Prognostic Value of p21<sup>WAF1</sup> and p53 Expression in Breast Carcinoma: An Immunohistochemical Study in 261 Patients with Long-Term Follow-Up

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ABSTRACT

p21 protein (p21) inhibitor of cyclin-dependent kinases is a critical downstream effector in the p53-specific pathway of growth control and can also be induced by p53-independent pathways in relation to terminal differentiation. We investigated p21 immunoreactivity in 261 breast carcinomas (141 node negative and 120 node positive) with long-term follow-up (median, 73 months; range, 37-119). p21 was seen in 214 (82%) infiltrating tumors, staining was nuclear and heterogeneous, and the p21 labeling index ranged from 0 to 90%. Sixty-eight (32%) patients showed p21 overexpression (>10% of reactive tumor cells). p21 overexpression was associated with large tumor size, positive nodal status, high histological grade, and high mitotic count and was related to short disease-free survival (DFS) in the whole series of patients (P = 0.04), in the node-negative subgroup (P = 0.004), and in the group of patients who did not undergo systemic adjuvant therapy (P = 0.003). In patients treated with systemic adjuvant therapy, bivariate analysis of the combined p21 and p53 phenotypes showed that p21+/p53+ tumors were associated with long DFS and overall survival (OS), whereas p21−/p53+ tumors had the worst prognosis. In treated patients, multivariate analysis showed that the p21−/p53− phenotype was independently associated with short DFS and OS. Our present data support the hypothesis that p21/p53 heterogeneous expression may be of clinical relevance for the therapeutic response to chemotherapy/ hormonotherapy. The p21−/p53+ phenotype could correspond to a situation where p53 overexpression really reflects complete abrogation of p53 function. These cases with disrupted p53 function should have impaired the G1 checkpoint and may not be able to activate the apoptotic cascade in response to DNA-damaging drugs.

INTRODUCTION

p21<sup>WAF1</sup> protein (p21) is an inhibitor of cyclin-dependent kinases and is a critical downstream effector in the p53-specific pathway of growth control in mammalian cells (1, 2). p21 can also be induced by p53-independent pathways (3-5), and its expression seems to be related to differentiation in several cell lines (6-8) and in some human tissues (9-11). p21 may not only be responsible for the p53-mediated growth arrest following DNA damage, but it may also play an important role in the maintenance of growth arrest in terminally differentiated cells (12).

p21 expression has been observed in various human epithelial neoplasms (9-11, 13). In a previous preliminary study on 91 breast carcinomas, we reported that high p21 expression was seen in one fourth of the cases, and was associated with high tumor grade and short relapse-free survival (13). However, in that study, because of the small number of cases, we could not address several questions regarding the value of p21 expression as an independent prognostic marker in different groups of homogeneously treated patients. The most interesting question about the possible role of p21 expression as a predictor of therapeutic response remained unanswered (13-17). Therefore, in the present study we investigated p21 immunoreactivity in a much larger series of breast carcinomas, a portion of which had been treated with adjuvant therapy, with long-term follow-up. p21 was evaluated in relation to clinicopathological characteristics of the tumors, ER<sup>2</sup> status, Ki67 proliferation-related antigen expression, p53 overexpression, and survival. Moreover, to evaluate whether p21 and p53 may predict a response to therapy, we separately analyzed the survival impact of p21 and p53 in patients who did or did not undergo adjuvant systemic therapy.

MATERIALS AND METHODS

Patients. Two hundred sixty-one consecutive patients were investigated from January 1984 to November 1990, 91 of which have been included in a preliminary study on p21 expression (13). The study period ended on December 31, 1993. Eligibility criteria were: histological diagnosis of infiltrating breast carcinoma, axillary lymph node dissection, no distant
Expression in Breast Carcinoma

metastasis, and unilateral tumor. One hundred twenty cases were node negative (N0) and 141 were node positive (N1 or N2). The median follow-up duration was 73 (range, 37–119) months. Two hundred fifteen patients underwent modified mastectomy and 46 underwent conservative breast surgery followed by radiation therapy. Adjuvant systemic chemotherapy (CMF Milan protocol, i.e., cyclophosphamide, methotrexate, and 5-fluorouracil) was given to 72 N1/2 and 14 N0 patients. Adjuvant hormonotherapy (tamoxifen, 20 mg daily) was given to 59 N1/2 and 16 N0 patients; 9 node-positive patients were treated with both hormonotherapy and chemotherapy.

Tumor Samples. Surgical samples were routinely processed; tumors were classified according to Rosen and Oberman (18) as follows: 227 ductal, 11 lobular, 6 medullary, 8 tubular, 7 mucinous, and 2 cribriform. Tumors were graded according to the method of Elston and Ellis (19).

Immunohistochemistry. p21 immunoreactivity was evaluated on primary tumors using the EA10 monoclonal antibody

Fig. 1 p21 and p53 expression in infiltrating ductal carcinomas: case A, p21+/p53+ phenotype; case B, p21+/p53− phenotype; case C, p21−/p53+ phenotype. A-C, immunostaining for p21; a-c, immunostaining for p53. All specimens were counterstained with hematoxylin.
(Oncogene Science, Cambridge, MA) with microwave antigen retrieval as described (10, 11, 13). Paraffin sections were incubated for 1 h with the primary antibody (1:100 dilution) and processed with the StreptABC technique (Duet kit; DAKO, Glostrup, Denmark). Positive controls were sections of lung tumors with known p21 mRNA and protein expression (13). ER immunohistochemical status was evaluated using the ER1D5 antibody. One hundred forty-three consecutive cases had already been immunostained with the MIB1 antibody against Ki67 proliferation-related antigen. There were no evident differences in stage or biological features between cases with MIB1 immunostaining and the whole series of cases. p53 protein immunoreactivity was assessed with the DO7 monoclonal antibody (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom); positive controls were sections of breast carcinomas with known p53 gene mutation and protein accumulation (13). Negative controls were obtained by omitting primary antibodies.

Cells were considered positive for p21, ER, MIB1, and p53 only when distinct nuclear staining was identified. Immunostaining of each marker was evaluated by scanning the whole sections at medium and high magnification and by counting at least 500 cells in the most densely stained tumor areas. The percentage of immunoreactive nuclei was considered as the LI for the given marker.

**Statistical Analysis.** Statistical analysis was performed using the SAS system (PROC FREQ, PROC LIFETEST and PROC PHREG), run on an IBM-compatible personal computer. The association between the variables has been assessed using the \( \chi^2 \) test, Fisher exact test, and Cochran-Mantel-Haenszel statistics. DFS and breast cancer-related OS were estimated for the given marker.

### RESULTS

**Immunohistochemistry.** p21 reactivity was seen in 214 (82%) cases. Staining intensity was variable and heterogeneous (Fig. 1). p21 LI ranged from 0 to 90%; mean ± SD and median were 9.7 ± 12 and 9.7, respectively. p21 overexpression, defined as p21 LI > 10% according to our previous study (13), was seen in 68 (32%) cases. High p21 LI was associated with large tumor size, \( N_{ij} \) status, high grade, high mitotic count, and high nuclear and tubules score (Table 1).

ER immunoreactivity was seen in 129 (49%) carcinomas; positive ER status (i.e., ER LI > 10%) was seen in 121 (46%) cases. MIB1 LI ranged from 2 to 90%, with a median value of 15; 64 cases with MIB1 LI higher than the median value were considered to be highly proliferative tumors. p53 immunoreactivity was seen in 68 (32%) cases; the p53 LI ranged from 0 to 95%, with a median value of 15%; 61 (23%) cases with p53 LI higher than 15% were considered as overexpressing p53.

**Clinical Outcome of the Patients.** Disease relapses were seen in 93 patients (25 in \( N_0 \) patients and 68 in \( N_{ij} \) patients). Seventy-four patients died of the disease (15 in \( N_0 \) and 59 in \( N_{ij} \)). Five-year DFS and OS were 68% and 76%, respectively. Among \( N_0 \) patients, 5-year DFS and OS were 82 and 89%, respectively. Among \( N_{ij} \) patients, DFS and OS were 57% and 66%, respectively. Five-year DFS and OS for 170 patients treated with any type of adjuvant therapy were 60 and 68%, respectively; 5-year DFS and OS for patients treated with adjuvant chemotherapy were 52 and 67%, respectively; five-year DFS and OS for patients treated with adjuvant hormone therapy were 69 and 69%, respectively; 5-year DFS and OS for patients not treated with adjuvant therapy were 83 and 91%, respectively.

### Univariate Survival Analysis Stratified by the Different Variables

Univariate survival analysis has been performed on the whole series of patients (Table 2), in the node-negative
and node-positive subgroups, and in the subgroups of patients with or without systemic adjuvant treatment.

In the whole series of patients p21 overexpression was related to short DFS (P = 0.04; Fig. 2); the presence of lymph node metastases, large tumor size, high tumor grade, high mitotic count, negative ER status, and p53 overexpression were all related to short DFS and OS (Table 2).

In N0 patients, p21 was associated with short DFS (23 positive versus 97 negative; 5-year DFS 65% versus 86%, P = 0.004; Fig. 3). Variables related to short DFS and OS were large tumor size (67 T1 versus 53 T2/T3; 5-year DFS 88% versus 75%, P = 0.04; 5-year OS 94% versus 84%, P = 0.04) and high tumor grade (25 G1 versus 43 G2 versus 52 G3; 5-year DFS 100% versus 88% versus 68%, P = 0.002; 5-year OS 100% versus 95% versus 80%, P = 0.01). Ductal histology was related to short OS (ductal versus other; 5-year DFS 79% versus 93%; P = 0.04).

In N12 patients, parameters related to short DFS and OS were large tumor size (50 T1 versus 91 T2/T3; 5-year DFS 80% versus 44%, P = 0.0002; 5-year OS 86% versus 59%, P = 0.0001), high tumor grade (16 G1 versus 26 G2 versus 92 G3; 7 missing values; 5-year DFS 87% versus 58% versus 52%, P = 0.01; 5-year OS 94% versus 77% versus 62%, P = 0.01), negative ER status (61 positive versus 80 negative; 5-year DFS 71% versus 45%, P = 0.003; 5-year OS 81% versus 58%, P = 0.01), and p53 overexpression (105 negative versus 36 positive; 5-year DFS 65% versus 22%, P = 0.0001; 5-year OS 78% versus 42%, P = 0.01).

In patients (90 N0 and 1 N12) who did not receive any adjuvant therapy, the only variables associated with short DFS were p21 overexpression (17 positive versus 74 negative; 5-year DFS 64% versus 87%; P = 0.003) and grading (22 G1 versus 34 G2 versus 35 G3; 5-year DFS 100% versus 85% versus 70%; P = 0.01). No variable was associated with short OS.

In patients treated with adjuvant therapy, the variables associated with short DFS and OS were large tumor size (58 T1 versus 112 T2/T3; 5-year DFS 82% versus 49%, P = 0.0001; 5-year OS 88% versus 61%, P = 0.00001), high grade (19 G1 versus 35 G2 versus 109 G3, 3 missing values; 5-year DFS 89% versus 67% versus 54%, P = 0.002; 5-year OS 94% versus 83% versus 63%, P = 0.002), ER negative status (69 positive versus 101 negative; 5-year DFS 75% versus 50%, P = 0.003; 5-year OS 93% versus 61%, P = 0.01), and p53 overexpression (122 negative versus 48 positive; 5-year DFS 68% versus 41%, P = 0.0002; 5-year OS 78% versus 48%, P = 0.0001). Positive

### Table 2 Five-year DFS and OS after stratifying by clinicopathological variables and univariate survival analysis

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<th>P*</th>
<th>OS %</th>
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<td>73</td>
<td>0.3</td>
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<tr>
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<td>192</td>
<td>71</td>
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<td>80</td>
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<sup>a</sup> Log rank test.

<sup>b</sup> Seven missing values; according to the method of Elston and Ellis (19).

<sup>c</sup> Eleven missing values.

<sup>d</sup> Immunohistochemical assay; cutoff, 10% reacting cells.

<sup>e</sup> Cutoff, 15% reacting cells.

<sup>f</sup> p21 overexpression as defined in the text (i.e., >10% reacting nuclei).
Fig. 2 DFS curves in the whole cohort of patients, stratified according to p21 expression: cases with p21 LI > 10% have shorter DFS ($P = 0.04$).

Fig. 3 DFS curves in the group of 120 node-negative patients, stratified according to p21 expression: cases with p21 LI > 10% have shorter DFS ($P = 0.004$).
nodosal status was associated only with short DFS (140 N1/2 versus 30 N0, 5-year DFS 57% versus 80%, P = 0.01).

**Bivariate Analysis.** The combined p21/p53 phenotype was evaluated in relation to DFS and OS. We analyzed separately the whole series of cases, the N0 and N1/2 subgroups, the subgroups of patients who did or did not undergo adjuvant chemotherapy and/or hormonotherapy (Table 3). Among N0 patients and in patients not treated with any adjuvant therapy, only six and five cases, respectively, with the p21+!p53+ phenotype. Among N1/2 subgroups confirmed the lack of independent prognostic value of high p21 expression per se (data not shown).

We also performed multivariate analysis using a second model where p21 and p53 were entered as covariates, and analyzed separately the groups of patients who did or did not undergo systemic adjuvant therapy. In patients not treated with any type of adjuvant therapy, the only variable independently associated with DFS was tumor grade (P = 0.0393). In patients who received adjuvant therapy, the variables independently associated with DFS and OS were tumor size and the p21−/p53+ phenotype (P = 0.0022 and 0.0148 for DFS, respectively, and P < 0.0001 and P = 0.0004 for OS, respectively); nodal status and ER status were independently associated with only DFS (P = 0.0001 and P = 0.0017, respectively).

**DISCUSSION**

Theoretical considerations and in vitro studies suggest that p53 plays a major role in determining cellular chemosensitivity/radiosensitivity (14, 20, 21). Chemotherapy/radiotherapy induces DNA damage which activates p53 function, which in turn blocks the cell cycle to allow DNA repair or apoptosis. However, in vivo studies relating p53 status and chemosensitivity/radiosensitivity are not conclusive. Elledge et al. (22), using p53 immunohistochemical overexpression as a marker of p53 mutation, reported a trend toward a larger benefit from therapy for p53-negative patients. However, in neoadjuvant trials (23, 24), no relationship was found between p53 overexpression and response to therapy. One of the main problems with p53 immunohistochemical studies is that we never know whether p53 overexpression really reflects p53 gene mutation and/or loss of p53 function. A way to investigate the functional status of p53 is to evaluate some of its downstream effectors such as p21.

p53, indeed, exerts its function inducing the transcription of several genes, among which is the p21 gene whose product acts by blocking cyclin-dependent kinases. Therefore, the evaluation of the combined expression of p53 and p21 may provide further insights into the p53-dependent metabolic pathway.

In the present article, we present evidence that the combined evaluation of p53 and p21 expression may provide prognostic information which is more accurate than the evaluation of p53 expression alone. We observed two different and apparently conflicting situations: in node-negative patients and in patients who did not undergo systemic adjuvant therapy, p21+ breast carcinomas were associated with short DFS. Conversely, in node-positive patients and in patients treated with systemic adjuvant therapy, p21+/p53+ tumors were associated with longer DFS and OS than p21−/p53− tumors, which had the worst prognosis.

The association between p21 expression and disease progression and shortened DFS in untreated patients could be related to the fact that, in these cases, p21 may be mainly regulated by factors which may interfere with disease progression in breast cancer. p21 expression can indeed be up-regulated by epidermal growth factor receptor and transforming growth factor B1 (25, 26), which are associated with higher tumor grade and disease progression in breast carcinoma (27, 28). Con-
Fig. 4  DFS (a) and OS (b) curves in the group of 170 patients (140 node positive and 30 node negative) treated with any adjuvant therapy, stratified according to the combined p21/p53 expression. There are 82 p21+/p53- cases (- - -), 36 p21-/p53+ (---), 40 p21+/p53- (-), and 12 p21+/p53+ (---). p21-/p53+ cases have the poorest prognosis ($P = 0.0002$ and $P = 0.00001$ for DFS and OS, respectively).
versely, p21 is suppressed by bcl-2 (29), which is expressed in low-grade tumors (30, 31).

Our present data showing that the p21−/p53+ phenotype may identify a subgroup of patients with the worst prognosis among patients who undergo systemic adjuvant therapy support the hypothesis that p21/p53 heterogeneous expression may be of clinical relevance concerning the response to chemotherapy/hormonotherapy. The p21−/p53+ phenotype could correspond to a situation where p53 is overexpressed but lacks transcriptional activity because of mutational or functional inactivation, and hence this phenotype may really reflect complete abrogation of p53 function. This hypothesis seems supported by the fact that, in a previous study, we could show that most breast carcinomas with the p53 gene mutation demonstrated low to absent p21 immunoreactivity (13). These p21−/p53+ cases, with presumably disrupted p53 function, could have an impaired G1 checkpoint (32) and may not be able to activate the apoptotic cascade in response to DNA-damaging drugs. In this view, p21−/p53+ tumors could be more prone to treatment failure with conventional therapy because of abrogated p53 function. It could be suggested that p21−/p53+ cases may probably be more susceptible to treatment with drugs that interfere with the G2-M checkpoint (33, 34). Cases with the p53+/p21+ phenotype could bear either a mutated p53 protein, which accumulates in the nucleus but still retains some transcriptional function and is able to induce p21 and hence probably also apoptosis, or a wild-type p53 protein, which accumulates in the nucleus because of nonmutational events (such as stabilization by cellular or viral products, altered degradation, high endogenous DNA damage, etc.). However, p21 expression per se should not be considered as predictive of p53-mediated apoptosis, since p53-mediated p21 induction is not directly involved in p53-induced apoptosis (35). Additional in vivo studies are needed to investigate the relationship between the p53 gene mutation and function and p21/p53 expression.

The fact that in untreated patients the p21+ phenotype is associated with short DFS but not with short OS could be explained, suggesting that, although p21 overexpression is a marker of poor prognosis per se, chemotherapy, which is given to patients when they relapse, may reverse the situation because most of these cases may have an intact p53 function, and this may contribute to a better response to chemotherapy.

The observed association of high p21 expression in high tumor and nuclear grades is in keeping with our previous study (13), but is at variance with some experimental models (36) and with data on p21 expression in some other human tumor types such as non-small cell lung carcinomas (10). A tempting hypothesis to explain the high p21 expression in tumors with a high nuclear grade is that nuclear atypia may be a function of the age of the cells, and, in several cell systems, p21 expression increases in an age-dependent way (37). Moreover, recent in vitro data on human breast carcinoma cell lines suggest that cellular atypia can be induced by high p21 expression (38).

The observed lack of an inverse association between p21 overexpression and the expression of the proliferation-related antigen Ki67 is difficult to explain, but is not an uncommon phenomenon. Similar data have been shown for colonic (11) and ovarian (39) carcinomas. The fact that tumors with high levels of Ki67, and hence with a high percentage of cycling cells, may express also high levels of p21 could be related to the presence of other cell cycle regulatory pathways which may bypass the p21-mediated cell cycle block, such as c-Myc or B-myb (40, 41). Induction of p21 may be perhaps necessary but probably not sufficient for inhibition of cell cycle in all cellular conditions (42).

In our previous study, we suggested that p21 could be an interesting prognostic or predictive marker (13). However, in that study the number of investigated cases was too small to perform a separate survival analysis of different groups of homogeneously treated patients, and the most interesting question about the possible role of p21 expression as a predictor of therapeutic response remained unanswered. Now, on the basis of our present data, we can support the hypothesis that the combined evaluation of p53 and p21 expression may provide valuable clinical information for the response to systemic adjuvant therapy: p21−/p53+ tumors may have an impaired p53 function and be less susceptible to conventional adjuvant systemic therapy.

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F. X. Transforming growth factor β induces the cyclin-dependent kinase 
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