

Expression of p27^{KIP1}, p21^{WAF1} and p53 does Not Correlate with Prognosis in Node-negative Invasive Ductal Carcinoma of the Breast

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Abstract. *The expressions of p27^{Kip1} (p27) and p21^{Waf1} (p21) cyclin-dependent kinase inhibitors and p53 were examined in a series of 170 node-negative breast carcinomas (NNBCs) to evaluate their prognostic significance. Low nuclear (p27^{TN}) and cytoplasmic (p27^{TC}) p27 expressions were noted in 66% and 81% of NNBCs, respectively. p21 and p53 overexpressions were detected in 56% and 26%, respectively. Low p27^{TN} was significantly associated with high grade ($p=0.001$), age ≤ 50 years ($p=0.01$), negative hormone receptors ($p<0.001$), low p27^{TC} ($p<0.001$) and p53 overexpression ($p=0.02$). Low p27^{TC} was associated with negative hormone receptors ($p<0.001$). p53 overexpression was associated with high grade ($p<0.001$) and negative hormone receptors ($p<0.001$). p21 overexpression, although not correlated with the examined parameters, was associated with increased disease-free survival in univariate analysis. In multivariate analysis, p27^{TN}, p27^{TC}, p21 and p53 were not associated with disease-free survival or overall survival. These findings argue against the prognostic value of p27, p21 and p53 in NNBC.*

Although breast carcinoma patients are stratified into different prognostic groups with the aid of classic clinicopathological parameters such as the lymph node status (1), tumor size (1, 2) grade (3-5) and histological type (6), the outcome of patients in each stratum is often quite divergent. In the node-negative breast carcinoma (NNBC) group, that

generally has a favorable outcome even without adjuvant therapy, 30% of the patients will die of their disease without adjuvant treatment (7). Since the classic clinicopathological parameters are often not adequate to identify these patients, and the benefit of adjuvant therapy in this setting is debatable (7), additional information provided by novel prognostic factors will assist in the fine-tuning of the prognostication process and the treatment decision-making.

Factors involved in cell cycle control have been shown to be involved in carcinogenesis. The cell cycle progression (reviewed by Cordon-Cardo, 8) occurs *via* the enzymatic function of protein kinases, the cyclin-dependent kinases (CDKs), which are activated after complexing with appropriate cyclins, the regulatory units of the holoenzymes. Each phase of the cell cycle shows a characteristic profile of cyclin-CDK complex expression. Specific inhibitors, the cyclin-dependent kinase inhibitors (CKIs), inactivate each complex. The CKIs are classified into two groups: the INK4 family, which includes p15^{INK4A}, p16^{INK4B}, p18^{INK4C} and p19^{INK4D} and the CIP/KIP family, which includes p21^{WAF1/Cip1} (p21), p27^{Kip1} (p27) and p57^{Kip2}. The INK4 family members inhibit CDK4/6 while the CIP/KIP members are universal CKIs affecting all cyclin-CDK complexes (9).

The first checkpoint in the progression of the cell cycle occurs at the G1-S transition, which is modulated by the function of the cyclin D/CDK and cyclin E/CDK2 complexes. At this point, p27, p21 and p53 proteins have principal regulatory effects (8). p27 was first identified as an inhibitor of cyclin E/CDK2 (10), the complex directly involved in the G1-S transition (11), thus arresting G1-S progression. In addition, low levels of p27 activate cyclin D/CDK complexes, promoting G1-S progression. Therefore, the stoichiometry of p27 binding to these complexes is crucial for S-phase progression (12).

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The first CKI to be identified is p21 (reviewed by Dotto and Coqueret, 13, 14). p21 affects G1-S and G2-M checkpoints as well as DNA transcription, when localized in the nuclei. In addition to cell cycle control, p21 modulates stress response and apoptosis when localized in the cytoplasm. Following DNA damage, p21 transcription is activated by p53 and results in CDK inhibition (15, 16) and transient G1 arrest (17). p53-independent mechanisms of p21 transcription are also well known (18).

The p53 tumor suppressor protein plays a major role in the G1-S transition, by arresting cells in G1. This allows for DNA repair to occur or, if repair is not feasible, p53 induces apoptosis (19-21).

Recently, studies on breast carcinoma have suggested the prognostic importance of p27 (22-29), although others had contradictory results (30-35). The prognostic significance of p53 detected by immunohistochemistry is even more controversial in NNBC, with certain studies advocating its importance (36-39), while others are against it (40-43). The prognostic relevance of p21 expression remains to be elucidated (31, 44-49).

The aim of this work was to evaluate, retrospectively, the expressions of p27, p21 and p53 by immunohistochemistry in a series of NNBCs in order: a) to detect protein expression profiles in the tumor; b) to evaluate any associations between the proteins and the classic clinicopathological parameters; and c) to assess the prognostic significance of these proteins in the group of NNBCs.

Materials and Methods

Patient and tissue samples. This retrospective study included 170 patients with T1-T3, N0, M0 invasive ductal breast carcinoma, for whom paraffin tissue blocks and clinical information were available. The pathology reports were reviewed for data regarding the type of surgery, the greatest tumor diameter, the status of surgical margins and the number of retrieved axillary lymph nodes. Information regarding the status of estrogen and progesterone receptors, adjuvant treatment (chemotherapy, hormonal or radiation therapy) and outcome, *i.e.*, recurrence, metastasis or death, disease-free survival (DFS) and overall survival (OS) was collected from the clinical records.

For each case, representative hematoxylin and eosin-stained slides were reviewed in order to assess the tumor grade, according to the Nottingham modification of the Bloom-Richardson grading system (3), the histological type and the presence/extent of an *in situ* component. The receptor status was examined by immunohistochemistry on paraffin-embedded tissue when such information was not available in the patient's records.

Immunohistochemical analysis. Formalin-fixed, paraffin-embedded 5-µm-thick sections were deparaffinized in xylene, rehydrated in graded alcohols and processed using the streptavidin-biotin-immunoperoxidase method. Briefly, the sections were submitted to antigen retrieval by microwave oven treatment for 10 minutes in 0.01 mol/L citric acid buffer at pH 6.0. This procedure was followed

Table I. *Clinical and histopathological data of patients (n=170).*

Parameter	No. of patients (%)	
Age (years) (median 50, range 24-92)	≤50 years	85 (50)
	>50 years	82 (48)
	Unknown	3 (2)
Surgical treatment	Mastectomy	99 (58)
	Partial mastectomy	19 (11)
	Tumor resection	35 (21)
	Unknown	17 (10)
Tumor size (TNM)	T1	80 (47)
	T2	86 (51)
	T3	1 (1)
	Unknown	3 (2)
Tumor grade	I	28 (16.5)
	II	76 (45)
	III	65 (38)
	Unknown	1 (1)
Histological type	Invasive ductal NOS	154 (90.5)
	Mucinous	6 (3.5)
	Papillary	3 (2)
	Medullary	2 (1.2)
	Apocrine	2 (1.2)
	Metaplastic	1 (1)
	Tubulolobular	1 (1)
	Tubular	1 (1)
Carcinoma <i>in situ</i>	Absent	41 (24)
	Present (≤25%)	49 (29)
	Extensive (>25%)	37 (22)
	Unknown	43 (25)
Hormonal receptor status	Positive	103 (61)
	Negative	42 (25)
	Unknown	25 (15)
Adjuvant treatment	CT only	3 (2)
	HT only	20 (12)
	RT only	2 (1.2)
	CT & HT	42 (25)
	CT & RT	7 (4)
	CT & HT & RT	17 (10)
	RT & HT	24 (14)
	Unknown	55 (32)
Outcome	NED	137 (81)
	Relapse	31 (18)
	Death	20 (12)
Disease-free survival	Median (mo) NRY	Range: 2.10 – 187.6+
Overall survival	Median (mo) NRY	Range: 14.8 – 187.6+

Abbreviations: CT, chemotherapy; HT, hormonal therapy; NED, no evidence of disease; NRY, not reached yet; RT, radiation therapy.

for all the antibodies studied. After cooling, the sections were incubated with 1% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity and, subsequently, with 1% bovine serum albumin (BSA) diluted in Tris-buffered saline (TBS) at pH 7.6 for 20 minutes, to block non-specific binding. The slides were wiped and incubated overnight at 4°C in a humid chamber with the appropriately diluted primary antibody. The antibodies used were anti-p53 protein (DO-7) mouse monoclonal antibody (NCL-p53-DO7; Novocastra Laboratories Ltd., UK; 1:50 dilution), anti-p21^{WAF1} mouse monoclonal antibody (WAF1 (Ab-1); Oncogene

Table II. Immunoreactivity for p27^{TN}, p27^{TC}, p27^{bn}, p21 and p53 in benign and neoplastic breast tissue.

	Score 0	Score 1+	Score 2+	Score 3+	Score 4+
p27 ^{TN} (n=168)		63 (37.5%)	48 (29%)	36 (21%)	21 (12.5%)
p27 ^{TC} (n=160)		115 (72%)	15 (9%)	5 (3%)	25 (16%)
p27 ^{bn} (n=139)		17 (12%)	39 (28%)	57 (41%)	26 (19%)
p53 (n=150)	111 (74%)	4 (3%)	6 (4%)	14 (9%)	15 (10%)
p21 ^{TN} (n=166)		46 (28%)	46 (28%)	55 (33%)	19 (11%)
p21 ^{bn} (n=138)		109 (79%)	24 (17%)	5 (4%)	-

Abbreviations: p27^{TN}, p27 expression in tumor nuclei; p27^{TC}, p27 expression in tumor cytoplasm; p27^{bn}, p27 expression in benign breast tissue; p21^{TN}, p21 expression in tumor nuclei; p21^{bn}, p21 expression in benign breast tissue.

Research Products/Calbiochem, USA; 1:20 dilution), anti-p27^{KIP1} mouse monoclonal antibody (p27 (Ab-2); Oncogene Research Products/Calbiochem; 1:100 dilution), anti-estrogen receptor mouse monoclonal antibody (ER1D5; Immunotech, France; 1:50 dilution), anti-progesterone receptor mouse monoclonal antibody (1A6; Immunotech; 1:30 dilution). The slides were then rinsed three times in TBS and incubated with the reagents of the StrAvigen Multilink HRP-concentrated detection kit (Biogenex Laboratories, USA; 1:80 dilution), according to the manufacturer's instructions. Following three washes with TBS, the peroxidase reaction was developed in freshly prepared 0.025% diaminobenzidine/0.1% hydrogen peroxide in TBS. Finally, the sections were counterstained with hematoxylin. Tissues previously known to be positive for p27^{KIP1}, p21^{WAF1} and p53 were used as positive controls. Sections prepared by substitution of the primary antibody with TBS were used as negative controls.

Immunohistochemical evaluation and scoring. Two pathologists (H.P.K. and V.Z.), blinded to the clinical, histopathological and other immunohistochemical results, independently evaluated the histological and immunohistochemical slides. Benign breast tissue was available for immunohistochemical examination, on the same or on a separate histological section, in 139 cases. The non-neoplastic tissue, which was evaluated for the immunohistochemical staining of the proteins, consisted of either terminal duct-lobular units present in the periphery of the tumor or normal/ectatic ductal structures entrapped within the tumor. The expression of the proteins was not assessed in hyperplastic breast tissue. Each histological section was screened and evaluated for the percentage of benign and neoplastic nuclei displaying immunostaining. p27 tumor cytoplasmic positivity was also recorded. p27 immunoreactivity was stratified as 1+, 2+, 3+ or 4+ if 0-25%, 26-50%, 51-75% and 76-100% of the cells, respectively, displayed nuclear or cytoplasmic staining. The p27 positivity cut-off was set at >50% nuclear or cytoplasmic p27 expression. In order to evaluate the potential participation of p27 intensity in prognosis, the intensity of nuclear p27 expression was also recorded as 1+, 2+ and 3+ for weak, moderate and strong staining, respectively, and a second scoring system was composed of the sum of the proportion and intensity points, ranging from 2 to 7 points. Using this system, a case was considered positive if the sum was 6 or 7, corresponding to tumors expressing strong p27 in >50% of the nuclei or at least moderate p27 in >75% of the nuclei. Immunoreactivity for p21^{WAF1} was stratified as 1+, 2+, 3+ or 4+ if <1%, 1-5%, 6-20% or >20% of the tumour nuclei, respectively, were positive. A carcinoma was considered p21-positive when ≥6% of the nuclei were immunoreactive. Immunoreactivity for p53 was stratified as 0, 1+, 2+, 3+ or 4+ if 0-9%, 10-25%, 26-50%, 51-75%

and 76-100% of the tumor cell nuclei, respectively, were positive. A carcinoma was classified as p53-positive when ≥10% of the nuclei were immunoreactive. Estrogen and progesterone receptor expressions were considered positive if they were noted in ≥10% of the neoplastic nuclei. When evaluation between the observers differed by ≥10% or led to a different stratum of immunoreactivity, the case was reviewed jointly in order to achieve consensus.

Statistical methods. Statistical analysis was performed using the SPSS 11.0 system (SPSS, Inc. Chicago, IL, USA). Fisher's exact test was used for comparing the cell cycle proteins and various demographic and clinical characteristics.

The overall survival (OS) was measured from the date of diagnosis until death from any cause. Surviving patients were censored at the date of last contact. Disease-free survival (DFS) was measured from the diagnosis date until local recurrence, distant relapse, or death from the disease without relapse. The time to event distributions were estimated using Kaplan-Meier curves. Cox proportional hazards models were used to assess the strength of the associations of DFS and OS with various histopathological variables and the expressions of various proteins.

A backward selection procedure, with removal criterion $p > 0.05$, identified the subclass of significant variables among the following: age group (≤ 50 vs. > 50), tumor grade (I vs. II vs. III), receptor status (negative vs. positive), TNM stage (T1 vs. T2-T3), expression of p27 in tumor nuclei (p27^{TN}) ($\leq 50\%$ vs. $> 50\%$), expression of p27 in benign tissue (p27^{bn}) ($\leq 50\%$ vs. $> 50\%$), cytoplasmic expression of p21 ($< 6\%$ vs. $\geq 6\%$), p53 overexpression ($< 10\%$ vs. $\geq 10\%$) and expression of p27 in tumor cytoplasm (p27^{TC}) ($\leq 50\%$ vs. $> 50\%$).

Results

The clinical and histopathological data are summarized in Table I. The median patient follow-up was 99 months (15-188 months). A median of 16 (range 2-38) lymph nodes were examined per case in 133 cases in which the number of examined lymph nodes was stated. The results of the immunohistochemical evaluations for the p27, p21 and p53 proteins are presented in Table II.

Figures 1 and 2 show the expression of p27 in tumor nuclei (p27^{TN}) and tumor cytoplasm (p27^{TC}), respectively. p27^{TN} was mainly heterogeneous in terms of both topography within a tissue section as well as staining intensity, an observation in agreement with previous reports (50). p27^{TC} was weak in all

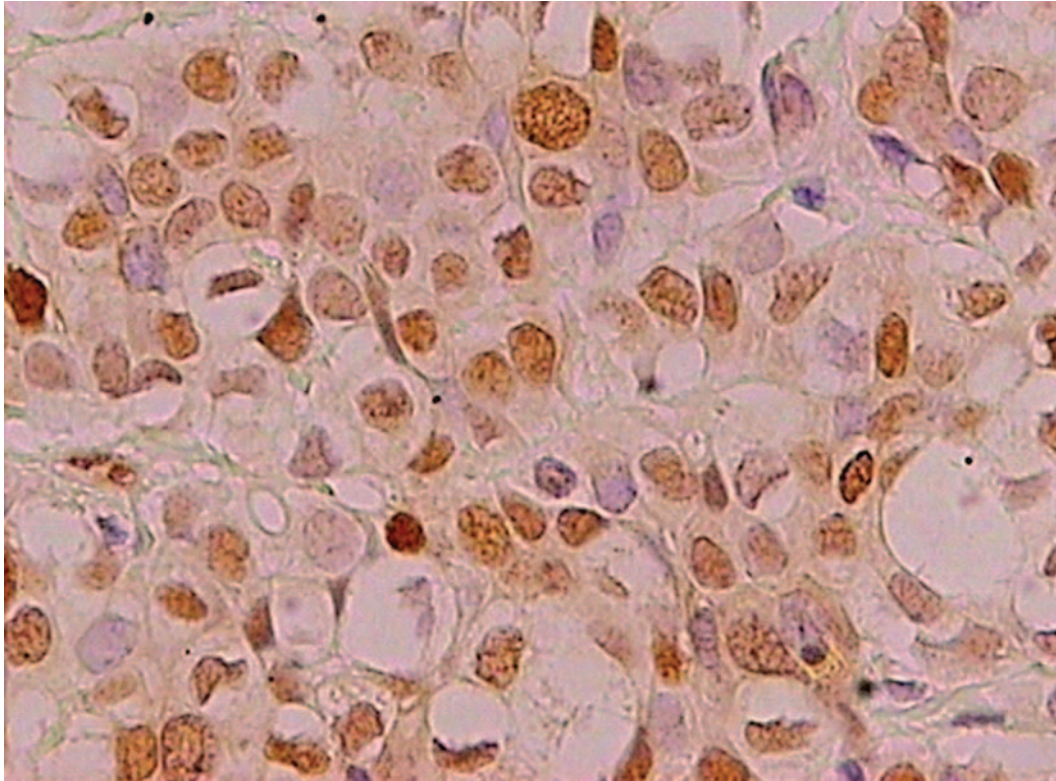


Figure 1. Breast carcinoma displaying weak to strong nuclear p27 expression in >75% of the neoplastic cells (4+).

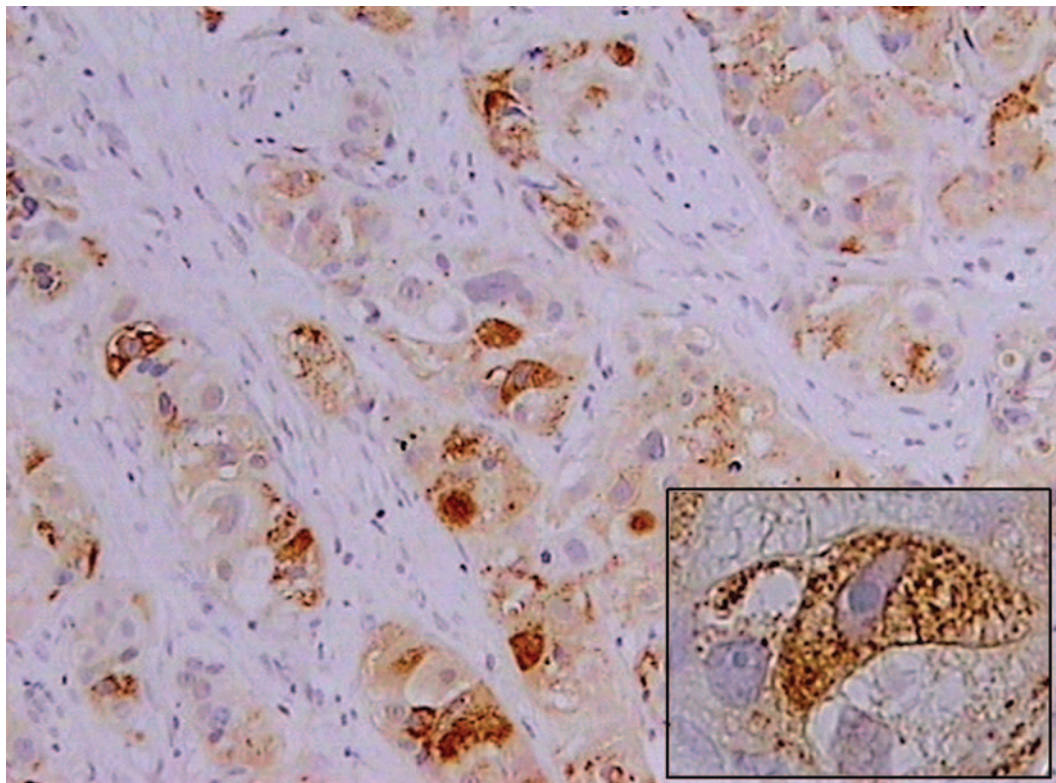


Figure 2. Breast carcinoma displaying weak, diffuse (4+) cytoplasmic p27 expression with strong expression in a minority of the neoplastic cells (inset).

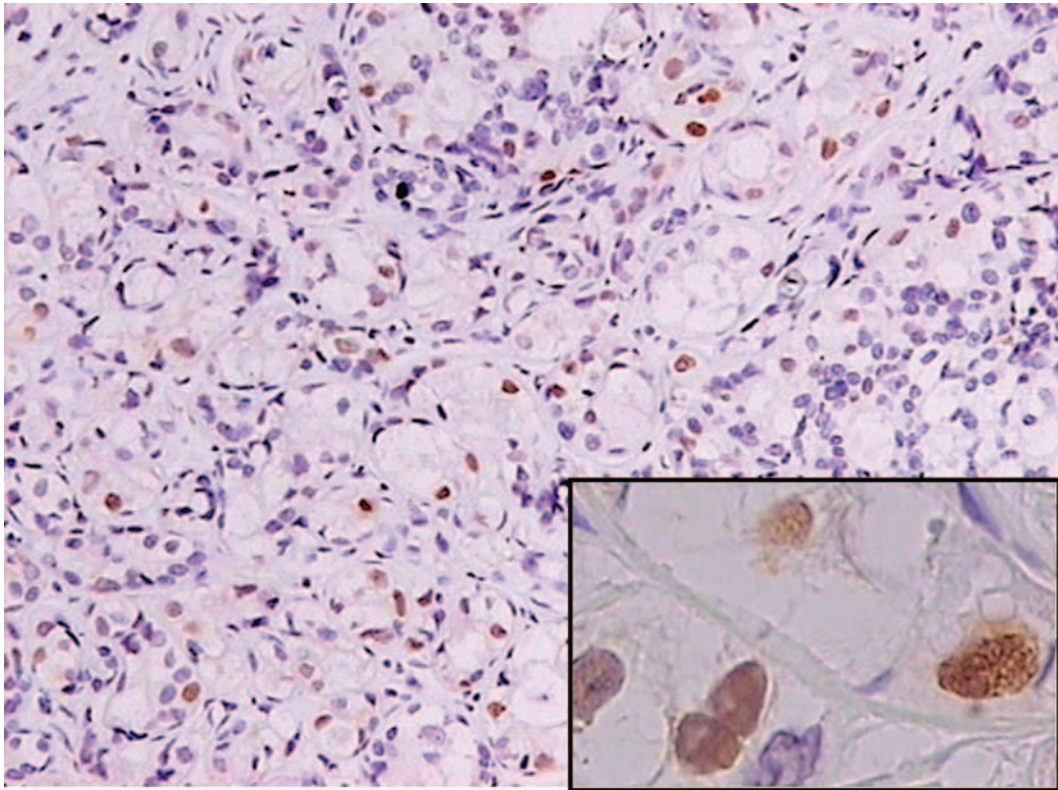


Figure 3. Breast carcinoma displaying weak to moderate nuclear p21 expression in >20% of the neoplastic cells (4+).

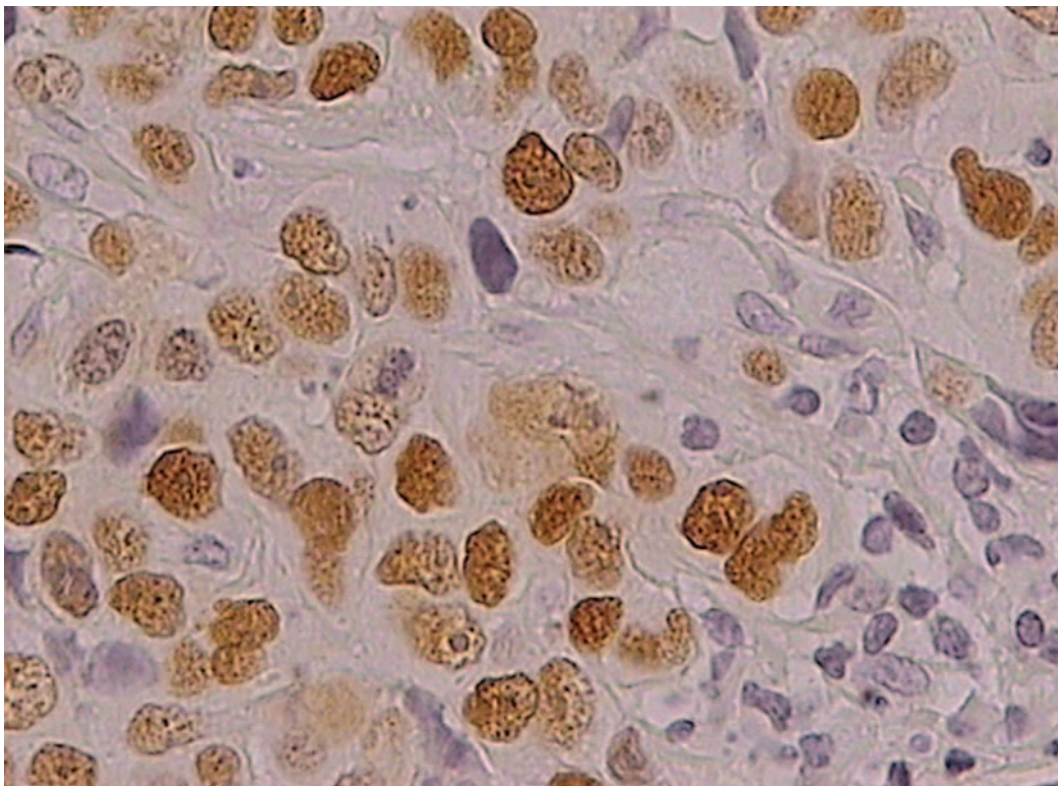


Figure 4. Breast carcinoma displaying moderate to strong, diffuse (4+) nuclear p53 expression.

but 6 tumors, which showed moderate to strong granular cytoplasmic staining. In contrast, p27TC was always more intense and extensive in the *in situ* component of the tumors. Neoplastic nuclei compared to adjacent benign tissues expressed higher levels of p27TN in 22, equal in 38 and lower in 78 cases. p27TC compared to adjacent p27bn was higher in 15, equal in 26 and lower in 90 cases. The median expressions of p27TN, p27TC and p27bn were 2+, 1+ and 3+, respectively. Low p27TN, p27TC and p27bn expressions (in $\leq 50\%$ of tumor nuclei, tumor cytoplasm or benign nuclei) were noted in 66% and 81% of the carcinomas and 40% of the benign breast tissue. Tables III and IV show the statistical analysis data for p27TN and p27TC. Low p27TN was significantly associated with grade III tumors (48% vs. 19%, $p=0.001$), age ≤ 50 years (58% vs. 37.5%, $p=0.01$), negative hormonal receptor status (40% vs. 8%, $p<0.001$), low p27TC (10% vs. 36.5%, $p<0.001$) and p53 overexpression (33% vs. 15%, $p=0.02$). The rate of patients with negative hormonal status was significantly higher among tumors with low p27TC expression (37% vs. 4%, $p<0.001$).

Figure 3 shows the expression of p21 by the tumor. Similarly to p27, p21 immunostaining was mainly heterogeneous. Low p21 ($<6\%$) was noted in 56% of the carcinomas and 96% (133/138) of the benign breast tissue. No cytoplasmic expression of p21 was observed. The statistical analysis data for the p21 protein are presented in Table V. No statistically significant associations were found between p21 protein levels in the tumor and the various demographic, clinical or histopathological covariates, or the other proteins examined. However, the rate of disease relapse was significantly higher among patients with low p21 protein levels in the tumor (25% vs. 11%, $p=0.03$).

Figure 4 shows the expression of p53 by the tumor. p53 overexpression was detected in 26% of the tumors, displaying some topographic heterogeneity. The tumor cytoplasm and benign breast epithelium were always negative for p53. The statistical analysis data for the p53 protein are presented in Table VI. p53 overexpression was significantly associated with grade III tumors (82% vs. 24%, $p<0.001$) and negative hormonal receptor status (56% vs. 16%, $p<0.001$).

Tables VII and VIII show the association of the examined parameters with DFS and OS in univariate analysis. High p21 levels in the tumor significantly decreased the hazard of disease progression by 63% compared to low levels (low vs. high: HR=0.37, 95% confidence interval (C.I) 0.17 – 0.83, $p=0.02$). No significant associations were found between the examined parameters and OS ($p>0.05$ in all cases).

The results of multivariate analysis including age group (≤ 50 vs. >50), tumor grade (I vs. II vs. III), receptor status (negative vs. positive), TNM stage (T1 vs. T2-T3), expression of p27 in the tumor nuclei (p27TN) ($\leq 50\%$ vs. $>50\%$), expression of p27 in the tumor cytoplasm (p27TC) ($\leq 50\%$ vs. $>50\%$), expression of p27 in benign tissue (p27bn) ($\leq 50\%$

vs. $>50\%$), cytoplasmic expression of p21 ($<6\%$ vs. $\geq 6\%$) and p53 overexpression ($<10\%$ vs. $\geq 10\%$), revealed that high p21 levels were still associated with a significantly decreased risk of disease progression (low vs. high: HR=0.4, 95% confidence interval (C.I) 0.14 – 1.13, $p=0.08$).

Using the summing score of proportion and intensity for p27TN expression, 25(15%) tumors were positive. The summing score of p27 intensity and proportion of immunoreactivity was, also, not associated with DFS and OS.

Discussion

In this study, the p27, p21 and p53 proteins were evaluated by immunohistochemistry, and their expressions were associated with clinicopathological parameters and outcome in NNBC.

The evaluation of p27 by immunohistochemistry is a reliable method for detection of cellular p27 protein status (22, 50). Benign epithelial breast elements, entrapped or surrounding the tumors, expressed high levels of p27 in most cases. By contrast, 66.5% of carcinomas expressed low levels of p27 in tumor nuclei (p27TN), a finding supporting the hypothesis that the reduction of p27 expression participates in breast carcinogenesis. Previous studies reported decreased p27 immunoreactivity in 30-87% of breast tumors (22-28, 30-33, 35). The decreased p27 immunoreactivity in this study is placed at the higher end of this range, in agreement with other investigators who stated that low levels of p27 were noted more frequently in NNBCs (32, 51) and contrary to studies showing an association of p27 loss with positive nodal status (26, 28).

In agreement with previous reports, low p27TN is associated with parameters indicative of an aggressive tumor profile such as high tumor grade (22, 25, 28, 30-32, 35), negative hormone receptors (22, 24, 25, 29-32, 34) and p53 overexpression (28, 29, 31). Yet, other authors have not associated p27 with hormone receptor status (28) or with p53 expression (25, 32). Decreased p27TN was also associated with young patient age, unlike a previous report (35). No association of p27TN with tumor size was noted, as in previous reports (25-27, 29, 32, 34, 35).

The prognostic significance of p27 in breast carcinoma has attracted the attention of investigators in recent years. These studies are summarized in Table IX. Three studies originally noted an independent prognostic significance for p27TN. Catzavelos and colleagues associated low ($<50\%$) levels of p27TN with decreased DFS but not with OS in multivariate analysis (22). Porter and colleagues noted that the loss of p27TN was associated with increased mortality in a large group of young women with breast carcinoma in multivariate analysis but, in NNBC patients, the association was significant in univariate analysis only (23). Tan and coworkers reported that T1a and b breast carcinomas lacking p27 were associated with lower OS in multivariate analysis, but this study does not allow

Table III. Clinicopathological parameters in relation to p27 expression in tumor nuclei (p27TN).

	p27TN ≤50%		p27TN >50%	
Age (years) (n=165)				
median (range)	49 (24-92)		55 (34-78)	
Tumor size (cm) (n=156)				
median (range)	2.5 (0.5-6)		2 (0.3-4)	
TNM stage (n=165)	No.	(%)	No.	(%)
T1	48	(44)	31	(56)
T2-T3	62	(56)	24	(44)
Histology grade (n=167)	No.	(%)	No.	(%)
I	15	(14)	13	(23)
II	42	(38)	33	(58)
III	53	(48)	11	(19)
Hormonal receptor status (n=143)	No.	(%)	No.	(%)
Negative	37	(40)	4	(8)
Positive	56	(60)	46	(92)
p27TC (n=159)	No.	(%)	No.	(%)
Negative (≤50%)	96	(90)	33	(63.5)
Positive (>50%)	11	(10)	19	(36.5)
p21 (n=165)	No.	(%)	No.	(%)
Negative (<6%)	59	(55)	33	(58)
Positive (≥6%)	49	(45)	24	(42)
p53 (n=149)	No.	(%)	No.	(%)
Negative (<10%)	64	(67)	46	(85)
Positive (≥10%)	31	(33)	8	(15)
Relapse (n=168)	No.	(%)	No.	(%)
Yes	20	(18)	11	(19)
No	91	(82)	46	(81)
Death (n=168)	No.	(%)	No.	(%)
Yes	15	(13.5)	5	(9)
No	96	(86.5)	52	(91)

Abbreviations: p27TN, p27 expression in tumor nuclei; p27TC, p27 expression in tumor cytoplasm.

definitive conclusions to be drawn for node-negative tumors, since node-negative and Nx carcinomas were analyzed together (24). In series of entirely or predominantly node-positive breast carcinoma (25, 27, 29), the prognostic significance of p27 for OS or DFS/OS was demonstrated by multivariate analysis. Wu and coworkers (28) noted a prognostic significance for DFS and OS by univariate and multivariate analysis in both node-negative and node-positive breast carcinomas. Contrary to these findings, Barbareshi and co-workers noted the absence of prognostic significance in a series of breast carcinomas, while in the subgroup of NNBCs, high rather than low levels of p27 indicated poor prognosis (32). Gillett and colleagues (30) noted an association of p27 expression with DFS and OS in univariate, but not in multivariate, analysis, while Han and coworkers (26) found an association of p27 with DFS, but not OS, in univariate analysis. In grade I invasive ductal breast carcinomas, p27 did not predict outcome (33). In a series of 77 NNBCs (31), the overexpression of p27 (in more than 25% of the neoplastic

Table IV. Clinicopathological parameters in relation to p27 expression in tumor cytoplasm (p27TC).

	p27TN ≤50%		p27TN >50%	
Age (years) (n=157)				
median (range)	50 (24-92)		55 (37-78)	
Tumor size (cm) (n=149)				
median (range)	2.3 (0.5-6.0)		2.5 (0.3-4.0)	
TNM stage (n=157)	N	(%)	N	(%)
T1	62	(49)	13	(43)
T2-T3	65	(51)	17	(57)
Histology grade (n=159)	N	(%)	N	(%)
I	18	(14)	6	(20)
II	56	(43)	16	(53)
III	55	(43)	8	(27)
Hormonal receptor status (n=136)	N	(%)	N	(%)
Negative	40	(37)	1	(4)
Positive	69	(63)	26	(96)
p27TN (n=159)	N	(%)	N	(%)
Negative (≤50%)	96	(74)	11	(37)
Positive (>50%)	33	(26)	19	(63)
p21 (n=157)	N	(%)	N	(%)
Negative (<6%)	70	(55)	17	(57)
Positive (≥6%)	57	(45)	13	(43)
p53 (n=143)	N	(%)	N	(%)
Negative (<10%)	80	(70)	24	(83)
Positive (≥10%)	34	(30)	5	(17)
Relapse (n=160)	N	(%)	N	(%)
Yes	21	(16)	8	(27)
No	109	(84)	22	(73)
Death (n=160)	N	(%)	N	(%)
Yes	16	(12)	3	(10)
No	114	(88)	27	(90)

Abbreviations: p27TN, p27 expression in tumor nuclei; p27TC, p27 expression in tumor cytoplasm.

cells) showed a trend towards better prognosis, but did not reach significance. A large prospective series of 286 NNBCs, receiving only locoregional treatment, noted the absence of a prognostic significance for p27 (34). Similarly, another large prospective series of 461 patients, including 198 NNBCs, did not demonstrate prognostic significance for p27 (35). In agreement with the latter studies, no association of p27TN expression with DFS or OS, either by univariate or multivariate analyses, was identified in this series of 170 NNBCs.

The contradictory prognostic results for p27 may be attributed to the number of patients studied, the administration of various treatment protocols, often not mentioned or analyzed, and short follow-up periods. Furthermore, these discrepancies may result from varying intracellular levels of p27. It is known that high levels of p27, as well as p21, inhibit the cell cycle by disrupting the CDK2 and G1-S transition, while low levels participate in the activation of CDK4/6 in the G1-phase (12). We hypothesized that the intensity of staining might serve as an indirect, although crude

Table V. Clinicopathological parameters in relation to p21 expression in tumor nuclei (p21TN).

	p21TN <6%	p21TN ≥6%
Age (n=163)		
median (range)	50 (24 – 80)	51 (26 – 92)
Tumor size (cm) (n=156)		
median (range)	2.5 (0.5 – 6)	2 (0.3 – 5)
TNM stage (n=163)	No. (%)	No. (%)
T1	41 (45)	36 (50)
T2-T3	50 (55)	36 (50)
Histology grade (n=165)	No. (%)	No. (%)
I	16 (18)	11 (15)
II	37 (41)	38 (51)
III	38 (42)	25 (34)
Hormonal receptor status (n=143)	No. (%)	No. (%)
Negative	31 (40)	17 (26)
Positive	47 (60)	48 (74)
Relapses (n=166)	No. (%)	No. (%)
Relapse	23 (25)	8 (11)
No relapse	69 (75)	66 (89)
Deaths	No. (%)	No. (%)
Dead	13 (14)	7 (9.5)
Alive	79 (86)	67 (90.5)

Table VI. Clinicopathological parameters in relation to p53 expression.

	p53 ≤10%	p53 >10%
Age (n=148)		
median (range)	51 (26 – 80)	50 (35 – 92)
Tumor size (cm) (n=142)		
median (range)	2.25 (0.3 – 6)	2.4 (0.8 – 5)
TNM stage (n=147)	No. (%)	No. (%)
T1	52 (48)	17 (44)
T2-T3	56 (52)	22 (56)
Histology grade (n=149)	No. (%)	No. (%)
I	21 (19)	2 (5)
II	63 (57)	5 (13)
III	26 (24)	32 (82)
Hormonal receptor status (n=129)	No. (%)	No. (%)
Negative	15 (16)	19 (56)
Positive	80 (84)	15 (44)
Relapses	No. (%)	No. (%)
Relapse	19 (18)	6 (15)
No relapse	87 (82)	33 (85)
Deaths	No. (%)	No. (%)
Dead	15 (13.5)	5 (13)
Alive	96 (86.5)	34 (87)

and not definitive, index of the intracellular levels of the protein. Previous studies have considered the staining intensity along with the percentage of positive nuclei (23, 30, 33). In this series, we also examined the effect of staining intensity along with the proportion of positive neoplastic cells in prognosis, but failed to prove a prognostic value for p27 expression by using the summing score of proportion and intensity of immunoreactivity. Evidently, before definitive conclusions on the prognostic significance of nuclear p27 expression are drawn, further examination of p27 in large prospective series with long-term follow-up is necessary.

Investigators have also focused on cytoplasmic p27 expression by the tumors (p27TC). The cytoplasmic localization of p27 may have oncogenic potential because it can initiate its degradation, activate CDK2 and promote the assembly and nuclear accumulation of D-type complexes (14). We observed strong cytoplasmic p27 expression in the majority of malignant peripheral nerve sheath tumors (MPNSTs) (54), a finding not previously commented on to the best of our knowledge. Viglietto *et al.* (52) and Liang and colleagues (53) noted p27TC in 27% and 40% of primary breast cancers and correlated p27TC with higher grade and poor outcome, but not with nodal or hormone receptor status (53) In this study, weak p27TC was observed in 19% of NNBCs and, although p27TC correlated with p27TN and hormone receptors, no association was observed with grade, DFS or OS. Because this study comprised NNBCs only, the discrepancy may be due to differences in the biological profiles of the tumor groups. Evidence of the prognostic significance of p27TC has been

noted in other tumors (54-56), thus the potential prognostic function of p27TC needs further evaluation. Some investigators (52, 53) implicated the phosphorylation of p27 by PKB/Akt and the disruption of p27 nuclear transport as the mechanisms responsible for the absence of p27 from the nucleus and p27 cytoplasmic accumulation in breast carcinomas. In our study, the parallel course of p27TN and p27TC and the frequent absence of p27 from the tumor cytoplasm suggested that the loss of p27 from both the nuclei and cytoplasm is probably related to an increased rate of degradation. Contrary to the findings in breast carcinomas, in a series of MPNSTs (54), we noted decreased nuclear p27 expression and increased strong cytoplasmic p27 accumulation, findings probably explained by the model suggested by Viglietto *et al.* and Liang *et al.* (52, 53).

In the present series, nuclear p21 (p21TN) overexpression was noted in 44% of the tumors, in agreement with other investigators showing p21TN protein overexpression in 25-68% of breast carcinomas (31, 44-47, 49, 57-59). The cut-off positivity levels for p21 varied in different studies, being set at 1% of the neoplastic nuclei (58, 31), 5% (47, 49, 59), 10% (44, 46, 57), or 25% (45), while some investigators analyzed p21 as a continuous variable using an intensity distribution score (60). Furthermore, the heterogeneity in p21 immunoreactivity observed in this and previous studies (44, 45, 49, 57) may also contribute to the variation in results. No association of p21TN with clinicopathological parameters was observed in this series, or in others (49, 58), while such data have varied in the literature. p21 expression has been directly (44, 47, 57, 59),

Table VII. Univariate analysis of examined parameters for disease-free survival.

	HR	95% CI	p-value
Disease-free survival			
Age			
≤50 years	-	-	-
>50 years	1.32	0.65 – 2.68	0.44
TNM stage			
T1	-	-	-
T2-T3	1.47	0.71 – 3.03	0.3
Grade			
I	-	-	-
II	0.78	0.3 – 2.03	0.61
III	0.75	0.28 – 2.03	0.58
p27bn			
≤50%	-	-	-
>50%	0.79	0.35 – 1.82	0.6
p53			
<10	-	-	-
≥10%	1.05	0.47 – 2.36	0.9
p21			
<6%	-	-	-
≥6%	0.37	0.17 – 0.83	0.02
p27TN			
≤50%	-	-	-
>50%	1.08	0.52 – 2.25	0.84
p27TC			
≤50%	-	-	-
>50%	1.53	0.67 – 3.45	0.31
Hormonal status			
Negative	-	-	-
Positive	1.21	0.51 – 2.87	0.66

Abbreviations: p27bn, p27 expression in benign tissue; p27TN, p27 expression in tumor nuclei; p27TC, p27 expression in tumor cytoplasm.

inversely (45, 46), or not (49, 58) related to histological grade. Low p21 expression was associated with lymph node involvement (45, 46), although other investigators observed no such association (44, 47) or even noted the contrary (57) Nuclear p21 expression has been associated with positive estrogen receptors in some studies (31, 46), but not in others (44, 45, 47, 49, 57, 58). p21 overexpression has been associated with large tumor size (57), or has not been correlated with size (44, 46, 47, 49, 58). Notably, the absence of an association between p21 and p27 in this and previous series (31) suggests that the two CKIs act independently of each other.

Data regarding the effect of p21TN on outcome are also contradictory in breast carcinomas. Nuclear p21 expression has been associated with longer DFS by both univariate and multivariate analyses (45, 46) and with longer OS (45). In contrast, p21TN was of no prognostic significance in other studies (31, 48, 49, 58-60), or was associated with shorter relapse-free survival only by univariate analysis (44, 57). In this series, p21TN was associated with DFS ($p=0.02$) on univariate analysis, while no association was observed with

Table VIII. Univariate analysis of examined parameters for overall survival.

	HR	95% CI	p-value
Overall survival			
Age			
≤50 years	-	-	-
>50 years	1.07	0.45 – 2.58	0.87
TNM stage			
T1	-	-	-
T2-T3	1.36	0.55 – 3.34	0.5
Grade			
I	-	-	-
II	0.53	0.15 – 1.89	0.33
III	1.1	0.34 – 3.44	0.9
p27bn			
≤50%	-	-	-
>50%	0.65	0.23 – 1.86	0.42
p53			
<10	-	-	-
≥10%	0.93	0.34 – 2.56	0.89
p21			
<6%	-	-	-
≥6%	0.6	0.24 – 1.5	0.28
p27TN			
≤50%	-	-	-
>50%	0.64	0.23 – 1.76	0.38
p27TC protein levels			
≤50%	-	-	-
>50%	0.73	0.21 – 2.51	0.62
Hormonal status			
Negative	-	-	-
Positive	1.97	0.56 – 6.85	0.29

Abbreviations: p27bn, p27 expression in benign tissue; p27TN, p27 expression in tumor nuclei; p27TC, p27 expression in tumor cytoplasm.

OS. p21 participates in multiple cellular pathways, its functions including G1-S and G2-M checkpoint control, DNA transcription, cellular stress responses and apoptosis (13, 14), varying according to its stoichiometry (61). It, thus, appears unlikely that direct conclusions can be drawn on the prognostic function of p21 using immunohistochemistry alone. No cytoplasmic p21 staining was observed in the present study because of the use of an acidic citrate buffer (pH 6.0) for antigen retrieval (48).

Overexpression of p53 has been noted in 14-50% of breast carcinomas (25, 28, 31, 33, 38, 39, 41, 46, 47, 49, 58, 59) while, in this series, p53 overexpression was detected in 26% of NNBCs. The cut-off positivity levels for p53 protein overexpression were established at 1%(31), 5% (41, 47), 10% (23-25, 32, 49, 59), 15% (28, 44, 45, 57), 20% (39) or 75% (38) of the neoplastic nuclei. The overexpression of p53 has been associated with an intense proliferation rate (39), high tumor grade (28, 31, 38, 39, 41, 45, 46, 49) and negative hormonal receptor status (28, 39, 41, 45, 46, 49). In this study, p53 overexpression was also associated with high grade and

Table IX. Review of data regarding prognostic significance of nuclear p27 expression in breast carcinoma.

Author/ yr	Study type	# pts	LN status	Adjuvant Rx	p27 antibody	Positivity cut-off	DFS	OS	Median F/U
Catzavelos, 1997	R	168	N-:75 N+:81	N-:HT N+:CT/HT	Mp27, Transduction	>50%	U:p=0.005 M:p=0.01	U:p=0.05 M:NS	NA
Porter, 1997	R, pts< 45 yrs	246	NA	NA	Polyclonal p27	Intermed. /high vs. low	NA	N-:U:p=0.01 N+:U:p=0.007 All:M:p<0.001	5.2 yrs
Tan, 1997	R, T1a,b tumors	202	N-:83 N+:19 Nx:100	N-,Nx:53% N+:79%	Mp27, Transduction	≥50%	NA	U:p=0.02 M:p=0.03	65.9 mos
Chu, 1999	NA	169	N-:69 N+:100	NA	Mp27, Transduction	>30%, 5-30%, <5%	NA	U:p=0.0001 M:p=0.0096	#69.6 mos
Gillett, 1999	NA	189	N-:63 N+:126	NA	Mp27, 53G8	>50% strong or >75% moderate	U:p=0.0025 M:NS	U:p=0.001 M:NS	16.7 yrs
Han, 1999	R	68	N-:33 N+:35	CT	Mp27, G173-524 Pharmingen	≥20% (median)	U:p=0.029 M:NP	U:p=0.052 M:NP	46 mos
Reed, 1999	P	77	N-:77	NA	K2502, Transduction	≥1 and ≥25	NS	NS	#163 mos
Tsuchiya, 1999	NA	102	N+:102	CT, HT	Mp27, Transduction	>50%	U:p=0.0023	U:p=0.0079 M:p=0.0218	NA
Wu, 1999	NA	181	N-:83 N+:98	CT:71%	Mp27 Pharmingen	>50%	U:p=0.0001 N-:M:p=0.003 N+:M:p=0.022	U:p=0.0012 N-:M:p=0.049 N+:M:p=0.02	5 yrs
Barbareshi, 2000	R	512	N-:249 N+:262	CT, HT, RT	K2502, Transduction	>50%	U:NS	N-:M:p=0.04* N+:M:p=NS	115 mos
Leong, 2000	Grade I	148	N-:81 N+:61 Nx:6	CT, HT	53G8	>50% strong or>75% moderate	NS	NS	11.3 yrs
Volpi, 2000	P	286	N-:286	+/- RT	Clone 57, Transduction	≥60% (median)	NS	NA	74 mos
Nohara, 2001	R	216	N-:11 N+:105	NA	Clone 57, Transduction	≥62.4% (median)	M:p<0.05	M:p<0.05	NA
Spataro, 2003	P	461	N-:198 N+:263		Clone 57, Transduction	≥50%	N-:M:NS N+:M:NS	NA	13.3 yrs

DFS, disease-free survival; F/U, follow-up period; LN, lymph node; M, multivariate analysis; Mp27, monoclonal p27; mos, months; N-, node-negative; N+, node-positive; NA, not available; NP, not performed; NS, not significant; OS, overall survival; P, prospective; R, retrospective; Rx, treatment; U, univariate analysis; yr(s), years(s). *High rather than low p27 levels were associated with poor prognosis, # mean value.

negative hormone receptors. The overexpression of p53 has been associated with decreased DFS and OS by multivariate analysis (28, 38, 45, 57). In contrast, the independent prognostic significance of p53 has not been proved in this or previous series of NNBCs (31, 39, 40, 42, 49, 59), or in others including both node-positive and -negative breast carcinomas (23-25, 41, 44, 46, 59). Despite the increasing number of studies disproving the prognostic significance of immunohistochemically-detected (p53) protein, it should be noted that the detection of p53 gene mutations has been shown to be of prognostic importance (62-64).

Following DNA damage, p53 induces p21 transcription, resulting in CDK inhibition (15, 16) and transient G1 arrest (17). Favoring the p53-dependent pathway of p21 transcription is the study of Jiang and colleagues (45), who noted an association of p53 overexpression with p21 loss. Other investigators, as well as the present study, noted an absence of

association between p53 and p21 (31, 44, 47, 49), or even the unexpected positive correlation of p53 overexpression with p21 (58, 59) indications of the induction of p21 by p53-independent mechanisms, as previously suggested (18). In this series, p53 overexpression was associated with low p27 expression, similarly to previous reports (28, 31). Both these findings are associated with, and may reflect, high-grade tumors. Unlike these results, Chu *et al.* (25) did not observe any association between p27 and p53 expressions.

The weaknesses of this study include its retrospective nature, the administration of adjuvant treatment in the majority of patients, altering the course of the disease, and the use of different treatment regimens. Due to the latter, we were not able to investigate the potential relationships between the expression of the proteins and therapeutic responses. Further research on the prognostic significance of these proteins, as well as on their potential implications in

standard (35) and novel therapeutic manipulations, such as with agents targeting HER-2 and EGFR (65), remain to be further defined.

In conclusion, although nuclear p27 and p53 expressions were associated with parameters of an aggressive tumor profile, such as high-grade and negative hormone receptor status, they did not affect the outcome in node-negative invasive ductal breast carcinoma. The expression of p21 was not associated with the clinicopathological parameters or outcome, possibly reflecting its complex function in several intracellular pathways appearing to function independently of p27 and p53. It is unclear, at this point, whether the inverse association of p27 with p53 resulted from the relationship of each protein with grade or from their ties in intracellular pathways.

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