p21^{waf} correlates with DNA replication but not with prognosis in invasive breast cancer

U-J Göhring, A Bersch, M Becker, W Neuhaus, T Schöndorf

Abstract

***Background/Aims*—p21^{waf} plays a central role both in the regulation of the cell cycle and in DNA replication. Accordingly, p21^{waf} is a putative tumour suppressor. The role of p21^{waf} expression in breast cancer is still unclear, particularly with respect to the clinical situation. Therefore, this retrospective study was designed to investigate the value of immunohistochemically detected p21^{waf} expression in invasive breast cancer.**

***Methods*—Cellular expression of p21^{waf} was assessed in 307 breast cancer tissues by immunohistochemistry using the monoclonal antibody, clone 4D10. The data were correlated to established and functional factors of prognosis (age, menopausal status, tumour size, nodal status, tumour grade, receptor status, proliferating cell nuclear antigen (PCNA) expression, Her-2/neu expression, and p53 expression), and to clinical follow up (median observation time, 82 months).**

***Results*—Ninety nine of 307 (32.2%) tumour tissues were considered p21^{waf} positive (nuclear staining). In the entire study group, p21^{waf} expression correlated only with increased PCNA expression (χ^2 test: p = 0.029), and with none of the other investigated markers. In node negative patients (n = 134), p21^{waf} expression correlated with increased tumour size and increased PCNA expression, whereas the node positive subgroup (n = 161) showed no correlation with these parameters (lymphonodectomy was done in 295 women). With respect to clinical outcome, p21^{waf} expression showed a definite favourable trend in both subgroups (N0: p21^{waf} negative, 23 of 87; p21^{waf} positive, nine of 43. N+: p21^{waf} negative, 63 of 107; p21^{waf} positive, 23 of 52), but this observation was not significant (p > 0.05). Multivariate analysis for disease free survival as indicated by Cox regression analysis included all factors investigated. The most striking parameters were nodal status (relative risk (RR), 1.74; p = 0.00001), receptor status (RR, 0.59; p = 0.0085), tumour size (RR, 1.42; p = 0.02), and Her2/neu expression (RR, 1.56; p = 0.033). p21^{waf} expression was not significant in the multivariate analysis (p > 0.05).**

***Conclusions*—p21^{waf} expression is an independent factor but fails to be of prognostic or predictive value in multivariate analysis. These data confirm the hypothesis of a p53 independent p21^{waf} induction and suggest a functional role in the inhibition of PCNA mediated DNA replication.**

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Keywords: p21^{waf}; proliferating cell nuclear antigen; p53; breast cancer; immunohistochemistry; prognosis

Department of Gynecology and Obstetrics, University of Cologne, 50924 Cologne, Germany
U-J Göhring
A Bersch
M Becker
W Neuhaus
T Schöndorf

Correspondence to:
Dr Schöndorf
thomas.schoendorf@medizin.uni-koeln.de

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Disorders of cell cycle control are the major causes of cancer. The defective function of regulatory cell cycle elements leads towards increased cell proliferation and, in addition, expansion of genome damaged cells.[1 2] Aberrations of the cell cycle are often accompanied by overexpressed nuclear kinases, such as the cyclin dependent kinases (CDKs).[3] Alternatively, the abnormal function of CDK inhibitors (for example, p16 and p27) or the retinoblastoma gene product (Rb), a central regulatory effector, causes the deregulated proliferative activities of the tumour cell.[4]

p21^{waf} (also termed CIP1 or SDI1) is a nuclear protein with a pivotal role in cell cycle regulation.[5] It acts as a universal inhibitor of CDKs,[6] thus directly arresting the cell cycle at the G1/S phase checkpoint. In particular, p21^{waf} mediates p53 induced cell cycle arrest resulting from DNA damage after irradiation.[7 8] This arrest is important in the process of DNA repair or, alternatively, the switch to apoptosis. Moreover, p21^{waf} is induced independently of p53.[8] BRCA1 mediated growth arrest operates through p21^{waf} expression.[9] Differentiation inducing agents such as transretinoic acid,[10] growth factors,[11] or prostaglandin A2[12] can also initiate p21^{waf} transcription. Cyclin D1 is associated with p21^{waf} expression[13] and acts as an p21^{waf} inducer.[14] In addition, p21^{waf} is involved in arrest of the cell cycle at the G2 phase, by inhibiting the c-myc oncogene.[15] Thus, p21^{waf} executes its numerous regulatory functions both intrinsic to and separated from the core cell cycle machinery. Accordingly, the p21^{waf} protein possesses tumour suppressive properties.[16 17]

These basic facts hold true for all cancer types, including carcinomas of the breast. However, the clinical value of p21^{waf} detection in breast tumour tissue remains unknown. In clinical practice, there are no determinants to separate patients with an unfavourable prognosis from those whose tumours are not prone to form occult metastases. Those with an unfavourable prognosis need intensified systemic treatment, whereas for those with a more favourable prognosis chemotherapy should be less intensive or avoided. At present, a large number of patients with node negative tumours are treated unnecessarily, and patients who

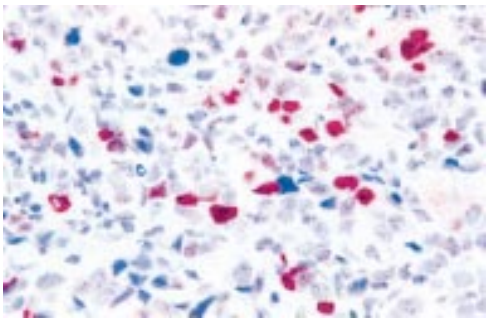

Figure 1 Positive p21^{waf} staining in the nuclei of tumour cells in an infiltrating ductal breast cancer (immunoreactive score, 6).

need dose intensification cannot be identified precisely. More specific tumour characteristics are needed so that patients can be offered individualised treatments according to the phenotype of their tumours.

Because of its functional properties, p21^{waf} is a potential marker. Our retrospective study on primary breast cancer tissues was designed to investigate whether p21^{waf} has prognostic impact and whether it correlates with other markers.

Material and methods

PATIENTS

Our study comprised 307 women with primary infiltrating breast carcinomas (T1–4, N0–2). The patients were treated between 1983 and 1989 at our department. All women had no evidence of metastases (M0) at the time of diagnosis. The patients underwent mastectomy (225 patients) or tumorectomy with postoperative irradiation (linear accelerator: 50 Gy and 10 Gy boost to tumour bed) of the breast (82 patients). Two hundred and ninety five patients underwent axillary lymphonodectomy with removal of more than nine (≥ 10) nodes to rule out nodal metastatic disease. No adjuvant systemic treatment was administered to node negative patients (n = 134). In the node positive patient subgroup, premenopausal or postmenopausal women without steroid hormone receptors received six cycles of a chemotherapy regimen consisting of a combination of either cyclophosphamide, methotrexate, and 5-fluorouracil (600/40/600 mg/m^2) or epirubicin and cyclophosphamide (60/600 mg/m^2). Postmenopausal women with positive steroid hormone receptors were treated with 20–30 mg tamoxifen for up to five years.

Physical, laboratory, and apparative checks (mammography, thorax *x* ray, and abdominal ultrasonography) on patients were carried out regularly as a part of an organised follow up programme. Follow up ranged from 24 to 114 months (median, 82). Histological classification and grading were based on the World Health Organisation criteria (1981) and the suggestions of Bloom and Richardson, respectively. Tumour stage followed the TNM system of UICC. Oestrogen receptor (ER) and progesterone receptor (PR) status were determined by immunohistochemistry (ER: ERICA-System, Abbott, Wiesbaden, Germany; PR: monoclonal antibody mPR1, Dianova, Hamburg, Germany). Steroid hormone receptor status was considered to be positive when ER and/or PR were positive, and negative when both ER and PR were negative. The proliferation marker proliferating cell nuclear antigen (PCNA; NA03; Dianova), the tumour suppressor p53 (MAb 1801; Dianova), and the Her2/neu protein (OPA 01/1; Medac, Hamburg, Germany) were determined immunohistochemically in adjacent sections from the same tumour tissue block. Technical procedures and the immunoreactive scoring system have been described previously.[18–20]

Table 1 Clinical, morphological, and biological data of the entire study group T1–4 N0–2 M0 (n = 307)

	p21^{waf} negative		*p21^{waf} positive*		*Total*		
	n	*%*	*n*	*%*	*n*	*%*	*p Value*
Age (years)							
< 50	73	23.8	35	11.4	108	35.2	
> 50	135	44.0	64	20.8	199	64.8	NS
Menopausal status							
Pre	59	19.2	28	9.1	87	28.3	
Peri	28	9.1	16	5.2	44	14.3	
Post	121	39.4	121	39.4	176	57.3	NS
Tumour size (cm)							
< 2	80	26.1	35	11.4	115	37.5	
> 2–5	102	33.2	45	14.7	147	49.9	
> 5	26	8.5	19	6.2	45	14.7	NS
Nodal status							
N0	90	30.5	44	14.9	134	45.4	
1–3 nodes	69	23.4	27	9.2	96	32.5	
> 4 nodes	40	13.6	25	8.5	65	22.0	NS
Tumour grade							
GI	33	10.7	17	5.5	50	16.3	
GII	110	35.8	53	17.3	163	53.1	
GIII	65	21.2	29	9.4	94	30.6	NS
Receptor status							
Negative	66	21.9	27	8.9	93	30.8	
Positive	139	46.0	70	23.2	209	69.2	NS
PCNA							
< 10%	95	31.6	41	13.6	136	45.2	
10–49%	71	23.6	26	8.6	97	32.2	
> 50%	37	12.3	31	10.3	68	22.6	0.029
Her2/neu							
Negative	153	51.3	79	26.5	232	77.9	
Positive	49	16.4	17	5.7	66	22.1	NS
p53							
Negative	123	41.1	57	19.1	180	60.2	
Positive	81	27.1	38	12.7	119	39.8	NS
p21^{waf}	208	67.8	99	32.2			

Univariate statistical calculation using χ^2 test.
Receptor status: positive, ER positive and/or PR positive; negative, ER negative and PR negative; detection by immunohistochemistry.
Lymph nodes were removed in 295 cases, steroid hormone receptor status was determined in 302 women, PCNA in 301, Her2/neu expression in 298, and p53 expression in 299 samples.
ER, oestrogen receptor; NS, not significant; PCNA, proliferating cell nuclear antigen; PR, progesterone receptor.

IMMUNOHISTOCHEMISTRY

Immunohistochemical analyses of p21^{waf} were performed on routinely processed blocks of formalin fixed, paraffin wax embedded surgical specimens of the primary carcinomas. The 3–4 μm sections of carcinoma tissues were mounted on 3-aminopropyltriethoxysilane (APES) covered glass slides. After drying, paraffin wax was removed with xylene (30 minutes), the sections were rehydrated, and the tissues digested with 0.1% trypsin (15 minutes). A modified three step avidin–biotin complex method to detect the p21^{waf} protein was used. All incubations with antibodies were performed in a moist chamber. The primary monoclonal antibody (clone 4D10, mouse IgG1 subtype; Novocastra, Newcastle upon Tyne, UK) was incubated at 4°C for 24 hours. Second and third antibodies were incubated at room temperature

Table 2 Clinical, morphological, and biological data of node negative women T1–4 N0 M0 (n = 134)

	$p21^{waf}$ negative		$p21^{waf}$ positive		Total		
	n	%	n	%	n	%	p Value
Age (years)							
< 50 years	34	25.4	13	9.7	47	35.1	
> 50	56	41.8	31	23.1	87	64.9	NS
Menopausal status							
Pre	25	18.7	10	7.5	35	26.1	
Peri	14	10.4	7	5.2	21	15.7	
Post	51	38.1	27	20.1	78	58.2	NS
Tumour size (cm)							
< 2	48	35.8	17	12.7	65	48.5	
2–5	41	30.6	19	14.2	60	44.8	
> 5	1	0.7	8	6.0	9	6.7	0.001
Tumour grade							
GI	15	11.2	10	7.5	25	18.7	
GII	53	39.6	21	15.7	74	55.2	
GIII	22	16.4	13	9.7	35	26.1	NS
Receptor status							
Negative	25	18.9	13	9.8	38	28.8	
Positive	63	47.7	31	23.5	94	71.2	NS
PCNA							
< 10%	45	34.4	20	15.3	65	49.6	
10–49%	33	25.2	11	8.4	44	33.6	
> 50%	9	6.9	13	9.9	22	16.8	0.017
Her2/neu							
Negative	67	51.5	37	28.5	104	80.0	
Positive	20	15.4	6	4.6	26	20.0	NS
p53							
Negative	59	45.0	26	19.8	85	64.9	
Positive	30	22.9	16	12.2	46	35.1	NS
$p21^{waf}$	90	67.2	44	32.8			

Univariate statistical calculation using χ^2 test.
Receptor status: positive, ER positive and/or PR positive; negative, ER negative and PR negative; detection by immunohistochemistry.
Steroid hormone receptor status was determined in 132 women, PCNA in 131, Her2/neu expression in 130, and p53 expression in 131 samples.
ER, oestrogen receptor; NS, not significant; PCNA, proliferating cell nuclear antigen; PR, progesterone receptor.

for 30 minutes. At intervals of different incubations, slides were washed with phosphate buffered saline. Antigen–antibody complexes were visualised using 3,3'-diaminobenzidine tetrahydrochloride (10 minutes). Cell nuclei were counterstained with haematoxylin (two minutes). A highly positive breast cancer and a negative control (non-specific immunoglobulins) were used in each run.

Specific staining was evaluated independently by two investigators semiquantitatively, yielding an immunoreactive score (IRS) ranging from 0 to 9. The IRS was calculated by multiplying the number of positive nuclear staining tumour cells (0, none; 1, < 10%; 2, 10–49%; 3, ⩾ 50% positive tumour cells) by the staining intensity (1, weak; 2, moderate; 3, strong). Tumours were considered $p21^{waf}$ positive with IRS ⩾ 2. When the scores of the two investigators differed, consensus was reached after examination using a teaching microscope. To demonstrate the reproducibility of $p21^{waf}$ detection, consecutive sections from 40 carcinomas were stained at one week intervals.

STATISTICS

We used SPSS 9.0 for Windows (Statistical Package for the Social Sciences; Munich, Germany) for statistical analysis. The χ^2 test was used for univariate comparison of data, whereas follow up data were analysed using the log rank test. Multivariate analyses were based on the Cox proportional hazards model and calculated relative risks.

Table 3 Clinical, morphological, and biological data of node positive women T1–4 N+ M0 (n = 161)

	$p21^{waf}$ negative		$p21^{waf}$ positive		Total		
	n	%	n	%	n	%	p Value
Age (years)							
< 50	34	21.1	22	13.7	56	34.8	
> 50	75	46.6	30	18.6	105	65.2	NS
Menopausal status							
Pre	29	18.0	18	11.2	47	29.2	
Peri	12	7.5	8	5.0	20	12.4	
Post	68	42.2	26	16.1	94	58.4	NS
Tumour size (cm)							
< 2	30	18.6	17	10.6	47	29.2	
> 2–5	57	35.4	25	15.5	82	50.9	
> 5	22	13.7	10	6.2	32	19.9	NS
Tumour grade							
GI	18	11.2	6	3.7	24	14.9	
GII	49	30.4	32	19.9	81	50.3	
GIII	42	26.1	14	8.7	56	34.8	NS
Receptor status							
Negative	39	24.7	14	8.9	53	33.5	
Positive	69	43.7	36	22.8	105	66.5	NS
PCNA							
< 10%	46	29.1	19	12.0	65	41.1	
10–49%	35	22.2	14	8.9	49	31.0	
> 50%	26	16.5	18	11.4	44	27.8	NS
Her2/neu							
Negative	78	50.0	39	25.0	117	75.0	
Positive	28	30.1	11	7.1	39	25.0	NS
p53							
Negative	59	37.8	29	18.6	88	56.4	
Positive	47	30.1	21	13.5	68	43.6	NS
$p21^{waf}$	109	67.7	52	32.3			

Univariate statistical calculation using χ^2 test.
Receptor status: positive, ER positive and/or PR positive; negative, ER negative and PR negative; detection by immunohistochemistry.
Steroid hormone receptor status was determined in 158 women, PCNA in 158, Her2/neu expression in 156, and p53 expression in 156 samples.
ER, oestrogen receptor; NS, not significant; PCNA, proliferating cell nuclear antigen; PR, progesterone receptor.

Results

The $p21^{waf}$ protein was detected in the nuclei of tumour cells. Intracytoplasmic reactions were very rare and much weaker than nuclear staining. The staining pattern in tumours was heterogeneous, revealing a mixture of positive and negative cells. Among the specimens with specific $p21^{waf}$ staining, most of the tumours expressed $p21^{waf}$ only in up to 20% of the cells. Non-diseased lobular or ductal epithelia found in the tumour periphery did not express detectable amounts of $p21^{waf}$ (fig 1). The staining of different consecutive series showed similar results. Thus, the immunohistochemical detection of $p21^{waf}$ expression was considered reproducible and reliable.

Ninety nine of 307 tumours (32.2%) were considered $p21^{waf}$ positive. This observation was independent of the nodal status (N0, 32.3%; N+, 32.8%).

Table 1 summarises the clinical, morphological, and biological data of the study group. The study group showed the expected distribution of established parameters. There was no significant correlation between $p21^{waf}$ and the parameters studied ($p > 0.05$), except for PCNA expression ($p = 0.029$). One of the major subdivisions of the patients concerned their nodal status. Therefore, the group was further divided into patients with node negative and node positive tumour tissues (tables 2 and 3). There was no significant correlation between $p21^{waf}$ and the other parameters in the node positive subgroup ($p > 0.05$), although $p21^{waf}$ expression correlated with PCNA expression ($p = 0.017$) and larger tumour size ($p = 0.001$) in the node negative subgroup.

Table 4 Number of events (relapses) in the node negative (known follow up n = 130) and node positive (n = 159) subgroups

	Node negative patients (T1-4 N0 M0)				Node positive patients (T1-4 N1-2 M0)			
	Total	Events	%	p Value	Total	Events	%	p Value
Age (years)								
< 50	47	9	19.2		55	25	45.5	
> 50	83	23	27.7	NS	104	61	58.7	NS
Menopausal status								
Pre	35	6	17.1		46	22	47.8	
Peri	21	4	19.1		20	10	50.0	
Post	74	22	29.7	NS	93	54	58.1	NS
Tumour size (cm)								
< 2	63	14	13.0		47	19	40.4	
2–5	59	16	27.1		80	43	53.8	
> 5	8	2	25.0	NS	32	24	75.0	< 0.00001
Tumour grade								
GI	23	3	13.0		23	9	39.1	
GII	72	20	27.8		80	35	43.8	
GIII	35	9	25.7	NS	56	42	75.0	< 0.00001
Receptor status								
Negative	38	12	31.6		52	38	73.1	
Positive	90	19	21.1	NS	104	47	45.2	0.00001
PCNA								
< 10%	62	13	21.0		65	27	41.5	
10–49%	43	10	23.4		47	28	59.6	
> 50%	22	8	36.4	NS	44	29	65.9	0.037
Her2/neu								
Negative	102	24	23.5		116	56	48.3	
Positive	25	7	28	NS	38	29	76.3	0.0017
p53								
Negative	82	18	22.0		87	40	46.0	
Positive	46	13	28.3	NS	67	44	65.7	0.005
$p21^{waf}$								
Negative	87	23	26.4		107	63	58.9	
Positive	43	9	20.9	NS	52	23	44.2	NS
Total	130	32	24.6		159	86	54.1	

Median observation time for disease free survival, 82 months. Statistical calculation using log rank test.
Univariate statistical calculation using χ^2 test.
Receptor status: positive, ER positive and/or PR positive; negative, ER negative and PR negative; detection by immunohistochemistry.
ER, oestrogen receptor; NS, not significant; PCNA, proliferating cell nuclear antigen; PR, progesterone receptor.

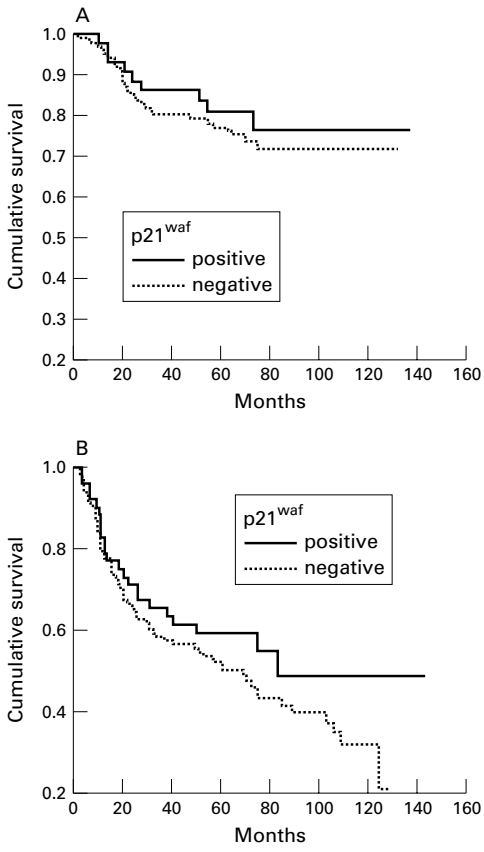

Figure 2 Kaplan-Meier curves for disease free survival (event = relapse) for (A) node negative (n = 130) and (B) node positive (n = 159) subgroups. Only trends could be observed (log rank test: p > 0.05).

Using the log rank test, tumour size, tumour grade, and the expression of steroid hormone receptors, PCNA, Her2/neu, and p53 correlated significantly with clinical outcome (table 4). In node negative patients, all markers failed to indicate recurrence. Disease free survival was calculated according to Kaplan and Meier (fig 2). There was a tendency to a favourable outcome for node positive patients with detectable $p21^{waf}$ expression, but this was not significant. Similarly, node negative patients seemed to benefit moderately from $p21^{waf}$ expression, although again the results were not significant ($p > 0.05$). Data for overall survival revealed similar results (not shown).

Multivariate analysis for disease free survival, as indicated by Cox regression analysis, found nodal status to be the strongest parameter (relative risk (RR), 1.74; $p = 0.00001$), followed by receptor status (RR, 0.59; $p = 0.0085$), tumour size (RR, 1.42; $p = 0.02$), and Her2/neu expression (RR, 1.56; $p = 0.033$) in the entire study group. In the subgroup of node positive patients, tumour size (RR, 1.83; $p = 0.0004$), steroid hormone receptor status (RR, 0.43; $p = 0.0002$), p53 expression (RR, 1.8; $p = 0.009$), and Her2/neu expression (RR, 1.74; $p = 0.019$) were independent parameters. Node negative patients showed no independent marker ($p > 0.05$).

Discussion

In selected breast cancer tissues, $p21^{waf}$ expression rates range from 32% to 57%.[13 21 22] In our study group, 32.2% of tumours had $p21^{waf}$ expression. With respect to the scoring system used we excluded spotted, weakly stained tumour cells from the $p21^{waf}$ positive group. Thus, $p21^{waf}$ detection in our study confirmed earlier results.

The role of $p21^{waf}$ expression in breast cancer is controversial: whereas some studies reveal an inverse correlation between $p21^{waf}$ expression and the apoptotic marker bcl-2,[23 24] the tumour suppressor p53,[22 25] and to histological grading,[22 26] other reports demonstrate p53 independent $p21^{waf}$ expression[13 27] and association with high histological grading,[13 28] positive nodal status,[28] and large tumour size.[28] It is postulated that $p21^{waf}$ expression is a prognostic marker for relapse free survival and improved overall survival.[22 25 28] In combination with nodal status, $p21^{waf}$ expression is thought to have predictive value. On the contrary, in more recent studies $p21^{waf}$ expression was not a prognostic factor[29] and did not correlate with clinical outcome.[13 30] Significant correlations were restricted to a lobular subtype.[13] Elledge and Allred summarised numerous clinical studies with respect to $p21^{waf}$ and concluded that $p21^{waf}$ expression plays a subordinate role in breast cancer prognosis.[31] Our study confirms these latter results. We show here that $p21^{waf}$ expression is an independent factor in

breast carcinoma progression, but lacks a clear predictive and prognostic relevance. Only trends could be seen with regard to clinical outcome.

Interestingly, in node negative women, $p21^{waf}$ expression correlated significantly with tumour size and PCNA expression (table 2). The subgroup of tumours > 5 cm showed an opposite distribution of $p21^{waf}$ positive and $p21^{waf}$ negative cells. Although more patients need to be investigated, this suggests that $p21^{waf}$ might have a protective effect on nodal involvement during tumour development. This is confirmed by earlier results that show a correlation between $p21^{waf}$ expression and negative nodal status,[26] and vice versa.[22] Further studies should be designed to prove the hypothesis of $p21^{waf}$ mediated node protection.

One of the main functions of $p21^{waf}$ uncoupled from cell cycle regulation is its inhibitory effect on the proliferation of tumour cells.[18] $p21^{waf}$ expression strongly correlates with Ki67 expression.[30] Furthermore, $p21^{waf}$ can affect DNA replication via physically binding to PCNA.[32] $p21^{waf}$ disrupts the PCNA–Fen1 complex, thereby prohibiting DNA replication.[33] This inhibitory role is concentrated on the operation of PCNA in DNA replication but not in DNA repair.[34] In our study, $p21^{waf}$ expression correlated with increased PCNA expression in node negative patients. To our knowledge, this is the first clinical study to deal with both parameters. We assume that increased PCNA expression induces $p21^{waf}$ expression in primary node negative breast cancer. Consistent with the reports discussed above, this might be a protective event that results in decreased nodal involvement. However, these effects are abolished if tumour cells are capable of inducing nodal spread.

In conclusion, the immunohistochemical detection of $p21^{waf}$ expression is of no use when making decisions about the treatment of breast cancer, although it is useful for understanding the biology of breast carcinogenesis.

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