Virchows Arch (2001) 439:132–140
DOI 10.1007/s004280100410

ORIGINAL ARTICLE

**Wenancjusz Domagala · Markus Welcker
Maria Chosia · Magdalena Karbowniczek
Barbara Harezga · Jirina Bartkova · Jiri Bartek
Mary Osborn**

p21/WAF1/Cip1 expression in invasive ductal breast carcinoma: relationship to p53, proliferation rate, and survival at 5 years

Received: 15 September 2000 / Accepted: 28 December 2000 / Published online: 31 March 2001

Abstract The p21/WAF1/Cip1 antibody, DCS-60, was characterized by means of immunoblotting and immunofluorescence on a variety of human breast cancer cell lines. Heterogeneous staining of nuclei was observed with strong staining of cells in early G_1. p21/WAF1/Cip1 expression in invasive ductal, not otherwise specified breast carcinomas was determined using immunohistochemistry with this antibody and computerized image analysis. Two hundred and twenty-two tumors, including 130 from patients with no axillary node involvement, were examined. p21-positive tumor cell nuclei were found in 30% of the breast carcinomas. The percentage of tumor cell nuclei that were positive ranged from less than 1% to greater than 10%. In the whole cohort of patients, p21 expression was significantly associated with a low histological grade. In the node-negative group, there was a significant negative correlation between p21 positivity and a high (>10%) MIB-1 score. The mean MIB-1 score was significantly lower in p21-positive tumors in the whole cohort of patients (P=0.03) and in the node-negative group (P=0.02). No association was found between p21 expression and overall survival at 5 years. With respect to p21/p53 phenotype, the significant difference in survival was noted only for the group of patients treated with adjuvant chemotherapy. The p21– p53+ phenotype had the worst survival (58% surviving 5 years), while the p21+ p53– phenotype had good survival (83% surviving 5 years; P<0.05). The results seem to suggest a correlation between p21/p53 phenotype and response to adjuvant chemotherapy.

Keywords p21/WAF1/Cip1 · Breast carcinoma · p53 · Prognosis

W. Domagala · M. Chosia · M. Karbowniczek
Department of Pathology, Faculty of Medicine,
Pomeranian Medical Academy, Szczecin, Poland

M. Welcker · J. Bartkova · J. Bartek
Institute of Cancer Biology, Strandboulevarden 49,
2100 Copenhagen, Denmark

B. Harezga
Department of Oncology, Medical Academy, Lodz, Poland

M. Osborn (✉)
Max Planck Institute for Biophysical Chemistry, Am Fassberg 11,
37077 Goettingen, Germany
e-mail: mosborn@gwdg.de
Tel.: +49-551-2011486, Fax: +49-551-2011578

Introduction

Poor prognosis in invasive breast cancer has been associated with both a high proliferation index of tumor cells [13] and with immunohistochemical p53 positivity [38]. The issue of p53 and prognosis in breast carcinoma is still controversial [3]. p53 mutations are found in 17–33% of breast carcinomas by DNA sequencing [2, 9, 26, 33, 37], and an accumulation of p53 is noted in 23–52% using histochemistry [1, 12, 20, 35, 38]. Such tumors also have a high growth fraction, presumably because tumor cells with p53 mutations that result in p53 accumulation are released from p53-mediated growth arrest. p53 can block the cell cycle by stimulating the *WAF1/Cip1* gene, which codes for a p21 protein that associates with and inhibits the cyclin-dependent kinases, the principal enzymes needed for cell cycle progression [17, 18, 41]. p21 is an important posttranscriptional effector in the p53-specific pathway of cell cycle control (reviewed in [10, 14, 15, 19]) and, therefore, it is reasonable to suppose that it may have prognostic significance in breast cancer. It seems, however, that p21 can be induced in cells by p53-dependent and p53-independent pathways [24, 27, 34, 36], and it is not clear whether this is also true for clinical specimens.

The clinical significance of p21 expression in breast cancer is still unknown. The few studies published to date on p21 expression in breast carcinomas reach different conclusions. Wakasugi et al. [40] and Jiang et al. [22] found a significant association of p21 expression with low histological grade, negative axillary lymph nodes, and better relapse-free survival, whereas Barbereschi et al. [4] and Caffo et al. [7] reported opposite results. Diab et al., [11] found no correlation between p21 expression

and survival in node-negative breast cancers. In two studies [22, 40], proliferation rate was not studied. Barbereschi et al. [4] and Caffo et al. [7] did not find a correlation between MIB-1 score and p21 expression, nor did Diab et al. [11] find a correlation between S-phase fraction and p21 expression. Finally, Rey et al. [32] reported a significant association of p21 positivity with an increased percentage of cells in S phase. The association of p53 accumulation with p21 expression in clinical specimens of breast cancer remains controversial. Positive [11], inverse [6, 22, 40], and no association [4, 7] have been reported in different studies.

One reason why p21 expression might be expected to have prognostic significance is its antiproliferative effect. In view of the controversies mentioned above and because the influence of the p53/p21 phenotype on proliferative activity of breast cancer cells has not yet been reported, the purpose of this study was threefold: (1) to explore the relationship between p21 expression, p53 accumulation, and tumor cell proliferation rate in invasive ductal not otherwise specified (NOS) breast carcinomas; (2) to look for correlations between p21 expression and clinicopathological prognostic factors, such as axillary lymph node status and tumor size; and (3) to assess the influence of p21 expression in tumor cells on the overall 5-year survival of patients with invasive ductal NOS breast cancer. To do so, we have used the largest cohort of invasive ductal carcinomas examined with p21 antibodies to date and a novel p21 antibody, DCS-60.

Materials and methods

Cell culture

The human diploid fibroblast cell line WI38, the simian virus (SV)40-transformed breast epithelial cell line HBL-100, and the human breast cancer cell lines MCF-7, BT-549, MDA-MB-134, MDA-MB-157, MDA-MB-231, MDA-MB-453, MDA-MB-468, ZR-75, BT-20, SK-BR-3, and T-47D were cultured in Dulbecco's modified Eagles' medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 10 U/ml penicillin, and 10 U/ml streptomycin.

Immunoblotting

Total cell extracts were prepared through direct lysis of exponentially growing cells with hot Laemmli sample buffer, and loading was adjusted according to Coomassie staining of control gels. Extracted proteins were separated on 12% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS), blotted onto nitrocellulose using a semi-dry method and probed for p21 using the enhanced chemiluminescence system (Amersham, Aylesbury, UK) according to the manufacturer's instructions. Immunofluorescence staining was performed as described [23], using biotinylated anti-mouse immunoglobulin antisera as the secondary reagent, followed by Texas red-conjugated Streptavidin (Vector Laboratories). Hoechst dye was used to counterstain the DNA.

Patients and tumor material

Formalin-fixed and paraffin-embedded tumor tissues from 222 unselected female patients with primary invasive ductal NOS breast carcinoma were retrieved from the files of the Department of Oncology, Medical Academy, Lodz, Poland. All patients underwent mastectomy with axillary lymph node dissection, and clinical follow-up was available for at least 60 months. Histological typing was performed according to the procedure of Millis and Girling [28], and histological grading was performed according to the procedure of Bloom and Richardson [5]. The computerized database contained the age of the patient, the number of positive lymph nodes, the size of the tumor, the histological type and grade, the stage of the disease at diagnosis, the treatment protocol, the date of operation, and the date of the last checkup or of death. Twenty-nine (32%) of the node-positive patients received adjuvant systemic chemotherapy (CMF) (cyclophosphamide, methotrexate, 5-fluorouracil), five (5%) received adjuvant hormonotherapy (tamoxifen), and 19 (21%) received adjuvant chemo- and hormonotherapy. In the node-negative group of patients, 36 (28%) received adjuvant chemotherapy (CMF), 12 (9%) received adjuvant hormonotherapy (tamoxifen), and eight (6%) received adjuvant chemo- and hormonotherapy. In addition, p53 status was available for 221 tumors tested with the monoclonal p53 antibody, DO1, from results published previously [12], while the MIB-1 score was known for 192 tumors from results also published previously [13].

Immunohistochemistry for p21

Sections were deparaffinized, immersed in citrate buffer (pH 6.0), and subjected to four microwave cycles according to Catoretti et al. [8]. The sections were incubated with the monoclonal p21 antibody DCS-60 used as an undiluted supernatant for 60 min at room temperature, washed, and reacted with biotinylated rabbit anti-mouse antibody and streptavidin peroxidase (Histostain-SP kit: Zymed Laboratories, Inc., San Francisco, Calif.). The sections were washed and then lightly counterstained with hematoxylin. Areas with the highest numbers of p21-positive cells were identified by scanning sections at low magnification. One such area was selected to start the counting and then additional contiguous fields were selected at random until at least 1000 tumor cells had been counted. Counts were made using the Leica Quantimet 600 S computerized image analysis system connected to an Axiophot microscope (Zeiss, Germany) and a 40× objective. The results were scored as the percentage of tumor cell nuclei that were p21-positive.

Statistical analysis

The association between p21 status and clinicopathological factors was assessed using the χ^2 test. The association between MIB-1 score and p21 and p53 statuses was assessed using the Student's *t* test. Differences between Kaplan–Meier survival curves were tested using the Wilcoxon test, as modified by Peto. All data were analyzed with the Statistica computer program.

Results

Characterization of the p21 antibody on cell lines

The isolation of the DCS-60 monoclonal antibody specific for human p21 protein has been described [39]. The specificity of the DCS-60 antibody for p21 was verified by means of immunoblotting and immunoprecipitation on a series of cell lines with known p53 status (Fig. 1). Immunoblotting of p21 in total cell extracts revealed a good overall correlation with the presence of wildtype p53 (Fig. 1). Relatively high levels of the p21 protein were found in the MCF-7 and ZR-75 cell lines, which both contain wildtype p53. An intermediate level of p21

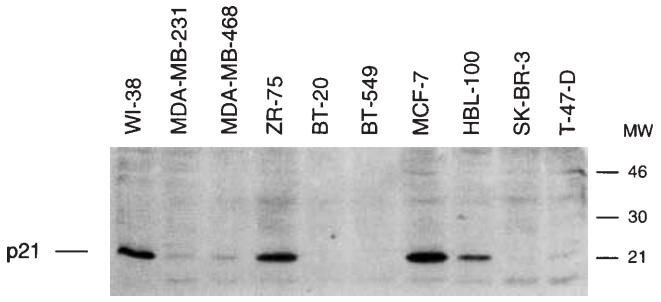

Fig. 1 Abundance of p21 correlates with p53 status in human breast cancer cell lines. Immunoblotting analysis of p21 in total cell extracts prepared from exponentially growing cells, using antibody DCS-60. Note that the highest levels of p21, comparable with that in control diploid fibroblasts (WI-38), are found in ZR-75 and MCF-7 breast cancer cell lines, which harbor wild-type p53. In contrast, a lower abundance of p21 is found in simian virus (SV)40-transformed HBL-100 cells, with very low or undetectable levels in the remaining six cell lines, which have different point missense mutations in the p53 gene. The cell lines are shown at the *top*, and the molecular weight of the markers on the *right* is given in kilodaltons

was found in the SV40-transformed HBL-100 cell line. Low or undetectable levels of p21 protein were found in the nine breast cancer cell lines which contain mutant p53, i.e., in MDA-MB-134, MDA-MB-157, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-20, BT-549, SK-BR-3, and T47D. It also recognizes p21 in mouse cells overexpressing exogenous human p21 (Welcker and Bartek, unpublished data). Immunoblotting with the mouse monoclonal antibody MO-1 against CDK7, a ubiquitously expressed nucleoprotein, showed that approximately equal amounts of extract had been loaded in all lanes in Fig. 1 (data not shown).

Immunofluorescence staining and evaluation of p21 at the single cell level showed that the p21 protein localized exclusively to cell nuclei. The abundance of p21 varied widely among individual cells in the MCF-7 or ZR-75 cell lines, with only a minor subpopulation of cells showing strong positivity. It was striking that the strongly stained cells were found often in pairs (Fig. 2), and their arrangements resembled cells in late telophase or early G1. In several breast cancer cell lines known to have mutant p53, there was a small subset of cells showing weak p21-specific staining (data not shown). In contrast, all nuclei were stained with the MO-1 antibody.

p21 positivity in histological specimens

Tumor cells were scored as positive for p21 if there was red nuclear staining (Fig. 3). Examination of normal breast tissue showed occasional p21-positive nuclei. The percentage of tumor cells that were p21-positive ranged from 0 to 16%. In 156 tumors, no tumor cells were stained. In 20 tumors, 0.1–1%, in 22 tumors 1–3%, in 20 tumors 3–10%, and in 4 tumors more than 10% of tumor cell nuclei were p21-positive. These 66 tumors constitut-

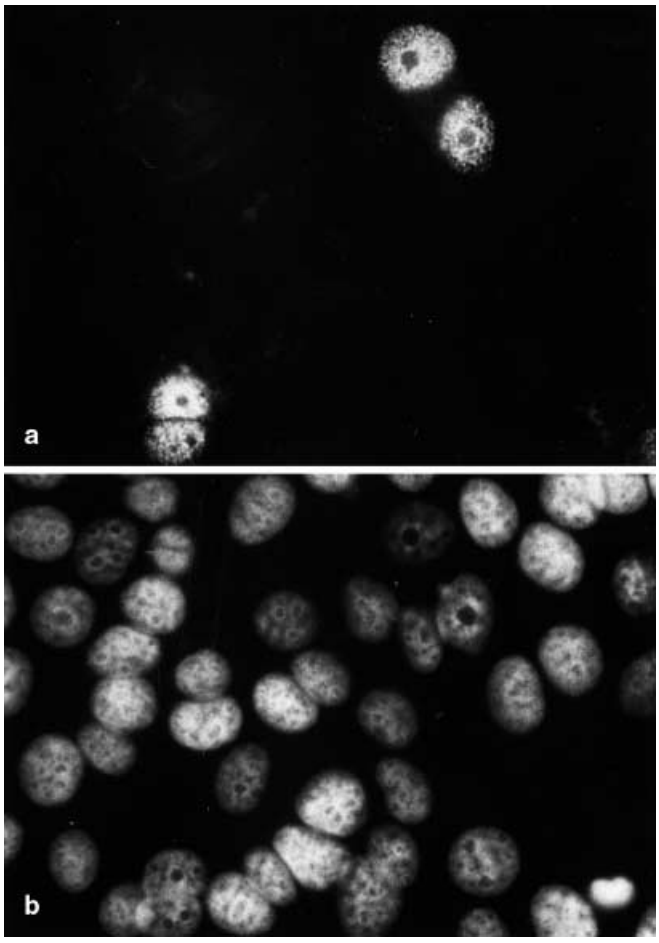

Fig. 2a,b Immunofluorescence visualization of p21 in the exponentially growing MCF-7 breast cancer cell line. **a** Strong nuclear positivity for p21 in a small subset of cells, as detected with the antibody DCS-60. Positive cells are usually found in pairs and are probably 'daughter cells' in late telophase or early G1. **b** Hoechst nuclear staining of the same cells as in (**a**). Magnification **a**, **b** ×800

ed the p21-positive group. The distribution of p21 tumor cells was heterogeneous, with p21 tumor cells often appearing in clusters (Fig. 3a, b), and sometimes in the tumor material it was easy to distinguish pairs of cells that were strongly stained with p21 antibody (Fig. 1b). The staining intensity of p21-positive tumor nuclei was heterogeneous. In some grade-III carcinomas, p21 expression was seen in very large nuclei (Fig. 3c) and in nuclei of multinucleated tumor cells (Fig. 3d). Tumor cells in 30% (66 of 222) of the invasive ductal NOS breast carcinomas expressed p21. In the node-negative subset, p21 was present in 32% (41 of 130), while in the node-positive subset, 27% (25 of 92) were p21-positive (Table 1).

Comparison of p21 status to other clinicopathological characteristics

The results in Table 1 show that p21 expression in the whole cohort of patients was associated with low histo-

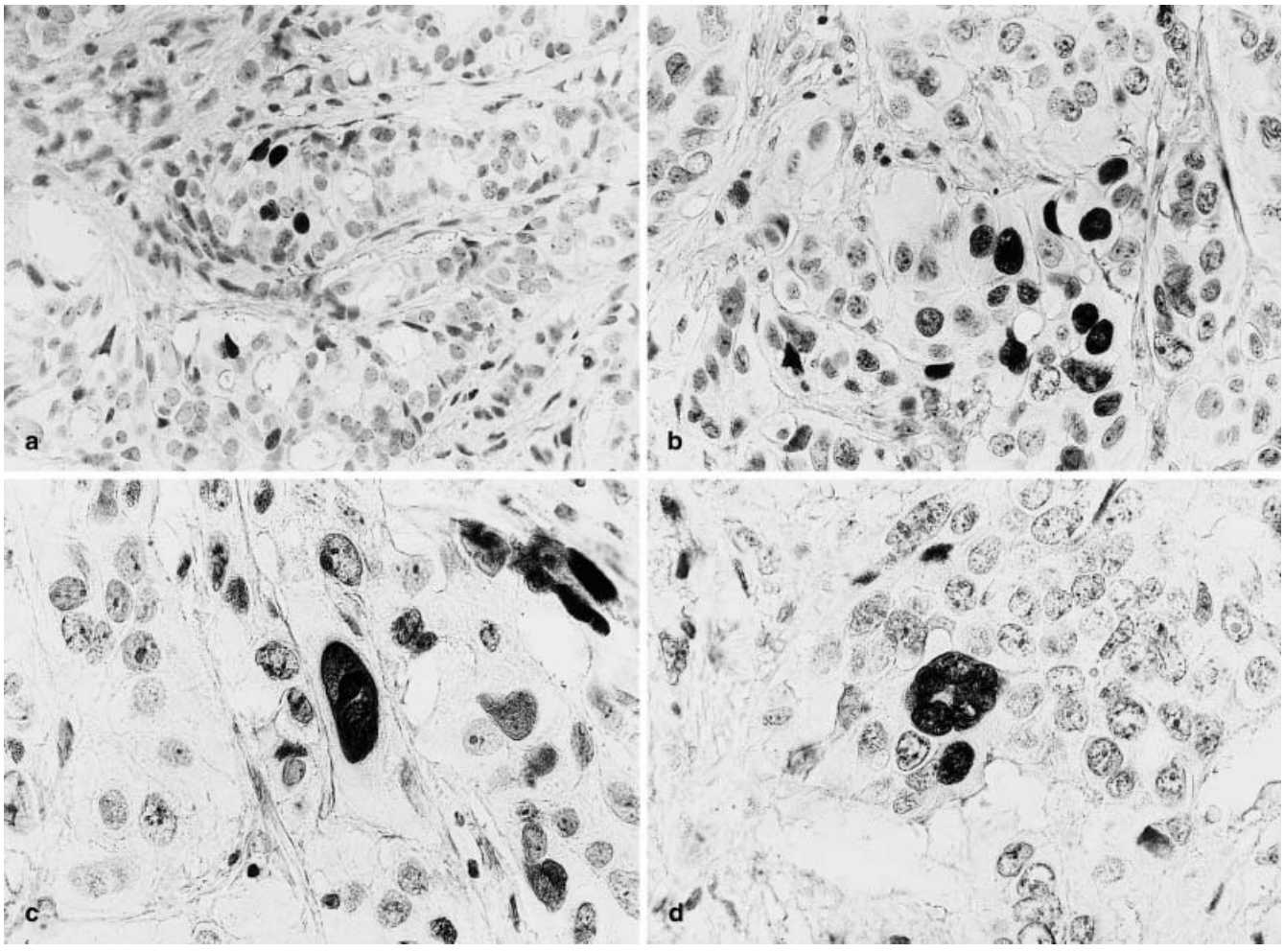

Fig. 3a–d p21 expression in invasive ductal not otherwise specified (NOS) breast carcinomas. **a** Grade-2 carcinoma with single p21-positive tumor cell nuclei. **b** Grade-3 carcinoma, with strong p21 reactivity in a group of tumor cell nuclei. **c** Huge p21-positive nucleus in a grade-3 carcinoma. **d** Multinucleated tumor cell with strong p21 expression. Streptavidin peroxidase method with 3-amino-9-ethylcarbazol (AEC) as the chromogen and light hematoxylin counterstain. Magnification: **a**, **b** ×250; **c**, **d** ×400

Table 1 p21 expression related to other clinicopathological factors. *NS* not significant

	All patients p21-positive		Node-negative p21-positive		Node-positive p21-positive	
	n (%)	*P*	*n* (%)	*P*	*n* (%)	*P*
Grade						
I+II	43/116 (37)	0.01	25/67 (37)	NS	18/49 (37)	0.03
III	23/106 (22)		16/63 (25)		7/43 (16)	
Size (mm)						
≤30	24/75 (32)	NS	12/49 (25)	NS	12/26 (46)	0.01
>30	42/147 (29)		29/81 (36)		13/66 (20)	
MIB-1						
≤10%	26/78 (33)	NS	17/41 (42)	0.01	9/37 (24)	NS
>10%	24/110 (22)		14/70 (20)		10/40 (25)	
p53						
Negative	53/158 (34)	(NS) 0.058	32/89 (36)	NS	21/69 (30)	NS
Positive	13/63 (21)		9/41 (22)		4/22 (18)	
Nodes						
Negative	41/130 (32)	NS				
Positive	25/92 (27)					

logical grade (grades I plus II vs grade III). p21 positivity showed a trend for an inverse correlation with p53 expression that, however, did not reach statistical significance ($P<0.058$). In node-negative patients, there was a significant negative correlation between p21 expression and a MIB-1 score greater than 10% ($P<0.01$). In node-positive patients, a significant correlation was found only between p21 expression and tumor size less than 30 mm ($P=0.01$) and between p21 expression and histological grade I plus II ($P=0.03$; Table 1).

p21 and proliferation rate of tumor cells

The mean MIB-1 score was significantly lower in p21-positive than in p21-negative tumors ($P=0.03$ for all and $P=0.02$ for axillary node-negative patients). In tumors from node-positive patients a similar trend was seen but it was not significant (Table 2). When tumors were divided into four groups according to p21 and p53 status, statistically significant differences in the MIB-1 score between the p21+ p53– group vs the p21– p53+ tumor group were found. This was true for all patients, for node-positive patients, and for node-negative patients (Table 3).

Prognostic significance of p21

One hundred and forty-two (64%) patients survived 5 years. The mean follow-up time for these patients was 69 months. When a cutoff level of less than 0.1% was used for p21 expression, Kaplan–Meier survival curves showed no statistically significant association between p21 expression in tumor cells and 5-year survival either in the whole cohort of patients or in the node-negative or node-positive subsets (data not shown).

When the p21 cutoff level was raised to more than 3%, and patients were stratified into four groups according to the p21/p53 status and further subdivided according to node status and therapy modality, five subsets could be analyzed, i.e., all, node-negative, node-positive, not treated with adjuvant therapy, and treated with adjuvant therapy. A significant ($P<0.035$) difference in Kaplan–Meier 5-year survival curves between patients with p21–/p53+ vs p21+/p53– tumors was found only in the group of patients treated with adjuvant therapy. In this group, only 58% of patients with p21–/p53+ tumors survived 5 years, whereas 83% of patients with p21+/p53– tumors survived 5 years. Thus, in patients treated with adjuvant therapy, the p21–/p53+ tumor phenotype was associated with the worst prognosis.

Table 2 p21 expression and proliferation rate of tumor cells in invasive ductal not otherwise specified (NOS) breast carcinomas

p21 status	*n*	MIB-1 (%)±SD	*P*
All patients	192		
p21-negative	140	18.3±14.6	
p21-positive	52	13.3±11.7	0.03
Node-negative	114		
p21-negative	82	19.5±14.1	
p21-positive	32	12.9±10.6	0.02
Node-positive	78		
p21-negative	58	16.2±15.1	
p21-positive	20	13.9±13.6	NS

Discussion

p21 expression in ductal carcinomas

In this report, we present immunohistochemical results on p21 expression in a homogeneous group of 222 invasive ductal NOS breast carcinomas. Previous studies have been concerned with p21 expression in histologically heterogeneous breast carcinomas and have usually not distinguished between the different histological types of breast carcinomas [4, 6, 7, 16, 22, 32, 40].

We found, using the DCS-60 antibody and a cut off level of 0.1%, that 30% of invasive ductal breast cancers expressed p21. p21 expression was heterogeneous both in terms of its distribution in the tumor tissue and intensity of expression, i.e., different percentages of p21-positive tumor cell nuclei were found in different tumors, and positive nuclei differed in staining intensity. In general, the level of expression of p21 was low. Only four tumors (6%) in our study expressed p21 in more then 10% of tumor cells. The highest percentage of p21-positive cells seen in a tumor was 16%.

Table 4 summarizes studies that have investigated p21 expression in breast carcinomas. Significant variation is

Table 3 The influence of p21 and p53 status on MIB-1 score in invasive ductal not otherwise specified (NOS) breast carcinomas

	p21	p53	All		Node negative		Node positive	
			n	MIB-1[a] (%±SD)	*n*	MIB-1[b] (%±SD)	*n*	MIB-1[c] (%±SD)
1	–	–	95	13.3±11	54	14.8±12	41	11.2±10
2	–	+	44	30.0±15	28	28.6±14	16	29.6±18
3	+	–	42	10.7±8	25	11.3±9	17	9.7±7
4	+	+	10	24.5±17	7	18.7±14	3	38.0±18

[a] 1 vs 2 $P<0.001$; 1 vs 4 $P>0.001$; 2 vs 3 $P<0.001$; 3 vs 4 $P<0.001$
[b] 2 vs 3 $P<0.001$; 1 vs 2 $P<0.001$
[c] 1 vs 2 $P<0.001$; 1 vs 4 $P>0.001$; 2 vs 3 $P>0.001$; 3 vs 4 $P>0.001$

Table 4 p21 expression in breast carcinomas. *F* formaldehyde; *Mw* microwave irradiation

	Series	Year	*n*	Histological type		Cutoff level p21+	p21+ MCF-7 cells	Antibody	p21+ nuclei (range)	p21+ Tumors	Fixation	Method of counting of p21+ cells
				Invasive ductal	Others							
1.	Barbareschi et al. [4]	1996	91	74	17	>10%		Oncogene EA10	0–50%	26%	F	Semi-quantitative[d]
2.	Caffo et al.[a] [7]	1996	261	164	34	>10%		Oncogene EA10	0–90%	32%	F	Semi-quantitative[d]
3.	Diab et al.[b] [11]	1997	115	?	?	>0%		Oncogene Ab3	0–60%	43%[c]	F	Not described
4.	Giannikaki et al. [16]	1997	102	88	14	>1%		Oncogene	0–25%	37%	F	Not described
5.	Jiang et al. [21]	1997	106	84	22	>25%		Santa Cruz clone 187	0–62%	32%	F?	Semi-quantitative[e]
6.	Wakasugi et al.[40]	1997	104	93	11	>10%	All[i]	Oncogene	?	49%	Mw	Semi-quantitative[g]
7.	Rey et al. [32]	1998	77	62	15	>5%		Oncogene EA10	?	57%	F	Semi-quantitative[f]
8.	Reed et al. [31]	1999	77	62	15	>0%		Oncog. Res. Prod. CC12	0–75%	68%	F	
9.	Mathoulin-Portier [25]	2000	162	162	–	≥1%		France Biochem. AB1	0–60%	53%	F?	Not described
10.	This study		222	222	0	>0.1%	Some[k]	DCS-60	0–16%	30%	F	Computerized image analysis[h]

[a] 91 cases from no. 1 included
[b] Node-negative only
[c] In 90% of positive tumors, the percentage of p21+ cells was low (>10%)
[d] At least 500 cells in the most densely stained areas
[e] 500 cells in five most representative areas
[f] In ten random high-power fields within the most stained areas
[g] In five different fields
[h] Count started in the most stained area and then additional contiguous fields were selected randomly until at least 1000 cells were counted
[i] All tumor cells p21-positive
[k] Only a fraction of tumor cells p21-positive

seen both in the percentage of p21-positive tumors, which varied from 26% [4] and 30% (this study) to 57% [6], and in the percentage of p21 nuclei that are positive in the different studies. In all studies, except one which used microwave fixation [40], formalin-fixed paraffin-embedded tissue was used and, therefore, the method of fixation is unlikely to be responsible for the differences. Factors that may explain the differences apparent in Table 4 include (1) the different histological material used in the different studies; (2) the different cutoff levels which ranged from 0% [11], to greater than 25% [22] (Table 4); (3) the different counting procedures. We started counts in a "hot spot" area and then additional contiguous fields were selected randomly until at least 1000 cells had been counted. Others counted p21-positive cells in "the most densely stained areas" [4, 7], in the "five most representative areas" [22], "in ten random high-power fields within the most stained areas" [32], or "in five different fields" [40]. We made counts using computerized image analysis, whereas others used semi-quantitative estimates; and (4) the different p21 antibodies. In this study, using the DCS-60 antibody, 0–16% of tumor cells were p21-positive. Diab et al. [11], using the Oncogene Ab3, found in almost all of their positive tumors (90%) that only a low proportion of cells (<10%) were stained for p21. However, in the other studies listed in Table 4, most of which used the EA10 antibody, the percentage of tumor cell nuclei showing p21 expression was higher. Thus, more than 10% of p21-reactive tumor cells were found in 26% [4], 32% [7], and 49% [40] of tumors, respectively. The highest percentage of p21+ nuclei in positive cases reached 90% in one study [7] and 62% in a second [22] (Table 4). Of the factors we list, probably the most important is the different p21 antibodies.

The DCS-60 antibody primarily detects cells in early G_1

Immunostaining of both cells in culture (Fig. 1) and of histological material (Fig. 3) suggests that cells in early G_1 are preferentially stained by the DCS-60 antibody. p21 has separate binding sites for the cyclins and cyclin-dependent kinase (CDK) and uses both of these contacts to bind the cyclin/CDK complexes with high affinity. The target epitope of the DCS-60 antibody has been located within the N-terminal domain of the p21 polypeptide, within the short region that physically interacts with the cyclins (Bartek et al., unpublished data). As a consequence, the DCS-60 antibody can immunoprecipitate free p21 or the transient complex of p21 with CDK but not the usually most abundant trimeric complex of

p21+cyclin/CDK, since the antibody competes for p21 with the cyclin. Thus, we speculate that the reason that immunostaining with DCS-60 reveals early G_1 cells is that this is the point in the cell cycle when the cyclins are still expressed at low levels, and p21 (if significantly expressed in a cell) is present in one of the two forms recognized by DCS-60. During other stages of the cell cycle, p21 is mainly found in a complex with cyclin/CDKs (and is lower in abundance as well).Therefore, the DCS-60 epitope is not accessible unless there is such a high level of p21 in the cell that some of it is in excess and thus in a free form which again is recognized by DCS-60. This latter scenario almost certainly explains the strongly p21-positive bizarre cells seen, for instance, in Fig. 3c. These may be damaged cells responding to a checkpoint with high levels of p21 or may be revealing a "senescence-like phenotype" in which p21 again plays a role and is expressed at high levels. A senescence-like phenotype has been shown to occur in tumor cells or diploid cells exposed to prolonged mitotic overload, and such a phenotype may represent one of the critical defense mechanisms against tumor development or progression. Such a mechanism may be able to stop or eliminate even tumor cells as long as they are able to respond by elevating p21.

p21 expression in p53-negative and in p53-positive breast carcinomas

Our results suggest a trend for an inverse relation between p21 expression and p53 accumulation in invasive ductal breast carcinomas, in keeping with previous findings showing a significant inverse relationship between p53 accumulation and p21 expression [22, 40] in breast cancer and a strong negative correlation between the presence of p53 mutations and p21 expression both in breast carcinoma [30] and in breast cancer cell lines [29, 30] on the messenger (m)RNA level. However, our results suggest that p21 could still be induced in tumors with mutant p53 since among p53-positive tumors those that were p21-positive had a lower mean MIB-1 score (24.5%) than did those that were p21-negative (30.0%). Although this difference was not statistically significant, it suggests a p53-independent pathway of p21-induced inhibition of tumor cell proliferation. These results are consistent with reports suggesting that p21, in breast cancer cell lines, may be induced by p53-dependent or p53-independent mechanisms. Thus, a few p53 mutant cell lines showed low levels of p21 expression [15], while some breast cancer cells expressed both p21 and (mutant) p53 [4, 32].

p21 expression and growth control in breast cancer

We found a significant association between p21 expression and a low MIB-1 score in all, and in node-negative invasive ductal NOS breast carcinomas (Table 2), which suggests p21-mediated growth control in breast cancer. This finding is consistent with data indicating that p21 suppresses tumor cell growth in vivo and in vitro [15]. Furthermore, we found striking differences in MIB-1 scores with p21+/p53– tumors having lower values than p21–/p53+ tumors. Barbareschi et al. [4] did not find a relationship between p21 expression and MIB-1 score, probably because they tested only 32 cases. However, using double staining, they documented that at a single cell level, p21 and MIB-1 were mutually exclusive. Also, Caffo et al. [7] saw no inverse association between p21 expression and MIB-1 score which, as they state, is difficult to explain. Rey et al. [32] and Mathoulin-Portier et al. [25] reported paradoxical results, i.e., a positive association between p21 expression and increased percentage of proliferating cells.

p21 expression and survival

High immunohistochemical p21 expression has been associated with short [4] and longer [22, 40] relapse-free and overall survival in breast carcinomas. However, in the studies, tubular, medullary, and mucinous carcinomas (which a priori have better prognosis) were included, and this may influence the results. Diab et al. [11], Reed et al. [31], and Mathoulin-Portier et al. [25] found no correlation with survival. In our series of breast carcinomas as well no statistically significant association was seen between immunohistochemical p21 expression or p21/p53 phenotype and overall survival at 5 years either in the whole cohort of patients or in the node-negative or node-positive subgroups. However, with respect to patients treated with adjuvant therapy, the p21–/p53+ phenotype was associated with the worst survival (only 58% surviving 5 years), while the p21+/p53– phenotype was associated with good survival (83% surviving 5 years). Caffo et al. [7] and Mathoulin-Portier et al. [25] also showed that a combined evaluation of p53 and p21 expression provides prognostic information in the group of patients given adjuvant therapy. However, they did not find an inverse association between p21 and the MIB-1 score, which would theoretically be expected and the lack of which is difficult to explain. We have significant (and theoretically expected) associations between p21 and MIB-1, but they do not seem to translate to differences in survival for node-negative, node-positive patients or for patients not given adjuvant therapy. A difference in survival is noted only for the group who received adjuvant therapy. Tumors with the p21–/p53+ phenotype presumably express mutated but not functional p53. Hence, they may not be able to activate apoptosis in response to chemotherapeutic DNA-damaging drugs. They also have a very high MIB-1 score of tumor cells, which may facilitate the emergence of clones of tumor cells resistant to chemotherapy. Since p21 is a key component of the DNA damage checkpoint, our results suggest a correlation between the p21/p53 phenotype and response to adjuvant chemotherapy. The results of Mathoulin-

Portier et al. [25] also suggest a correlation between the p53/p21/mdm2 phenotype and survival of patients with breast carcinomas treated by chemotherapy. These correlations are intriguing and suggest that in order to delineate better the group of breast cancer patients who may benefit from adjuvant chemotherapy, further studies are required of the relationship between the expression of p53 and some of its downstream effectors and chemotherapy and survival.

References

1. Allred DC, Clark GM, Elledge R, Fuqua SA, Brown RW, Chamness GC, Osborne CK, McGuire WL (1993) Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J. Natl Cancer Inst 85:200–206
2. Andersen TI, Holm R, Nesland JM, Heimdal KR, Ottestad R, Borresen AL (1993) Prognostic significance of TP53 alterations in breast carcinoma. Br J Cancer 68:540–548
3. Barbareschi M (1996) Prognostic value of the immunohistochemical expression of p53 in breast carcinomas. Appl Immunohistochem 4:106–116
4. Barbareschi M, Caffo O, Doglioni C, Fina P, Marchetti A, Buttitta F, Leek R, Morelli L, Leonardi E, Bevilacqua G, Dalla Palma P, Harris AL (1996) p21WAF1 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 74:208–215
5. Bloom HJG, Richardson WW (1957) Histologic grading and prognosis in breast cancer. Br. J. Cancer 11:359–377
6. Bukholm IK, Nesland JM, Karesen R, Jacobsen U, Borresen AL (1997) Relationship between abnormal p53 protein and failure to express p21 protein in human breast carcinomas. J Pathol 181:140–145
7. Caffo O, Doglioni C, Veronese S, Bonzanini M, Marchetti A, Buttitta F, Fina P, Leek R, Morelli L, Palma PD, Harris AL, Barbareschi M (1996) Prognostic value of p21(WAF1) and p53 expression in breast carcinoma: an immunohistochemical study in 261 patients with long-term follow-up. Clin Cancer Res 2:1591–599
8. Cattoretti G, Becker MHG, Key G, Duchrow M, Schlüter C, Galle J, Gerdes J (1992) Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB1 and MIB3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 168:357–363
9. Coles C, Condie A, Chetty U, Steel CM, Evans HJ, Prosser J (1992) p53 mutations in breast cancer. Cancer Res. 52:5291–5298
10. Cordon-Cardo C (1995) Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. Am J Pathol 147:545–560
11. Diab SG, Yu YY, Hilsenbeck SG, Allred DC, Elledge RM (1997) WAF1/CIP1 protein expression in human breast tumors. Breast Cancer Res Treat 43:99–103
12. Domagala W, Striker G, Szadowska A, Dukowicz A, Harezga B, Osborn M (1994) p53 protein and vimentin in invasive ductal NOS breast carcinoma – relationship with survival and sites of metastases. Eur J Cancer 30A:1527–1534
13. Domagala W, Markiewski M, Harezga B, Dukowicz A, Osborn M (1996) Prognostic significance of tumor cell proliferation rate as determined by the MIB-1 antibody in breast carcinoma: Its relationship with vimentin and p53 protein. Clin Cancer Res 2:147–154
14. El-Deiry WS (1998) p21/p53, cellular growth control and genomic integrity. Curr Top Microbiol Immunol 227:121–137
15. Gartel AL, Serfas MS, Tyner AL (1996) p21-negative regulator of the cell cycle. Proc Soc Exp. Biol Med 213:138–149
16. Giannikaki E, Kouvidou C, Tzardi M, Stefanaki K, Koutsoubi K, Gregoriou M, Zois E, Kakolyris S, Mavroudi C, Delides G, Georgoulias V, Kanavaros P (1997) p53 protein expression in breast carcinomas. Comparative study with the wild type p53 induced proteins mdm2 and p21/waf1. Anticancer Res 17: 2123–2127
17. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75:805–816
18. Hunter T (1993) Braking the cycle. Cell 75:839–841
19. Hunter T, Pines J (1994) Cyclins and cancer. II: cyclin D and CDK inhibitors come of age. Cell 79:573–582
20. Isola J, Visakorpi T, Holli K, Kallioniemi OP (1992) 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 84:1109–1114
21. Jiang H, Lin J, Su ZZ, Collart FR, Huberman E, Fisher PB (1994) Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. Oncogene 9:3397–3406
22. Jiang M, Shao ZM, Wu J, Lu JS, Yu LM, Yuan JD, Han QX, Shen ZZ, Fontana JA (1997) p21/waf1/cip1 and mdm-2 expression in breast carcinoma patients as related to prognosis. Int J Cancer 74:529–534
23. Lukas J, Pagano M, Staskova Z, Draetta G, Bartek J (1994) Cyclin D1 oscillates and is essential for cell cycle progression in human tumour cell lines. Oncogene 4:707–718
24. Marchetti A, Doglioni C, Barbareschi M, Buttitta F, Pellegrini S, Bertacca G, Chella A, Merlo G, Angeletti CA, Palma PD, Bevilacqua G (1996) p21 RNA and protein expression in non-small cell lung carcinomas: evidence of p53-independent expression and association with tumoral differentiation. Oncogene 12:1319–1324
25. Mathoulin-Portier M-P, Viens P, Cowen D, Bertucci F, Houvenaeghel G, Geneix J, Puig B, Bardou VJ, Jacqumier J (2000) Prognostic value of simultaneous expression of p21 and mdm2 in breast carcinomas treated by adjuvant chemotherapy with antracyclin Oncology Reports 7:675–680
26. Mazars R, Spinardi L, BenCheikh M, Simony-Lafontaine J, Jeanteur P, Theillet C (1992) p53 mutations occur in aggressive breast cancer. Cancer Res. 52:3918–3923
27. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D (1994) Induction of WAF1/CIP1 by a p53-independent pathway. Cancer Res 54:3391–3395
28. Millis R, Girling A (1989) The breast. In: Sternberg SS (ed) Diagnostic surgical pathology. Raven, New York, pp 253–313
29. Musgrove EA, Lilischkis R, Cornish AL, Lee CS, Setlur V, Seshadri R, Sutherland RL (1995) Expression of the cyclin-dependent kinase inhibitors p16INK4, p15INK4B and p21WAF1/CIP1 in human breast cancer. Int J Cancer 63: 584–391
30. Özcelik H, Mousses S, Andrulis IL (1995) Low levels of expression of an inhibitor of cyclin-dependent kinases (CIP1/WAF1) in primary breast carcinomas with p53 mutations. Clin Cancer Res 1:907–912
31. Reed W, Fllrenes VA, Holm R, Hannisdal E, Nesland JM (1999) Elevated levels of p27, p21 and cyclin D1 correlate with positive oestrogen and progesterone receptor status in node-negative breast carcinoma patients. Virchows Arch 435:116–124
32. Rey MJ, Fernandez PL, Jares P, Munoz M, Nadal A, Peiro N, Nayach I, Mallofre C, Muntane J, Campo E, Estape J, Cardesa A (1998) p21WAF1/Cip1 is associated with cyclin D1CCND1 expression and tubular differentiation but is independent of p53 overexpression in human breast carcinoma. J Pathol 184:265–271
33. Runnenbaum IB, Nagarajan M, Bowman M, Soto D, Sukumar S (1991) Mutations in p53 as potential molecular markers for human breast cancer. Proc Natl Acad Sci USA 88:10657–10661

34. Sheikh MS, Li XS, Chen JC, Shao ZM, Ordonez JV, Fontana JA (1994) Mechanisms of regulation of WAF1/Cip1 gene expression in human breast carcinoma: role of p53-dependent and independent signal transduction pathways. Oncogene 9:3407–3415
35. Silvestrini R, Benini E, Daidone MG, Veneroni S, Boracchi P, Cappelletti V, Di Fronzo G, Veronesi U (1993) p53 as an independent prognostic marker in lymph node-negative breast cancer patients. J Natl Cancer Inst 85:965–970
36. Steinman RA, Hoffman B, Iro A, Guillouf C, Liebermann DA, el-Houseini ME (1994) Induction of p21 (WAF-1/CIP1) during differentiation. Oncogene 9:3389–3396
37. Thompson AM, Anderson TJ, Condie A, Prosser J, Chetty U, Carter DC, Evans HJ, Steel CM (1992) p53 allele losses, mutations and expression in breast cancer and their relationship to clinico-pathological parameters. Int J Cancer 50:528–532
38. Thor AD, Moore DH, II, Edgerton SM, Kawasaki ES, Reihsaus E, Lynch HT, Marcus JN, Schwartz L, Chen LC, Mayall BH (1992) Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. J Natl Cancer Inst 84:845–855
39. Thullberg M, Welcker M, Bartkova J, Kjerulff AA, Lukas J, Högberg J, Bartek J (2000) Monoclonal antibody probes for p21 WAF1/CIP1 and the INK4 family of cyclin-dependent kinase inhibitors. Hybridoma 19:63–72
40. Wakasugi E, Kobayashi T, Tamaki Y, Ito Y, Miyashiro I, Komoike Y, Takeda T, Shin E, Takatsuka Y, Kikkawa N, Monden T, Monden M (1997) p21(Waf1/Cip1) and p53 protein expression in breast cancer. Am J Clin Pathol 107:684–691
41. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R (1993) p21 is a universal inhibitor of cyclin kinases. Nature 366: 701–704