ORIGINAL ARTICLE

p21^{WAF1} expression in invasive breast cancer and its association with p53, AP-2, cell proliferation, and prognosis

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Aims: To evaluate the expression and prognostic relevance of p21^{WAF1} in breast cancer and to investigate its association with p53, activator protein 2 (AP-2), and cell proliferation (as assessed by Ki-67 expression).

Methods: p21^{WAF1} expression was analysed immunohistochemically in a large prospective, consecutive series of 420 patients with breast cancer diagnosed and treated between 1990 and 1995 at Kuopio University Hospital, Kuopio, Finland. Inter-relations between p21^{WAF1} expression and p53, AP-2, and Ki-67 were evaluated. The expression of p21^{WAF1} was also compared with clinicopathological parameters and the patients' survival.

Results: In general, nuclear p21^{WAF1} expression was low in carcinomas (median, 2.5%; range, 0–70%). Expression was lowest in lobular carcinomas ($\chi^2 = 7.4$; $p = 0.025$). p21^{WAF1} positive tumours were more often p53 positive ($\chi^2 = 4.2$; $p = 0.041$) but expression of p21^{WAF1} did not correlate with AP-2 expression or Ki-67 in the whole patient group. In addition, the combined expression of p21 and p53 was not associated with AP-2 expression. High nuclear p21^{WAF1} positivity ($n = 160$; 38%) was associated with poor differentiation ($\chi^2 = 8.1$; $p = 0.017$). In the univariate analyses, p21^{WAF1} expression had no prognostic value for predicting breast cancer related survival (BCRS) or recurrence free survival (RFS) in the whole patient group or in the subgroups investigated. However, in postmenopausal patients with lymph node metastases, and oestrogen receptor (ER) and/or progesterone receptor (PR) positive tumours, high p21^{WAF1} expression predicted response to adjuvant hormonal treatment with antioestrogens. In the univariate analysis, the significant factors for predicting BCRS were Ki-67 expression, stage, lymph node status, histological grade, ER and PR status, and those for RFS were Ki-67 expression, stage, and lymph node status. In the multivariate analysis, the independent predictors of shorter BCRS were high cell proliferation activity measured by Ki-67 expression ($p < 0.001$), advanced stage ($p < 0.001$), and poor differentiation ($p = 0.048$). Shorter RFS was independently predicted by high cell proliferative activity ($p < 0.001$) and advanced stage ($p < 0.001$).

Conclusions: The regulation of p21^{WAF1} seems to occur independently of p53 or AP-2 and analysing p21^{WAF1} expression provided no prognostic information for patients with breast cancer.

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The p21^{WAF1} molecule, also known as CIP1¹ or SDI1,² was identified as a mediator of p53 dependent growth suppression.³ In response to DNA damaging agents, wild-type p53 induces the expression of p21^{WAF1}, which blocks the progression of the cell cycle at the G1/S transition by inhibiting cyclin dependent kinases (CDKs).^{1–4} In addition, several other factors can induce p21^{WAF1} expression and cell cycle arrest independent of p53.^{5–7} The expression of p21^{WAF1} is induced during terminal differentiation both in vivo and in vitro,^{6–9} and has also been linked with cell senescence (reviewed in Roninson¹⁰). Recently, it has been shown that p21^{WAF1} may also mediate G2 arrest.^{11–12}

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To date, a consensus has not been reached concerning the prognostic value of p21^{WAF1} in breast cancer.^{13–17} Similarly, the regulatory and functional mechanisms of p21^{WAF1} in breast cancer are still unclear.^{14–16–18} Recently, the in vivo association of p21^{WAF1} and activator protein 2 (AP-2) has been described in breast cancer, in addition to melanoma and colorectal

carcinoma.^{19–21} The AP-2 transcription factor is a new potential tumour suppressor, the reduced expression of which has been correlated with increased malignancy in some cancers.^{19–20–22–23} Members of the AP-2 protein family (AP-2 α , AP-2 β , and AP-2 γ) regulate gene expression during development, cell growth, and differentiation (reviewed in Hilger-Eversheim and colleagues²⁴). Several molecules, such as HER2,²⁵ the oestrogen receptor (ER),²⁶ and matrix metalloproteinase 2,²⁷ are transcriptionally regulated by AP-2. Indeed, because AP-2 has been shown to induce p21^{WAF1} expression in human cancer cells,²⁸ we hypothesised that the expression of p21^{WAF1} in breast cancer might be partly mediated by AP-2.

As far as we are aware, no previous study has compared the relations between p21^{WAF1}, p53, AP-2, and Ki-67 in breast cancer. Therefore, we analysed the expression of p21^{WAF1}, p53, AP-2, and Ki-67 by immunohistochemistry (IHC) in a large

Abbreviations: ABC, avidin–biotin–peroxidase complex; AP-2, activator protein 2; BCRS, breast cancer related survival; CDK, cyclin dependent kinase; ER, oestrogen receptor; IHC, immunohistochemistry; PBS, phosphate buffered saline; pN+, node positive; pN–, node negative; PR, progesterone receptor; pRb, retinoblastoma protein; RFS, recurrence free survival

prospective series of 420 patients with breast cancer with long term follow up data (median, 57.2 months; range, 1.2–115.1). Inter-relations between p21^{WAF1} and other IHC variables, clinicopathological data, and the patients' survival were investigated.

METHODS

Patients

Our prospective study consisted of 520 patients with breast cancer from the Kuopio breast cancer project.^{29–32} All women with a suspicious breast lump in the project's catchment area between April 1990 and December 1995 were invited to Kuopio University Hospital for further examinations and final diagnosis. Women willing to participate in the project were interviewed and examined by a trained nurse before diagnostic procedures were carried out. In total, the project comprised 479 invasive and 41 non-invasive carcinomas. After surgical treatment the patients were offered adjuvant chemotherapy and/or hormonotherapy and radiotherapy, depending on the mode of surgery, the patient's menopausal status, and the stage of the disease, according to national guidelines.³³ In brief, postoperative radiotherapy was given to all patients treated with breast conserving surgery and to all patients with axillary node positive status (pN+), irrespective of the mode of surgery. All premenopausal patients with pN+ and some with axillary node negative status (pN–) presenting with other adverse prognostic factors (ER/progesterone receptor (PR) negative or poorly differentiated tumour) were given adjuvant chemotherapy (CMF) for six cycles. All postmenopausal women with ER and/or PR positive tumours were adjuvantly treated with either tamoxifen or toremifene for three years within another study protocol. Thus, within a stage the postoperative treatment was relatively uniform, with only a few exceptions resulting from—for example, concurrent conditions. The stage was assessed by means of the UICC classification.³⁴ Patients with non-invasive carcinomas, an earlier history of breast cancer, metastatic disease, or insufficient tumour material were excluded from our study. Thus, 420 patients with sufficient primary tumour and complete clinical histories were available for p21^{WAF1} analysis, 418 for p53 analysis, 415 for AP-2 analysis, and 414 for Ki-67 analysis. The mean age of the 420 patients was 59.0 years and the median 56.5 (range, 23.3–91.6). The mean follow up time was 54.9 months and the median 57.2 (range, 1.2–115.1). The mean and median follow up times for patients with p53, AP-2, or Ki-67 expression were 55.0 and 57.3 months, 54.9 and 57.2 months, and 54.8 and 57.2 months (range, 1.2–115.1 for all), respectively. During the first five years of follow up, 76 of the 420 patients (18%) had a recurrence, 50 patients (12%) died of breast cancer and 36 patients (9%) died of other causes. The overall five year survival rate was 77%. The five year recurrence free survival rate of the patients was 79% and the breast cancer related survival was 85%. The five year survival of excluded stage IV patients (n = 17) was 24%. Table 1 summarises the clinicopathological data of the 420 patients.

Histology

The tumour samples were fixed in 10% buffered formalin and embedded in paraffin wax. The histological diagnosis was confirmed by reviewing one to four original sections of the primary tumour. All tumours were simultaneously re-evaluated for histological type and grade by two senior pathologists, who were unaware of the clinical data. The most representative blocks were selected to be cut into new 5 µm thick sections for immunohistochemical analysis.

Immunohistochemical staining for p21^{WAF1}

The sections were dewaxed in xylene, rehydrated in ethanol, and washed twice with distilled water. For better antigen retrieval the samples were boiled in a microwave oven for

Table 1 The clinicopathological data of the patients

Characteristic	n	%
Number of patients	420	100
Mean age in years (range)	59.0 (23.3–91.6)	
Tumour size		
T1	221	53
T2	166	39
T3	23	6
T4	10	2
Lymph node status		
Negative	247	59
Positive	166	39
Unknown	7	2
Stage		
I	163	39
II	212	50
III	38	9
Unknown	7	2
Histological type		
Ductal	273	65
Lobular	65	15
Other	82	20
Histological grade		
I	108	26
II	192	46
III	120	28
ER status		
Positive	323	77
Negative	95	22
Data not available	2	1
PR status		
Positive	261	62
Negative	157	37
Data not available	2	1
Menopausal status		
Premenopausal	132	31
Postmenopausal	288	69
Recurrence at 5 years		
No	344	82
Yes	76	18
Cause of death at 5 years		
Alive	334	79
Breast cancer	50	12
Other	36	9

ER, oestrogen receptor; PR, progesterone receptor.

5 × 5 minutes in citrate buffer (pH 6.0). Endogenous peroxidases were blocked by 5% hydrogen peroxidase treatment for five minutes. The samples were washed with phosphate buffered saline (PBS; pH 7.2) and incubated in 1.5% normal horse serum for 25 minutes to prevent non-specific antigen binding. The samples were incubated with a mouse monoclonal antibody specific for human p21^{WAF1} (NCL-WAF-1; clone 4D10; Novocastra Laboratories, Newcastle upon Tyne, UK), at a working dilution of 1/20, overnight at 4°C. Before applying the secondary antibody the samples were washed twice with PBS. The slides were incubated for 35 minutes with the biotinylated secondary antibody, followed by washing and a 45 minute incubation in an avidin-biotin-peroxidase complex (ABC) reagent (Vectastain rabbit ABC Elite kit; Vector Laboratories, Burlingame, California, USA). p21^{WAF1} expression was visualised by treatment with diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, Missouri, USA) for five minutes. The slides were counterstained with Mayer's haematoxylin, dehydrated, and mounted with DePex (BDH, Poole, Dorset, UK). Each staining series had positive (p21^{WAF1} positive colorectal carcinoma) and negative (breast cancer without primary antibody) control slides, which stained as was expected.

Immunohistochemical staining for p53, AP-2, and Ki-67

The expression of p53, AP-2, and Ki-67 was detected following the same procedure as that used for p21^{WAF1}, with a few modifications. The primary antibody for p53 detection was a mouse

Table 2 Relations between nuclear p21^{WAF1} expression and p53, AP-2, cell proliferation (Ki-67), and clinicopathological variables

Variable	Sample n	p21 ^{WAF1} expression		χ^2	df	p Value
		Low <5%	High ≥5%			
Stage				0.3	2	0.844
I	163	62	38			
II	212	61	39			
III	38	66	34			
Lymph node status				3.1	1	0.079
Negative	247	58	42			
Positive	166	67	33			
Histological type				7.4	2	0.025
Ductal	273	59	41			
Lobular	65	77	23			
Other	82	59	41			
Histological grade				8.1	2	0.017
I	108	73	27			
II	192	57	43			
III	120	60	40			
ER status				3.1	1	0.077
Positive	323	59	41			
Negative	95	70	30			
PR status				1.6	1	0.205
Positive	261	59	41			
Negative	157	66	34			
Recurrence at 5 years				0.6	1	0.441
No	344	61	39			
Yes	76	66	34			
Nuclear p53				4.2	1	0.041
Low <10%	225	66	34			
High ≥10%	193	57	43			
Nuclear AP-2				<0.1	1	0.785
Low <80%	211	62	38			
High ≥80%	204	61	39			
Cell proliferation				2.5	1	0.113
Low <20%	230	65	35			
High ≥20%	184	58	42			

For p21^{WAF1} expression the median value was used as the cut off value.

AP-2, activator protein 2; df, degrees of freedom; ER, oestrogen receptor; PR, progesterone receptor.

monoclonal antibody directed against human p53 (DO-7; Dako, Glostrup, Denmark), used at a dilution of 1/1000. Antigen retrieval was achieved by boiling in a microwave oven for 3 × 5 minutes. A larynx carcinoma specimen was used as a positive control.

AP-2 expression was visualised using a rabbit polyclonal antibody for human AP-2α (C-18; specific for AP-2α, AP-2β, and AP-2γ; Santa Cruz Biotechnology, Santa Cruz, California, USA) at a dilution of 1/2000. In addition, horse serum was replaced by goat serum and the microwave oven treatment lasted for 3 × 5 minutes. An AP-2-positive melanoma sample served as a positive control.

Ki-67 staining was performed using the Sequenza™ immunostaining centre (Shandon Scientific Limited, Astmoor, UK). Consequently, the incubation periods were longer: 35 minutes for normal serum, 40 minutes for secondary antibody, and 50 minutes for the ABC reagent. The primary antibody in Ki-67 staining (MIB-1; Immunotech, Marseille, France) was used at a working dilution of 1/600. The positive control for Ki-67 was a lung carcinoma.

Scoring of immunoreactivity

The specimens were analysed by three observers (p21^{WAF1} by TP, KR, and V-MK; p53 by JP, TP, and KR; and AP-2 and Ki-67 by JP, KR, and V-MK), who were unaware of the patients' clinical outcome. Discrepancies between the observers were found in less than 10% of the slides examined, and consensus was reached on a further review. All tumour cells with detectable nuclear staining were considered to be positive. For statistical analysis, the samples were divided according to per cent distribution of stained tumour cell nuclei into low and high

expression groups using the median value as a cut off point. Other cut off values were also tested, but the median value was chosen because it does not introduce a bias that could be caused by the use of a minimum p value approach.³⁵ The cut off values were as follows: p21^{WAF1}, low < 5% and high ≥ 5%; p53, low < 10 % and high ≥ 10%; AP-2, low < 80% and high ≥ 80%; and Ki-67, low < 20% and high ≥ 20%.

Statistical analyses

The statistical analyses were carried out using the SPSS for Windows 9.0 programme (SPSS Inc, Chicago, Illinois, USA). First, the relations between the degree of p21^{WAF1}, p53, AP-2, and Ki-67 expression were calculated using a Spearman correlation test, in which the variables were considered as continuous. The inter-relations between categorised IHC variables and their associations with clinicopathological parameters were tested with contingency tables and a χ^2 test. The univariate survival analyses were performed using the Kaplan Meier's log rank analysis and the independent prognostic value of variables was further examined with Cox's regression analysis. Probability values ≤ 0.05 were considered to be significant. In the Cox's multivariate analysis, the Enter method was used with an additional removal limit of $p < 0.10$. Both breast cancer related survival (BCRS) and recurrence free survival (RFS) were examined. Patients who died of reasons other than breast cancer were censored at the time of death. The recurrence free time was defined as the time between the diagnosis and the date of the first local recurrence or a distant metastasis, which ever one appeared first. Patients who remained healthy or died without breast cancer during the follow up were censored at the time of the last control examination or death.

Table 3 Immunohistochemical and significant clinicopathological variables of breast cancer related and recurrence free survival in univariate survival analysis

Variable	Surviving breast cancer at 5 years			Recurrence free at 5 years		
	n	%	p Value	n	%	p Value
Stage			<0.001			<0.001
I	163	92		163	87	
II	212	86		212	77	
III	38	54		38	54	
Lymph node status			<0.001			<0.001
Negative	247	92		247	86	
Positive	166	77		166	68	
Histological grade			0.043			0.071
I	108	92		108	85	
II	192	85		192	78	
III	120	81		120	74	
ER status			<0.001			0.124
Positive	323	89		323	80	
Negative	95	72		95	74	
PR status			0.001			0.183
Positive	261	89		261	80	
Negative	157	78		157	76	
Nuclear p21 ^{WAF1}			0.398			0.388
Low <5%	260	84		260	78	
High ≥5%	160	87		160	80	
Nuclear p53			0.837			0.678
Low <10%	225	86		225	79	
High ≥10%	193	84		193	78	
Combined p21/p53			0.461			0.782
p21+/p53+	84	84		84	80	
p21+/p53-	76	91		76	79	
p21-/p53+	109	85		109	76	
p21-/p53-	149	83		149	79	
Cell proliferation			<0.001			<0.001
Low <20%	230	93		230	89	
High ≥20%	184	75		184	64	

ER, oestrogen receptor; PR, progesterone receptor.

RESULTS

Expression of p21^{WAF1}

The expression of p21^{WAF1} in carcinomas was predominantly nuclear but faint cytoplasmic staining was quite often present. The adjacent benign tissue was mainly negative, although some scattered positive cells were constantly present, as has been described previously.¹³ In general, the expression of p21^{WAF1} in carcinomas was quite low (median, 2.5%; range, 0–70%), and 29 % of the samples were negative (< 1% stained nuclei). The fraction of samples considered to be high expressers (≥ 5%) was 38%. Expression was lowest in lobular carcinomas ($\chi^2 = 7.4$; $p = 0.025$; table 2).

Expression of p53, AP-2, and Ki-67

All three proteins were localised in the nuclei of the tumour cells. The expression of p53 was high (≥ 10%) in 46% of the tumours; the expression of AP-2 was high (≥ 80%) in 49% of the tumours, and the proportion of Ki-67 high expressing (≥ 20%) tumours was 44%.

Relation of p21^{WAF1} to p53 and AP-2

p21^{WAF1} positive tumours were more often p53 positive ($\chi^2 = 4.2$; $p = 0.041$; table 2). High p21^{WAF1} expression in p53 positive tumours was not related to AP-2 expression. Indeed, the expression of p21^{WAF1} and AP-2 was not related in the whole patient group (table 2) or in the node positive and negative subgroups either. Furthermore, we investigated whether the presence of ER would affect the ability of AP-2 to activate p21^{WAF1}, but the factors were not related to each other in ER positive or negative cases. In ER positive tumours, high p21^{WAF1} expression was associated with p53 positivity, but p21^{WAF1} expression was not related to AP-2. The expression of p21^{WAF1} was not associated with AP-2 in p53 positive ER negative cases either.

Relation of p21^{WAF1} to cell proliferation and clinicopathological factors

High p21^{WAF1} expression was associated with Ki-67 positivity only in grade I carcinomas ($\chi^2 = 7.0$; $p = 0.008$), in hormone receptor positive disease ($\chi^2 = 10.2$; $p = 0.001$ for ER and $\chi^2 = 4.1$; $p = 0.042$ for PR), and in patients with recurrent disease ($\chi^2 = 4.5$; $p = 0.035$). The association was not seen in the whole patient group (table 2). p21^{WAF1} high expressing tumours were more often poorly differentiated in the whole patient group ($\chi^2 = 8.1$; $p = 0.017$; table 2) and in node positive patients only ($\chi^2 = 6.3$; $p = 0.044$). No other association between p21^{WAF1} expression and clinicopathological parameters was apparent in the whole group (table 2) or in the node positive and negative subgroups.

Relation of combined p21^{WAF1} and p53 expression to AP-2, cell proliferation, and clinicopathological factors

For further analyses we combined the expression of p21^{WAF1} and p53. The proportions of the different groups in our samples were: p21+/p53+ ($n = 84$; 20.0%), p21+/p53- ($n = 76$; 18.2%), p21-/p53+ ($n = 109$; 26.1%), and p21-/p53- ($n = 149$; 35.6%). Combined p21 and p53 expression was not related to AP-2 or cell proliferation in the whole group or in the node negative or node positive subgroups. In all patients and in the node negative subgroup the highest proportion of p21-/p53+ cases was seen in the ER negative ($\chi^2 = 14.5$; $p = 0.002$) and poorly differentiated ($\chi^2 = 18.1$; $p = 0.006$) carcinomas.

Univariate survival analysis

p21^{WAF1}

The expression of p21^{WAF1} had no prognostic significance for survival in the whole group (table 3) or in the subgroups of patients according to lymph node status. Furthermore, the

Table 4 Results of Cox's multivariate analysis for breast cancer related and recurrence free survival

Category (variable)	β (SE)	Relative risk (95% CI)	p Value
<i>Breast cancer related survival</i>			
Stage			<0.001
I	Reference		
II	0.80 (0.41)	2.23 (1.00 to 5.01)	0.051
III	2.03 (0.45)	7.63 (3.18 to 18.30)	<0.001
Cell proliferation			
Low <20%	Reference		
High $\geq$ 20%	1.26 (0.38)	3.53 (1.68 to 7.44)	<0.001
Histological grade			0.048
I	Reference		
II	0.40 (0.47)	1.49 (0.59 to 3.75)	0.397
III	-0.51 (0.55)	0.60 (0.21 to 1.76)	0.355
<i>Recurrence free survival</i>			
Stage			<0.001
I	Reference		
II	0.53 (0.29)	1.70 (0.97 to 2.99)	0.066
III	1.34 (0.35)	3.81 (1.90 to 7.63)	<0.001
Cell proliferation			
Low <20%	Reference		
High $\geq$ 20%	1.26 (0.26)	3.54 (2.11 to 5.95)	<0.001

CI, confidence interval; SE, standard error.

prognostic significance was explored using combined p21 and p53 expression, but this combination also seemed to lack prognostic value because no impact on BCRS or RFS was demonstrated (table 3). In the subset of patients treated with adjuvant hormonotherapy (postmenopausal patients with lymph node and hormone receptor positive tumours), high p21^{WAF1} expression predicted longer BCRS ($p = 0.0179$) and RFS ($p = 0.0433$). The effect of combined p21 and p53 expression did not reach significance.

p53, Ki-67, and clinicopathological data

p53 expression had no prognostic value in both the whole group (table 3) or in the node positive and negative subgroups. High proliferation activity (as assessed by Ki-67 expression) was a strong predictor of shorter BCRS ($p < 0.001$) and RFS ($p < 0.001$) in both the whole group (table 3) and the node negative and positive patients. The other prognostic factors of BCRS and RFS in the whole study group were stage ($p < 0.001$ for both; table 3) and lymph node status ($p < 0.001$ for both; table 3). In addition, histological grade ($p = 0.043$), ER status ($p < 0.001$), and PR status ($p = 0.001$) predicted BCRS (table 3).

Multivariate survival analysis

There were 398 patients with complete sets of data available for BCRS and 404 for RFS analyses. Cox's regression model included all significant variables derived from the univariate survival analyses (stage, histological grade, ER and PR status, and Ki-67 expression in BCRS analyses, and stage and Ki-67 expression in RFS analyses). The independent predictors of shorter BCRS (table 4) were advanced stage ($p < 0.001$), poor differentiation ($p = 0.048$), and high proliferation activity ($p < 0.001$). Shorter RFS (table 4) was independently predicted by advanced stage ($p < 0.001$) and high proliferation activity ($p < 0.001$).

In the node negative patients, the only independent predictor of shorter BCRS ($n = 221$) and RFS ($n = 240$) was high cell proliferation rate ($p = 0.022$ for BCRS and $p < 0.001$ for RFS). In the node positive patients ($n = 162$ for BCRS and $n = 163$ for RFS), both advanced stage ($p = 0.001$ for BCRS and $p = 0.039$ for RFS) and high cell proliferation ($p = 0.032$ for BCRS and $p = 0.003$ for RFS) independently predicted poor survival.

DISCUSSION

In our study, we investigated the expression of the cell cycle inhibitor p21^{WAF1} by means of IHC in a large prospective series of patients with breast cancer. The expression of p21^{WAF1} was also examined in relation to clinicopathological parameters, patients' survival, cell proliferation (Ki-67), and the expression of p53 and the transcription factor AP-2. Our results indicate that in breast cancer the regulation of p21^{WAF1} occurs mostly via p53 or AP-2 independent pathways. In addition, p21^{WAF1} does not seem to have prognostic value in breast cancer.

The expression pattern of p21^{WAF1} seen in our study is in line with previous studies concerning the expression of p21^{WAF1} in breast cancer.^{13-18 36-46} In general, the expression of p21^{WAF1} has been low, with a median value of 3–5%,^{13 14 38 41 42} which is very close to that reported here (3%). We found that 38% of the samples expressed p21^{WAF1} highly, a number which falls well within recently reported values (range, 25–91%; mean, 43%^{13-16 18 36-46}). Rarely, cytoplasmic p21^{WAF1} expression has also been reported.^{13 15 17 42} Cytoplasmic staining was so faint in our series that it was hard to discern from background staining. For this reason, we only estimated the nuclear expression of p21^{WAF1}.

The reports concerning the regulation of p21^{WAF1} by p53 have been contradictory and the experiments performed on clinical tumour samples have failed to reach a consensus in determining the role of p53 in p21^{WAF1} regulation in breast cancer.^{14 18 36 37 46} In our study, we found a positive association between the expression of nuclear p21^{WAF1} and p53, which is in agreement with the studies of Bankfalvi and colleagues¹⁸ and Diab *et al.*³⁷ Cytoplasmic p21^{WAF1} positivity has also been associated with high p53 expression.¹⁷ Even though the DO-7 antibody used in the present study recognises both the wild-type and mutated p53 proteins, the accumulation of p53 in tumours has often been a good predictor of the presence of p53 mutation.^{47 48} Therefore, our results favour the theory of p53 independent pathways of p21^{WAF1} regulation in breast cancer.^{15 44} Accordingly, p53 may be responsible for the induction of p21^{WAF1} expression in some conditions but the overall regulation of p21^{WAF1} seems to be far more complex.

One potential regulator of p21^{WAF1} is AP-2, a transcription factor involved in cell growth and differentiation.²⁴ AP-2 can induce p21^{WAF1} expression *in vitro*,²⁸ and has been positively associated with p21^{WAF1} expression *in vivo*.¹⁹⁻²¹ In our study, we

found no association between the expression of AP-2 and p21^{WAF1}. The value of AP-2 as an inducer of the expression of p21^{WAF1} in p53 positive cases was also investigated, but the factors were not related to one another. Accordingly, AP-2 may not play such a major role in p21^{WAF1} regulation as was previously assumed, even though it may be partly responsible for the expression of p21^{WAF1}. Indeed, in epithelial ovarian cancer these proteins were not related to one another.⁴⁹ However, it should be noted that the antibody used in our study recognises all three AP-2 proteins, thus reducing the value of a possible association between certain AP-2 isoforms and p21^{WAF1}. Therefore, the role of different AP-2 proteins in p21^{WAF1} regulation needs to be investigated.

"p53 may be responsible for the induction of p21^{WAF1} expression in some conditions but the overall regulation of p21^{WAF1} seems to be far more complex"

We found that high p21^{WAF1} expression was related to high proliferative activity in well differentiated and in hormone receptor positive carcinomas. Increased cell proliferation in p21^{WAF1} positive cases has been described previously,^{15 41 44 45} although conflicting results also exist.^{13 14 38 42} High amounts of p21^{WAF1} in highly proliferating cells may reflect an unsuccessful effort to halt proliferation. This may result from the presence of other cell cycle regulatory pathways, which bypass the p21^{WAF1} mediated cell cycle block, such as c-Myc or B-myb.^{50 51} In addition, overexpression of CDK2 and cyclin A has been reported to abrogate the inhibitory effect of high p21^{WAF1} expression.^{52 53} Mutation of the retinoblastoma protein (pRb) may also result in increased p21^{WAF1} expression via deregulation of the E2F-1 transcription factor,^{54 55} the effect of which, however, may be overridden by the loss of pRb.⁵⁶ In contrast, the expression of p21^{WAF1} is induced by factors whose expression is known to be associated with disease progression in breast cancer, such as transforming growth factor β 1 and the epidermal growth factor receptor.^{57–60} Recently, p21^{WAF1} has been proposed to mediate the activation of the oestrogen signalling pathway in ER negative tumour cells.⁶¹ Thomas *et al* suggested a p21^{WAF1} mediated pathway as a possible mechanism for the growth inhibitory effects of oestradiol.⁶² Because we found that high p21^{WAF1} expression is associated with high proliferative activity in ER positive cancer, it is possible that high p21^{WAF1} expression is partly related to oestrogens.

The prognostic role of p21^{WAF1} in breast cancer is unclear because of the contradictory results from previous studies.^{13–18 37–39 41–43 45 46 63} Some authors have associated high p21^{WAF1} expression with poor differentiation^{14 44} and decreased survival,^{13 14 45} whereas others have found it to be associated with increased differentiation^{16 38 46} and better disease outcome.^{16 42 46} Yet other investigators have found p21^{WAF1} expression to be non-significant in breast cancer.^{15 18} The different patient samples, antibodies, analysing methods, and cut off values used in these p21^{WAF1} studies probably explain the discrepancies between studies and make comparisons of findings difficult. In our study, high expression of p21^{WAF1} was associated with reduced differentiation. In addition, high p21^{WAF1} expression was significantly associated with longer BCRC and RFS in postmenopausal patients with receptor and node positive tumours who were on antioestrogen treatment. Previously, combined p21 and p53 expression has been associated with response to adjuvant treatment.^{14 38 41} However, the low number of patients and events in each p21/p53 subgroup limits the analysis in our study. We also found that p21^{WAF1} expression lacks prognostic value in the whole patient group or in the subgroups investigated. Recently, Göhring *et al*,¹⁵ using the same p21^{WAF1} specific antibody (clone 4D10), found no prognostic significance of p21^{WAF1} expression in a large series of breast cancer cases (n = 307). Other studies have also confirmed this result.^{18 41} Combining the expression of p21 and

Take home messages

- p53 and AP-2 do not appear to be involved in the regulation of p21^{WAF1} in tumour tissue from patients with breast cancer
- The analysis of p21^{WAF1} expression provides no prognostic information for patients with breast cancer

p53 has given additional information on disease outcome.^{14 16 41} However, this was not the case in our study. Although p21^{WAF1} expression has predicted disease outcome in many malignancies,^{64–69} it seems to have no prognostic significance in breast cancer.

In conclusion, the expression of the cell cycle inhibitor p21^{WAF1} does not seem to provide additional information on the clinical outcome in breast cancer. This may stem from the extremely complex regulatory network of p21^{WAF1} in breast cancer, which reduces its prognostic value.

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