyclin B1 or Cdc2 expression and lymph node involvement, large tumour size (> 2 cm) or ER negativity (⩽10 fmol/mg). Increasing cytoplasmic Cdc2 expression was associated with loss of tumour differentiation ($P = 0.02$). A multinomial logistic regression showed a significantly higher cytoplasmic Cdc2 expression in grade III tumours ($P = 0.03$). Nuclear $p21^{WAF1/CIP1}$ showed no consistent trend in relation to the established prognostic indicators, such as nodal involvement, tumour differentiation and tumour size. Similarly, cytoplasmic $p21^{WAF1/CIP1}$ expression demonstrated no significant association with lymph node-positivity or tumour size.

3.3. p53 immunoreactivity and relationship to subcellular localisation of $p21^{WAF1/CIP1}$, cyclin B1 and Cdc2

p53 immunoreactivity was seen in 40 (55%) cases, with 15 (21%) women assessed as having moderate p53 positivity and only 6 (8%) tumours classified as having the highest level of p53 expression (3+). Consequently, the 2+ and 3+ p53-expressing breast cancers were combined and studied as a single group. A significant trend of increasing cytoplasmic $p21^{WAF1/CIP1}$ expression was seen with increasing levels of p53 immunoreactivity ($P = 0.03$). Cytoplasmic $p21^{WAF1/CIP1}$ staining was significantly higher in the p53 2+/3+-expressing breast cancers (median IDS of 84, range 7.5–187) compared with p53 non-expressing (0) tumours (median IDS = 38,

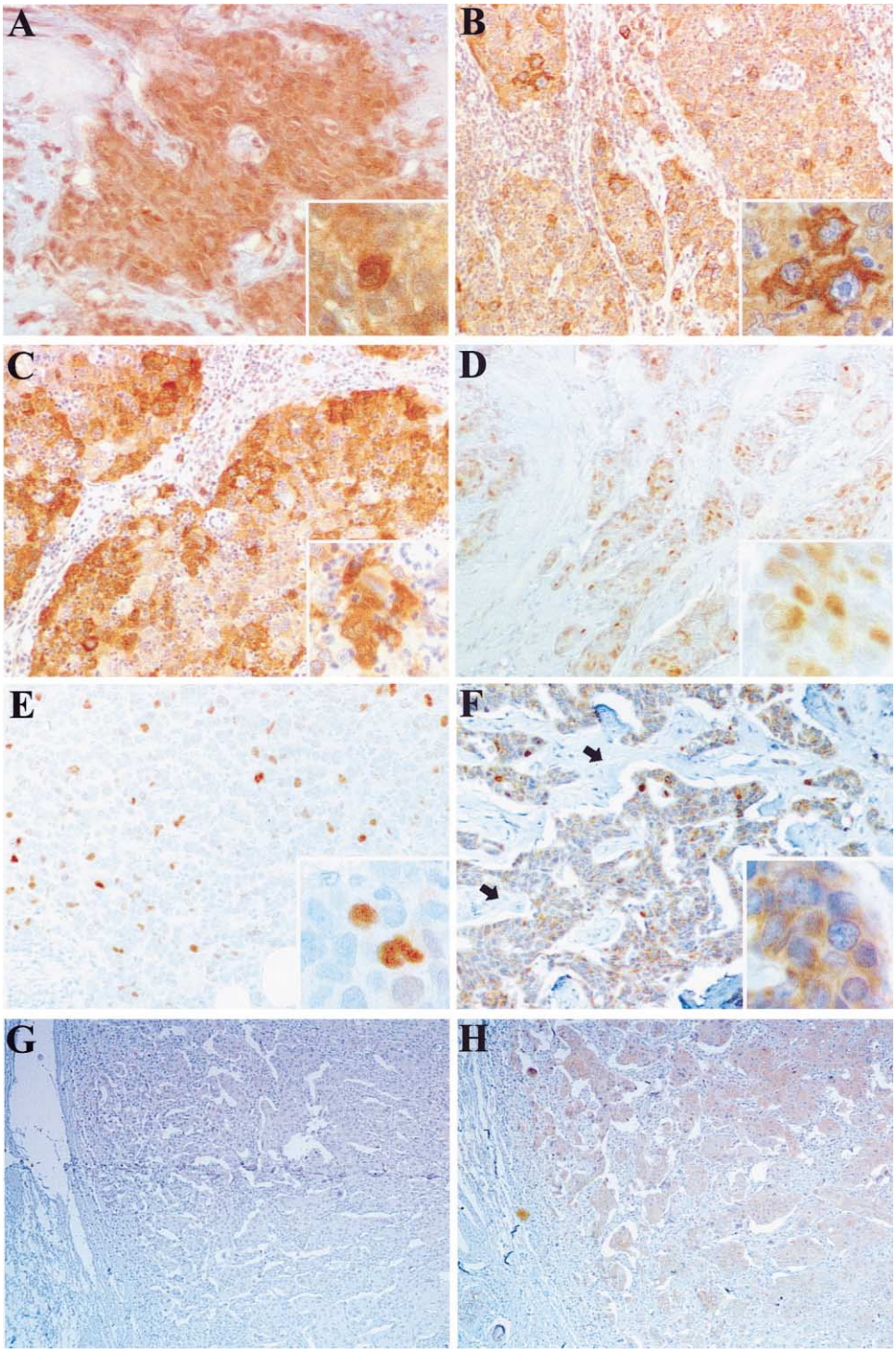

Fig. 1. Examples of cyclin B1 (a and b), Cdc2 (c and d) and p21$^{WAF1/CIP1}$ (e–h) immunoreactivity in infiltrating ductal carcinoma of the breast. Immunostaining was performed using peroxidase conjugates and 3,3 diaminobenzidine as described in Methods, and nuclei were counterstained with haematoxylin. Insets in panels a–f show higher power views of the same fields. (a) A tumour showing both nuclear and cytoplasmic distribution of cyclin B1; (b) a tumour showing prominent cytoplasmic cyclin B1 staining; (c) nuclear and cytoplasmic Cdc2 expression; (d) prominent nuclear Cdc2 expression; (e) prominent nuclear p21$^{WAF1/CIP1}$ expression; (f) p21$^{WAF1/CIP1}$-reactivity in both the nucleus and cytoplasm. Arrows indicate the lack of reactivity in stromal cells within the same section; (g) and (h) parallel sections of a single tumour illustrating the lack of p21$^{WAF1/CIP1}$ immunostaining obtained with the citrate pH 6.0 antigen retrieval protocol (g) in comparison with that obtained with the Tris/ethylene diamine tetra acetic acid (EDTA) pH 9.0 method (h).

Table 2
Intensity distribution scores (IDS) of cyclin B1, Cdc2 and p21$^{WAF1/CIP1}$

	p21$^{WAF1/CIP1}$	Cdc2	Cyclin B1
Nuclear IDS			
Median (range)	6.95 (0, 88.5)	118.5 (22.5, 182)	123 (19.5, 205.5)
Cytoplasmic IDS			
Median (range)	58.5 (0, 154.50)	154.50 (103.50, 213.50)	177.5 (122, 239)
P value	<0.0001	<0.0001	<0.0001
Nuclear count ⩾ Cytoplasmic count (number of patients)	(14/66)	(1/69)	(4/72)

range 0–180; $P=0.03$). There was no significant relationship between cyclin B1 or Cdc2 expression and p53 reactivity. p21$^{WAF1/CIP1}$ in the cytoplasm was significantly associated with cytoplasmic cyclin B1 ($P=0.006$), but not with cytoplasmic Cdc2.

3.4. Relationship of clinicopathological factors to prognosis and the predictive potential of p53, p21$^{WAF1/CIP1}$, cyclin B1 and Cdc2 expression

Disease relapses were seen in 26 patients (18 of the 49 node-negative (N0) patients and 8 of the 24 node-positive (N1/2) patients); 20 women died from breast carcinoma. Five year relapse-free survival (RFS) and overall survival (OS) were 63 and 75%, respectively. As expected, poor prognostic factors such as the presence of lymph node metastases, high tumour grade, large tumour size, negative ER status and high p53-expressing (2+/3+) tumours, were all associated with a lower percentage of patients remaining disease-free (RFS) and alive (OS) at 5 years. The log-rank comparison of the RFS and OS survival curves for each of these clinicopathological factors did not reach statistical significance, presumably because of the relatively small study size (data not shown). Univariate and multivariate analyses using the continuous IDS variables were used to investigate possible relationships between patient survival data and the subcellular distribution of p21$^{WAF1/CIP1}$, Cdc2 and cyclin B1 (Table 3). Univariate analysis showed that cytoplasmic p21$^{WAF1/CIP1}$ was significantly associated with a decreased OS ($P=0.02$) and RFS ($P=0.05$). Cyclin B1 in the nucleus was predictive for reduced OS ($P=0.05$) and RFS ($P=0.02$), with cytoplasmic cyclin B1 demonstrating a similar predictive significance for reduced OS ($P=0.02$) and RFS ($P=0.001$).

The multivariate analysis was adjusted for lymph node involvement (no/yes), tumour grade (grade I and II versus grade III versus non-ductal), and size (⩽2 cm/>2 cm), ER receptor status (⩽10 fmol/mg/>10 fmol/mg), age and treatment groups and p53 expression (0 versus 1+ versus 2+/3+). Cytoplasmic p21$^{WAF1/CIP1}$ expression was an independent predictor for reduced OS ($P=0.002$) and RFS ($P=0.01$), while nuclear p21$^{WAF1/CIP1}$ was not predictive for prognosis. Nuclear cyclin B1 was significantly predictive for OS ($P=0.02$) and RFS ($P=0.005$) and similarly, cytoplasmic cyclin B1 predicted OS ($P=0.005$) and RFS ($P<0.001$). Cdc2 expression was not an independent prognosticator for OS or RFS on uni- or multivariate analyses.

The results obtained using the continuous IDS system and summarised above were compared with log-rank analyses in which cytoplasmic or nuclear staining was classed as high (⩾median) or low (<median); examples for RFS in relation to cyclin B1 or cytoplasmic

Table 3
Relationship between p21$^{WAF1/CIP1}$, cyclin B1 and Cdc2 localisation and overall survival/relapse-free survival[a]

Intensity distribution score (IDS)	Univariate *P*	HR per 10 unit IDS	95% CI	Multivariate *P*
	Overall survival			
p21 nucleus	0.90	0.95	0.71–1.26	0.73
p21 cytoplasm	**0.02**	**1.38**	**1.13–1.68**	**0.002**
Cyclin B1 nucleus	**0.05**	**1.22**	**1.03–1.43**	**0.02**
Cyclin B1 cytoplasm	**0.02**	**1.36**	**1.10–1.69**	**0.005**
Cdc2 nucleus	0.99	0.87	0.72–1.05	0.15
Cdc2 cytoplasm	0.65	0.89	0.72–1.10	0.27
	Relapse-free survival			
p21 nucleus	0.81	0.97	0.75–1.25	0.81
p21 cytoplasm	**0.05**	**1.22**	**1.05–1.41**	**0.01**
Cyclin B1 nucleus	**0.02**	**1.23**	**1.06–1.42**	**0.005**
Cyclin B1 cytoplasm	**0.001**	**1.57**	**1.26–1.95**	**<0.001**
Cdc2 nucleus	0.80	0.96	0.81–1.13	0.61
Cdc2 cytoplasm	0.88	0.95	0.57–0.78	0.57

[a] *P* values are given for the results of both the univariate and multivariate analyses. For multivariate analysis, the hazard ratio (HR) for each 10-unit change in IDS is given, along with the 95% confidence interval (CI). Relationships that reached significance ($P\leqslant0.05$) are highlighted in bold.

p21$^{WAF1/CIP1}$ are shown in Fig. 2. Using this approach, the only significant association detected was that between nuclear cyclin B1 and RFS ($P = 0.007$).

4. Discussion

Cell culture studies have shown that altered p53 function can result in altered regulation of the cyclin B1/Cdc2 complex [3,4], and both components of this complex have been found to be overexpressed in human cancers [6–12]. As suggested by a previous study [9], Cdc2 expression appeared to be a useful indicator of the proliferation rate, with high levels of Cdc2 reactivity in high-grade (III) tumours. The relationship between cytoplasmic Cdc2 expression and lack of tumour differentiation would suggest that this subcellular distribution might be more sensitive than nuclear Cdc2 as a predictor of tumour proliferation. Another study has shown a significant difference between cyclin B1/Cdc2 expression in benign/pre-malignant lesions of the breast compared with invasive carcinomas [6]. Assessment of the percentage of positively stained cells, together with a continuous system of scoring staining intensity in the nucleus and cytoplasm as used here, has not previously been applied to cyclin B1 and Cdc2 immunoreactivity in breast cancers, however. Our findings suggest that this methodology may be more sensitive than more conventional log-rank analyses in which immunohistochemical data are arbitrarily split based on the median into 'high' and 'low' groups (compare Table 3 and Fig. 2).

In this study, cyclin B1 and Cdc2 showed increased nuclear and, more strikingly, cytoplasmic expression in a series of breast cancers relative to stromal cells. Cyclin B1 and cytoplasmic p21$^{WAF1/CIP1}$ were significant prognostic markers, in contrast to nuclear p21$^{WAF1/CIP1}$, p53 and Cdc2 expression. This series of breast cancers represents mixed populations of cells with varying cell cycle distributions, which may contribute to variations in the cytoplasmic or nuclear distribution of cyclin B1 and Cdc2.

Tumour p21$^{WAF1/CIP1}$ expression was high in the cytoplasm and was associated with high levels of p53 staining, suggesting that cytoplasmic p21$^{WAF1/CIP1}$, presumably expressed independently of p53, may be a marker for abnormal p53 function. However, p53 immunostaining is not necessarily tightly correlated with *TP53* gene function. Previous studies of breast carcinoma have reported the exclusive nuclear localisation of p21$^{WAF1/CIP1}$ [13,14,26]. In our study, cytoplasmic p21$^{WAF1/CIP1}$ staining was much more commonly seen. This difference reflects the method of microwave antigen retrieval (Fig. 1g and h), performed here using an alkaline buffer (Tris/EDTA pH 9.0), rather than the acidic citrate buffer (pH 6.0) used by others [14]. In many cases, tumour sections (as well as the adjacent stromal tissue in all cases) stained using the alkaline buffer microwave protocol were negative for cytoplasmic p21$^{WAF1/CIP1}$ (Fig. 1e), strongly suggesting that the cytoplasmic positivity seen in other sections (Fig. 1f and h) is genuine. In line with our data, nuclear and cytoplasmic p21$^{WAF1/CIP1}$ were recently demonstrated in

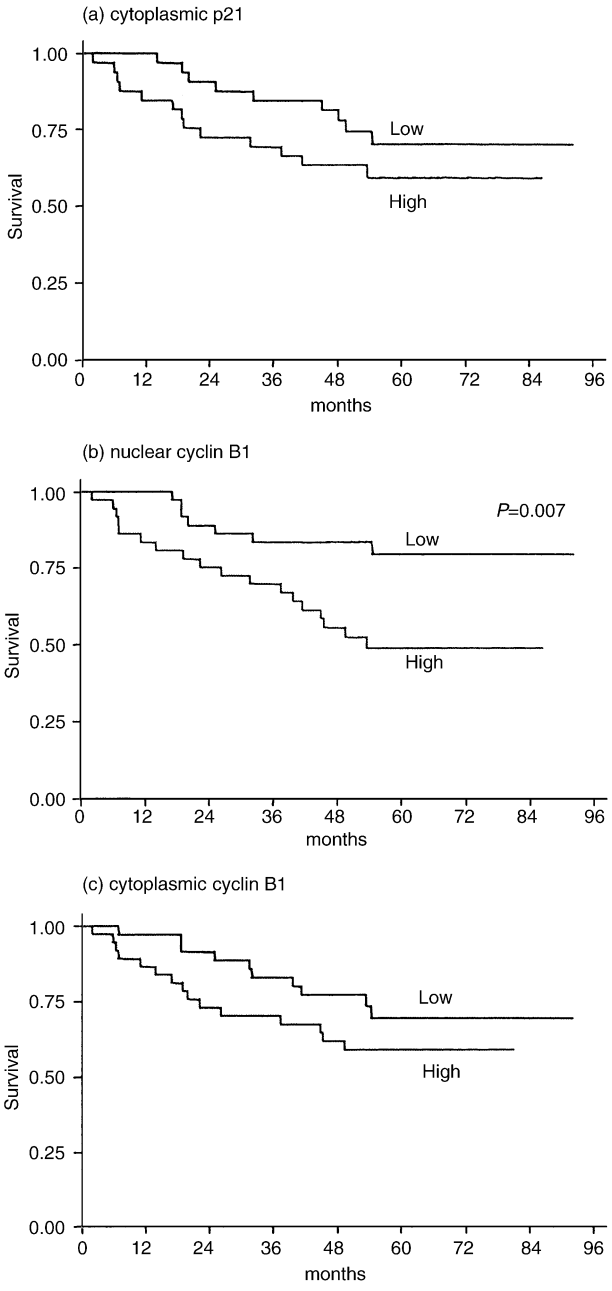

Fig. 2. Relationship between p21$^{WAF1/CIP1}$ or cyclin B1 localisation and relapse-free survival (RFS). RFS curves according to cytoplasmic p21$^{WAF1/CIP1}$ (a) and cyclin B1 in the nucleus (b) and cytoplasm (c). The classification of 'high' and 'low' p21$^{WAF1/CIP1}$ and cyclin B1 expression was determined using the median IDS as an arbitrary 'cut-off' point. 'Low' and 'high' cytoplasmic p21$^{WAF1/CIP1}$ expression was determined by an IDS of 58.5 (<58.5; $\geqslant 58.5$); nuclear cyclin B1 by an IDS of 123 (<123; $\geqslant 123$) and cytoplasmic cyclin B1 by an IDS of 177.5 (<177.5; $\geqslant 177.5$). The P value is given for the one log-rank comparison that reached significance (b).

primary breast cancers [17] and in ovarian cancer cell lines and tumour biopsies [27]. The latter study used a different antibody from that used here, and the apparent cytoplasmic localisation was confirmed independently by cell fractionation. Unfortunately, cell fractionation of the tumours used in this study was not possible, since only formalin fixed blocks were available.

Cytoplasmic $p21^{WAF1/CIP1}$ may be more sensitive than nuclear $p21^{WAF1/CIP1}$ staining as a marker of tumour aggression and p53 function, as suggested by its association with high p53 expression and its ability to predict prognosis (Table 3). Cytoplasmic localisation could reflect binding of $p21^{WAF1/CIP1}$ to the cytoplasmic cyclin/Cdk complexes [28] or $p21^{WAF1/CIP1}$ phosphorylation by the protein kinase Akt, as a result of HER-2/neu overexpression [17]. Cytoplasmic $p21^{WAF1/CIP1}$ may inhibit apoptotic death by binding and inhibiting the apoptosis signal-regulating kinase 1 (ASK1) [16]. Such an anti-apoptotic function could underlie the association between cytoplasmic $p21^{WAF1/CIP1}$ and poor prognosis found in this study. It will be interesting to determine whether this cytoplasmic $p21^{WAF1/CIP1}$ is also associated with HER-2/neu overexpression.

Cytoplasmic $p21^{WAF1/CIP1}$ may in addition be unable to target cyclin B1/Cdc2 to the nucleus [29], potentially explaining the significant co-localisation of $p21^{WAF1/CIP1}$ and cyclin B1 to the cytoplasm in the breast cancer series studied here. It is intriguing that there was no correlation between cytoplasmic $p21^{WAF1/CIP1}$ and cytoplasmic Cdc2. Cdc2 is probably present in molar excess over cyclin B1 in these tumour cells, however, and in this case the localisation of most Cdc2 molecules is likely to be independent of cyclin B1.

In conclusion, these findings, if confirmed in a more extensive survey, indicate that the combined evaluation of p53, cytoplasmic $p21^{WAF1/CIP1}$ and cyclin B1 expression in breast cancer could provide valuable prognostic information to supplement that obtained using more conventional parameters. Increasingly precise molecular diagnosis of this sort is ultimately expected to contribute to improved therapeutic outcome.

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