Low p27 Expression Predicts Early Relapse and Death in Postmenopausal Hormone Receptor–Positive Breast Cancer Patients Receiving Adjuvant Tamoxifen Therapy

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Abstract

Purpose: Previously, we have shown that p27 may be a potential predictive biomarker for the selection of premenopausal women with early-stage hormone-responsive breast cancer for adjuvant endocrine therapy. The purpose of the present study was to assess the clinical relevance of p27 expression in postmenopausal hormone receptor–positive breast cancer patients who were treated with adjuvant tamoxifen therapy.

Experimental Design: We determined the expression of p27 by immunohistochemistry in the surgical specimens of breast carcinoma patients who had been enrolled in Austrian Breast and Colorectal Cancer Study Group Trial 06 and received tamoxifen for 5 years. Early relapse and death within the first 5 years of follow-up were analyzed using Cox models adjusted for clinical and pathologic factors.

Results: p27 expression was high (>70% p27-positive tumor cells) in 252 of 483 (52%) tumor specimens and was associated with favorable outcome of the patients. Women with high p27 expression had a significantly longer disease-free survival (adjusted hazard ratio for relapse, 0.22; 95% confidence interval, 0.11-0.42; $P < 0.001$) and overall survival (adjusted hazard ratio for death, 0.39; 95% confidence interval, 0.21-0.72; $P = 0.002$) as compared with women with low p27 expression.

Conclusion: Low p27 expression independently predicts early relapse and death in postmenopausal women with early-stage, hormone receptor–positive breast cancer who received adjuvant tamoxifen for 5 years. (Clin Cancer Res 2009;15(18):5888–94)

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Note: Other investigators of the Austrian Breast and Colorectal Cancer Study Group who participated in the study are listed in the Appendix.

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The cell cycle regulator $p27^{Kip1}$ (p27) is a key factor of the G_1- to S-phase transition in the cell cycle and contributes to tumor progression (1). p27 is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors and is present at high levels in quiescent cells (2, 3). The gene encoding p27 is rarely mutated but p27 is functionally inactivated in a majority of human cancers through enhanced p27 proteolysis, through sequestration by cyclin D/cyclin-dependent kinase complexes, and by cytoplasmic mislocalization (4–10). p27 proteolysis is mediated by SKP2, a component of the SCF^{Skp2} ubiquitin ligase (5). Data from several population-based and clinical studies indicate that abnormal expression of p27, as determined by immunohistochemistry, is associated with poor clinical outcome in many human cancers, including breast cancer (11). The prognostic effect of p27 could depend on the patients' treatment because it is likely that this protein may affect the patients' response to various anticancer drugs (12–14). Moreover, there is evidence from preclinical studies that p27 is essential for cell cycle arrest by tamoxifen and other antiestrogenic drugs (15,

Translational Relevance

Tamoxifen has been the standard of care for women with hormone receptor–positive breast cancer. Although aromatase inhibitors are a slightly more effective endocrine strategy against hormone-dependent breast cancer, tamoxifen still plays an important role in the adjuvant endocrine treatment. Therefore, the ability to predict outcome of tamoxifen treatment may improve the management of hormone-dependent breast cancer. Previously, we have shown that p27 may be a potential predictive biomarker for the selection of premenopausal women with early-stage hormone-responsive breast cancer for adjuvant endocrine therapy. In the present study, we showed that low p27 expression independently predicts early relapse and death in postmenopausal women with early-stage, hormone receptor–positive breast cancer who received adjuvant tamoxifen for 5 years.

16). Previously, we reported that high p27 expression was independently associated with improved relapse-free and overall survival among patients treated with combined hormonal therapy (17). These findings were recently confirmed by results from a large translational research study of the Southwest Oncology Group-Intergroup Trial S9313 (18). In this particular study of the Intergroup, low p27 expression was associated with poor prognosis, especially among patients with hormone receptor–positive tumors. As part of the S9313 trial, 5 years of tamoxifen treatment was prescribed for all postmenopausal women and all hormone-responsive premenopausal women after chemotherapy (18). In another smaller study, low p27 expression was associated with poor survival in tamoxifen-treated patients but not in patients treated with surgery alone (19). These data in patients with hormone-responsive breast cancer indicated that p27 expression was at least prognostic and may also be predictive of response to hormonal therapy.

In the present study, we evaluated the association of p27 protein expression with disease-free and overall survival in patients with early-stage hormone-responsive breast cancer who were enrolled in an Austrian Breast and Colorectal Cancer Study Group (ABCSG) randomized clinical trial and treated with adjuvant tamoxifen therapy.

Experimental Design

Patients. The present investigation is part of the ABCSG translational research program (abcsg.research). All patients included in this study had participated in ABCSG Trial 06 and received tamoxifen for 5 y. The objective of ABCSG Trial 06 was to determine whether addition of aminoglutethimide to tamoxifen improves the outcome of postmenopausal patients with hormone receptor–positive, early-stage breast cancer. In brief, a total of 2021 postmenopausal breast cancer patients were randomly assigned to receive either tamoxifen for 5 y or tamoxifen in combination with aminoglutethimide for the first 2 y of treatment. The primary end points were disease-free survival and overall survival.

Inclusion criteria and the main clinical results were reported previously (20).

Specimen collection and immunostaining for p27. A block containing representative formalin-fixed, paraffin-embedded tumor tissue was available from 483 of 996 patients allocated to the tamoxifen arm of ABCSG Trial 06 and sent to the central lab of abcsg.research at the Medical University of Vienna. In total, 19 centers contributed tumor samples (see Appendix A). All tumor specimens were obtained at the time of surgery before adjuvant therapy. Paraffin blocks were stored at room temperature and were identifiable only by an identification number assigned to each patient at randomization. Approval was obtained from the local institutional review boards. From each tumor block, sections were cut at 4 μm. One section was stained by H&E to confirm the presence of invasive carcinoma histologically, and further sections were used for immunohistochemical analyses as described previously (21).

Immunohistochemistry was done and evaluated in the abcsg.research central lab at the Medical University of Vienna by means of a standard protocol (17).

Briefly, tissue sections were deparaffinized and rehydrated. After heating for 10 min in 10 mmol/L citrate buffer (pH 6.0) in a pressure cooker for epitope retrieval, the tissue sections were incubated for 30 min at room temperature with a mouse monoclonal antibody specific for p27 (clone SX53G8, Dako; dilution 1:100). Antibody binding was detected by means of the UltraVision LP detection system according to the manufacturer's recommendations (Lab Vision Corporation). Color development was done by 3,3′-diaminobenzidine and counterstaining by hematoxylin. Sections of breast carcinoma specimens known to express p27 served as external positive controls. Lymphocytes and normal breast epithelium served as internal positive controls.

p27 immunostaining was evaluated by an experienced breast pathologist (M.R.) who was unaware of the patients' clinical data. All invasive tumor cells on each slide were evaluated. Interpretation of the results was limited to the invasive part of the tumor and only nuclear staining was scored as positive. The results were documented as the percentage of p27-stained nuclei regardless of staining intensity.

Statistical analyses. The primary end points of the statistical analyses were disease-free survival and overall survival. Disease-free survival was defined as the interval between the date of surgery and the first evidence of relapse at any site or incidence of

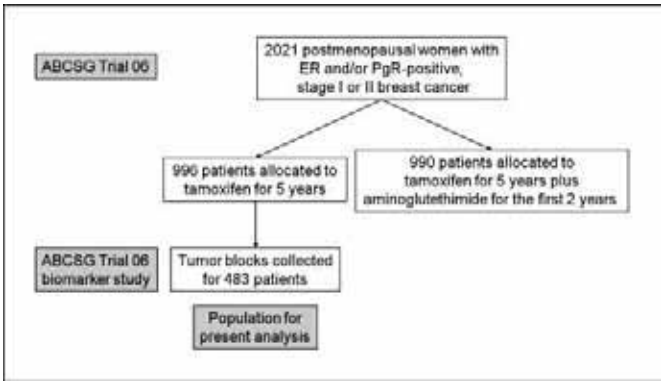

Fig. 1. Study flowchart for the process of tumor block and patient selection.

Table 1. Baseline characteristics of patients with and without tumor blocks and of the 483 patients of the tamoxifen arm of ABCSG Trial 06 analyzed for p27 immunoreactivity

Characteristic	Tamoxifen arm ABCSG Trial 06, *n* = 996 [*n* (%)]	Patients without tumor block, *n* = 513 [*n* (%)]	Patients with tumor block, *n* = 483 [*n* (%)]	*P**	Low p27 expression, *n* = 231 [*n* (%)]	High p27 expression, *n* = 252 [*n* (%)]	*P*
Age, y				0.10			0.18
<51	24 (2)	11 (2)	13 (3)		10 (4)	3 (1)	
51-60	291 (29)	148 (29)	143 (30)		70 (30)	73 (29)	
61-70	411 (41)	229 (45)	182 (38)		85 (37)	97 (39)	
>70	270 (27)	125 (24)	145 (30)		66 (29)	79 (31)	
Tumor size				0.57			0.18
pT_1 (≤2 cm)	576 (58)	290 (57)	286 (59)		127 (55)	159 (63)	
pT_2 (>2 to ≤5 cm)	391 (39)	206 (40)	185 (38)		97 (42)	88 (35)	
pT_3 (>5 cm)	29 (3)	17 (3)	12 (3)		7 (3)	5 (2)	
Lymph node metastases				0.25			0.40
None	620 (62)	332 (65)	288 (60)		134 (58)	154 (61)	
1-3 nodes	252 (25)	120 (23)	132 (27)		61 (26)	71 (28)	
4-10 nodes	92 (9)	48 (9)	44 (9)		24 (10)	20 (8)	
>10 nodes	32 (3)	13 (3)	19 (4)		12 (5)	7 (3)	
Tumor grade				0.12			0.008
G1, G2	779 (78)	391 (76)	388 (80)		174 (75)	214 (85)	
G3	217 (22)	122 (24)	95 (20)		57 (25)	38 (15)	
Estrogen receptor				0.14			0.49
Negative	38 (15)	7 (1)	13 (3)		5 (2)	8 (3)	
Positive	976 (98)	506 (99)	470 (97)		226 (98)	244 (97)	
Progesterone receptor				<0.001			0.09
Negative	195 (20)	74 (14)	121 (25)		66 (29)	55 (22)	
Positive	801 (80)	439 (86)	362 (75)		165 (71)	197 (78)	
Cyclin D1				—			<0.001
Low (≤10%)	—	—	185 (38)		125 (54)	60 (24)	
High (>10%)	—	—	298 (62)		106 (46)	192 (76)	

NOTE: Percentages may not total 100 because of rounding.
**P* values were calculated with the χ^2 test.

contralateral breast cancer. As in the parent trial, patients who died because of confirmed reasons other than breast disease, without having experienced breast cancer recurrence, were considered as censored for all analyses. The early period was defined by censoring all observation times more than 5 y with a value of 5 y.

To identify any selection bias, the baseline characteristics of patients with or without tumor blocks were compared using the χ^2 test. All multiple regression models (i.e., logistic and Cox) mentioned below were adjusted for age, tumor size, lymph node status, tumor grade, estrogen receptor status, progesterone receptor status, and cyclin D1 expression.

Baseline data according to dichotomized p27 status (≤70%, >70%) were compared in univariate analyses using the χ^2 test and in a multiple logistic model. Survival rates were estimated with the use of the Kaplan–Meier method. The prognostic value of p27 was studied using univariate and multiple Cox models. To understand the functional form of the p27 effect and, subsequently, to justify a potential cutoff point for p27, restricted cubic spline functions were used to represent p27 in the Cox model adjusted for clinical and pathologic factors (22). Five so-called "knots" were placed at or close to p27 percentiles 5, 27.5, 50, 72.5, and 95.

All reported *P* values are results of two-sided tests. $P \leq 0.5$ was considered statistically significant. All statistical analyses were done using SPSS software version 15.0 (SPSS, Inc.) and SAS statistical software system version 9.1 (SAS, Institute Inc.). For spline modeling, the SAS macro[14] of Heinzl and Kaider (23) was used.

Results

The study group consisted of 483 postmenopausal women with early-stage, hormone receptor–positive breast cancer who had been enrolled in the tamoxifen arm of ABCSG Trial 06. Figure 1 shows the study flowchart. All patients received adjuvant tamoxifen for 5 years. The 483 evaluable patients with tumor blocks and the remaining 513 evaluable patients from the tamoxifen arm of ABCSG Trial 06 without tumor blocks had similar baseline characteristics with the exception of progesterone receptor expression (Table 1).

p27 immunoreactivity ranged from 0% to 100% of the tumor cell nuclei. Figure 2 shows representative examples of p27 immunostaining. Comparisons of p27 expression with clinical parameters including survival of the patients were done with p27 expression as a dichotomized variable classified as high (>70% p27-positive tumor cells) or low (≤70% p27-positive tumor cells). The 70% cutoff was determined on the basis of the spine fitting for p27 as described in Experimental Design.

Of the 483 tumors, 252 (52%) showed high p27 expression. Table 1 compares the characteristics of the patients according to

[14] http://www.meduniwien.ac.at/msi/biometrie/programme/Rcs.htm

p27 expression in a univariate analysis. A multiple logistic regression model showed that p27 expression was significantly associated with tumor grade [odds ratio (OR), 0.51; 95% confidence interval (95% CI), 0.31-0.85; $P = 0.008$] and cyclin D1 expression (OR, 4.19; 95% CI, 2.79-6.29; $P < 0.001$). Data on cyclin D1 expression are reported in detail elsewhere (21).

Within the first 5 years of follow-up, 55 of 483 (11%) patients had relapsed (12 patients with high p27 and 43 patients with low p27) and 54 of 483 (11%) patients had died (19 patients with high p27 and 35 patients with low p27). Tumor size [hazard ratio (HR) for relapse, 2.82; 95% CI, 1.82-4.39; $P < 0.001$], lymph node status (HR for relapse, 2.28; 95% CI, 1.79-2.92; $P < 0.001$), tumor grade (HR for relapse, 2.55; 95% CI, 1.47-4.42; $P = 0.001$), progesterone receptor (HR for relapse, 0.51; 95% CI, 0.30-0.87; $P = 0.02$), cyclin D1 (HR for relapse, 2.12; 95% CI, 1.14-3.94; $P = 0.02$), and p27 (HR for relapse, 0.24; 95% CI, 0.13-0.45; $P < 0.001$) were significantly associated with disease-free survival in the univariate analyses. Age (HR for death, 1.48; 95% CI, 1.06-2.07; $P = 0.02$), tumor size (HR for death, 1.94; 95% CI, 1.23-3.05; $P = 0.004$), lymph node status (HR for death, 1.88; 95% CI, 1.46-2.42; $P < 0.001$), tumor grade (HR for death, 2.14; 95% CI, 1.21-3.77; $P = 0.008$), estrogen receptor (HR for death, 0.23; 95% CI, 0.09-0.57; $P = 0.002$), cyclin D1 (HR for death, 1.84; 95% CI, 1.00-3.38; $P = 0.05$), and p27 (HR for death, 0.48; 95% CI, 0.28-0.84; $P = 0.01$) were significantly associated with overall survival in the univariate analyses, as well.

The independent effect of p27 expression on disease-free survival and overall survival was assessed by Cox models adjusted for age, tumor size, lymph node status, tumor grade, estrogen receptor status, progesterone receptor status, and cyclin D1 expression. In these multivariate analyses, p27 expression was significantly associated with disease-free survival (adjusted HR for relapse, 0.22; 95% CI, 0.11-0.42; $P < 0.001$) and overall survival (adjusted HR for death, 0.39; 95% CI, 0.21-0.72; $P = 0.002$) of the patients (Fig. 3A and B; Table 2).

Because low p27 expression and high cyclin D1 expression independently predict early relapse and death in our study, we combined the two biomarkers. In the multivariate analyses, low p27/high cyclin D1 was significantly associated with shorter disease-free survival (adjusted HR for relapse, 4.65; 95% CI, 2.66-8.13; $P < 0.001$) and overall survival (adjusted HR for death, 2.68; 95% CI, 1.52-4.74; $P = 0.001$) compared with the other three groups (Fig. 3C and D; Table 2).

In addition, we explored the 50% cutoff point from our previous study (17). Using this 50% cutoff, 386 of 483 patients (80%) had tumors with ≥50% p27-positive tumor cells and similar results were observed if p27 was included with this 50% cutoff into the multiple Cox models (adjusted HR for relapse, 0.48; 95% CI, 0.24-0.97; $P = 0.04$; adjusted HR for death, 0.46; 95% CI, 0.22-0.95; $P = 0.04$). Thus, we confirmed the previous results obtained in patients enrolled in ABCSG Trial 05 in an independent patient population from ABCSG Trial 06. In conclusion, p27 independently predicts early relapse and death in postmenopausal women with early-stage, hormone receptor–positive breast cancer who had been treated with tamoxifen for 5 years.

Discussion

In the adjuvant treatment of hormone-responsive breast cancer, 5 years of tamoxifen almost halves the annual recurrence rate and reduces the annual breast cancer death rate by a third (24). Whereas aromatase inhibitors have recently been shown to be even more effective, tamoxifen remains an important part of the endocrine treatment (25–30). However, a considerable fraction of patients do not respond to tamoxifen despite having estrogen receptor–positive tumors (31). These patients may be identified using additional predictive biomarkers and also may need other therapeutic interventions. Therefore, the ability to predict outcome of tamoxifen treatment should significantly improve the management of early-stage breast cancer.

In this study, we showed that low protein expression of p27 predicted early relapse and death of breast cancer patients who had received adjuvant tamoxifen therapy. Women with low p27 expression had a significantly shorter disease-free survival and overall survival than women with high p27 expression. These findings are in line with the results of most previous studies that showed decreased disease-free or overall survival among patients whose tumors had low p27 expression, even though these studies differed in the antibodies used, the scoring systems, and the patient population (32–42). In some studies, p27 expression was not an independent prognostic marker in multivariable analyses or did not maintain statistical significance after long-term follow-up (43–46).

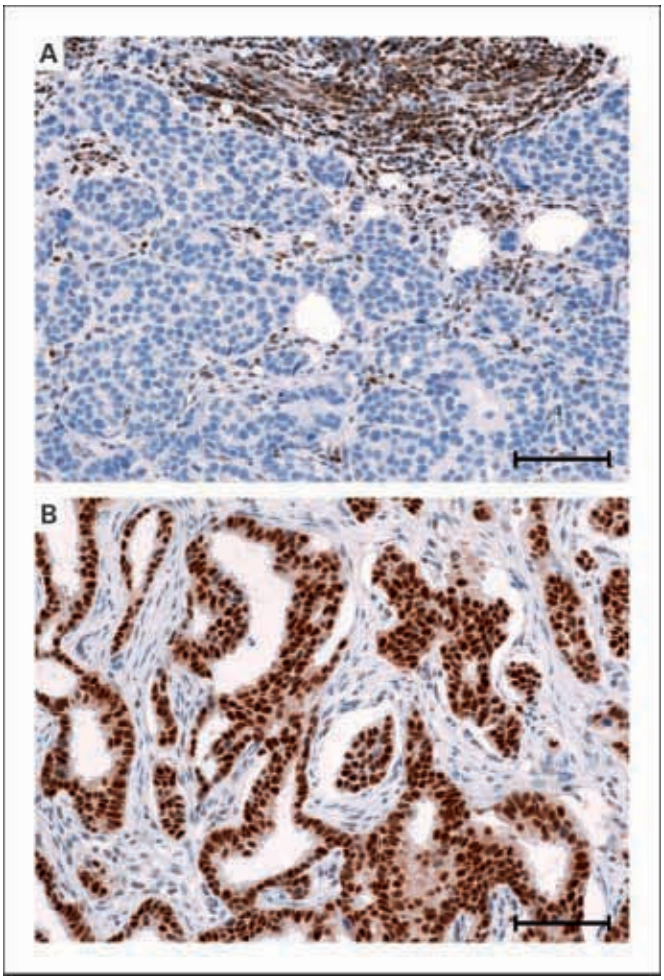

Fig. 2. Examples of p27 immunostaining. A p27-negative breast carcinoma (*A*) and a breast carcinoma with high p27 expression (*B*). Bar, 100 μm.

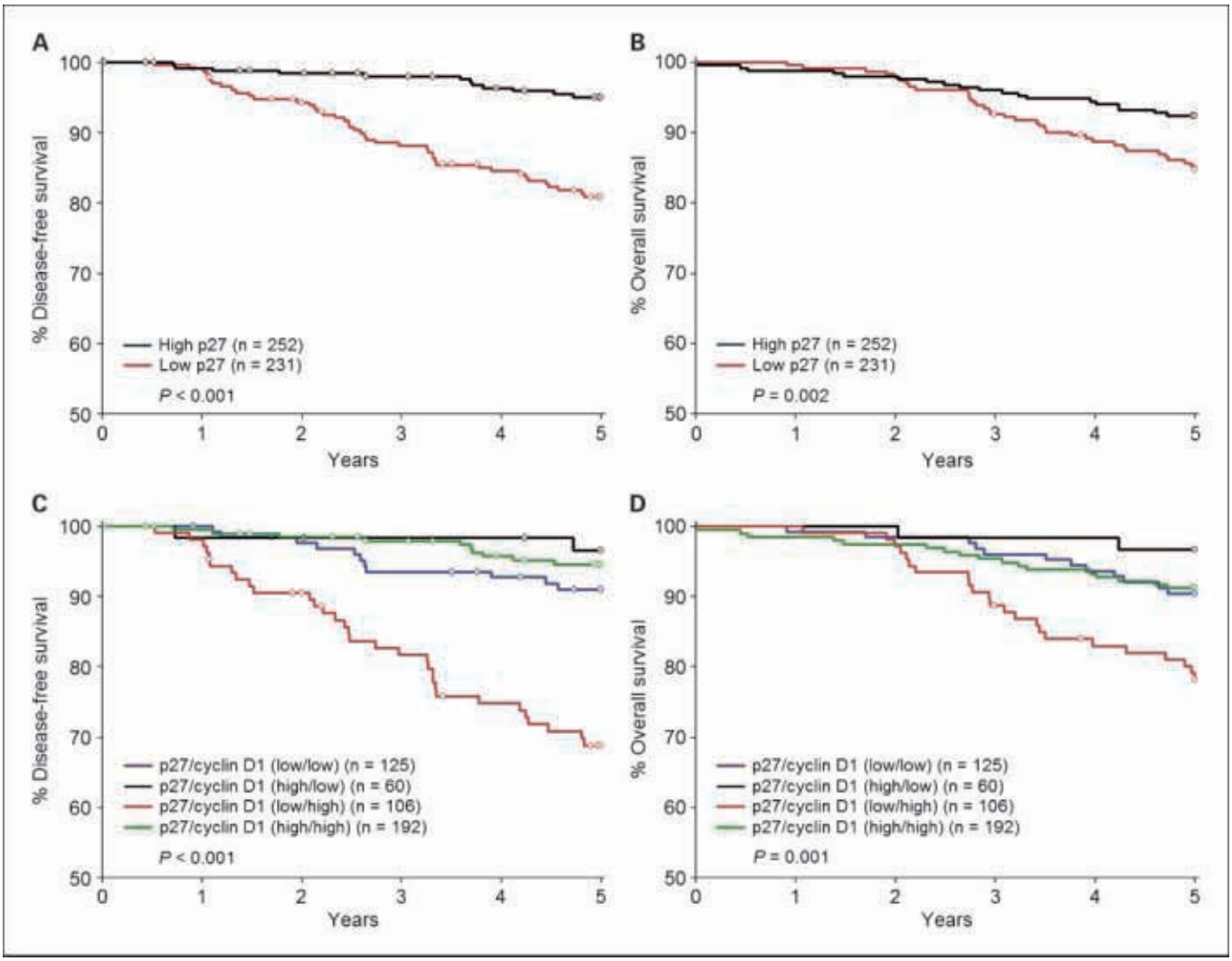

Fig. 3. Kaplan-Meier estimates of the probability of disease-free survival (*A*) and overall survival (*B*) according to dichotomized p27 protein expression (≤70%, >70%) of 483 breast cancer patients enrolled in ABCSG Trial 06 with tissue available for analysis. Patients with low p27/high cyclin D1 expression had a shorter disease-free survival (*C*) and overall survival (*D*) compared with patients with high p27/low cyclin D1, high p27/high cyclin D1, and low p27/low cyclin D1. Adjusted *P* values from multiple Cox models are shown.

The predictive role of p27 has been retrospectively evaluated in the setting of adjuvant clinical trials by us and others. In the ABCSG Trial 05, the analysis focused on premenopausal hormone receptor–positive breast cancer patients with stage I to II disease enrolled into a randomized trial comparing goserelin plus tamoxifen to cyclophosphamide, methotrexate, and fluorouracil (CMF) chemotherapy (17). High expression of p27, as determined by immunohistochemistry, predicted improved relapse-free and overall survival and was also an independent predictor of responsiveness to endocrine therapy. Furthermore, in patients with high p27 expression, endocrine therapy was superior to chemotherapy, although the benefit was statistically significant only for relapse-free survival. An unfavorable outcome was observed in patients with low p27 expression regardless of the adjuvant systemic treatment. In the International Breast Cancer Study Group Ludwig Trial V, p27 protein expression was assessed in lymph node–negative and lymph node–positive breast cancer patients, but it did not show any significant prognostic value with regard to disease-free and overall survival (47). However, in the lymph node–negative population, the benefit from one course of perioperative chemotherapy (CMF i.v.) was confined exclusively to patients with tumors showing low p27 expression. In the Southwest Oncology Group-Intergroup Trial S9313, low p27 expression was associated with shorter disease-free and overall survival in the total patient population and among hormone receptor–positive patients. No association between p27 expression and survival was observed in hormone receptor–negative patients (18). In International Breast Cancer Study Group Trials VIII and IX, p27 expression did not have any prognostic or predictive value, neither in patients with endocrine responsive tumors nor in patients with endocrine nonresponsive tumors (43). The results of these large retrospective trials indicate that the usefulness of p27 in predicting response to systemic treatment needs to be investigated in large prospective trials. Moreover, the role of p27 as a predictive marker is further supported by the results of *in vitro* studies showing a causal relationship between p27 and response to tamoxifen (15, 16). In the MCF-7 breast cancer cell line, estrogen stimulated cell cycle progression through loss of p27. Treatment with tamoxifen caused cell cycle arrest and up-regulation of

Table 2. Multivariate analysis using Cox models (n = 483)

Variable	HR for relapse (95% CI)	P	HR for death (95% CI)	P
Age	0.91 (0.66-1.25)	0.57	1.54 (1.11-2.15)	0.01
Tumor size	1.65 (0.99-2.72)	0.051	1.16 (0.69-1.95)	0.59
Lymph node metastases	1.87 (1.40-2.50)	<0.001	1.62 (1.21-2.17)	0.001
Tumor grade	0.98 (0.51-1.88)	0.96	1.37 (0.72-2.58)	0.34
Estrogen receptor	0.52 (0.12-2.23)	0.38	0.18 (0.07-0.49)	0.001
Progesterone receptor	0.52 (0.30-0.92)	0.02	0.75 (0.42-1.36)	0.35
Cyclin D1	3.13 (1.64-5.97)	0.001	2.26 (1.19-4.31)	0.01
p27	0.22 (0.11-0.42)	<0.001	0.39 (0.21-0.72)	0.002
Age	0.92 (0.67-1.27)	0.63	1.56 (1.12-2.17)	0.009
Tumor size	1.70 (1.03-2.81)	0.04	1.20 (0.71-2.01)	0.50
Lymph node metastases	1.84 (1.38-2.45)	<0.001	1.59 (1.19-2.12)	0.002
Tumor grade	1.01 (0.53-1.94)	0.97	1.42 (0.75-2.68)	0.28
Estrogen receptor	0.50 (0.12-2.13)	0.35	0.17 (0.06-0.46)	<0.001
Progesterone receptor	0.51 (0.29-0.89)	0.02	0.74 (0.41-1.32)	0.31
Low p27/high cyclin D1	4.65 (2.66-8.13)	<0.001	2.68 (1.52-4.74)	0.001

p27 levels. Antisense inhibition of p27 expression led to abrogation of cell cycle arrest (15). These data show that p27 is a critical mediator of the therapeutic effects of tamoxifen in breast cancer and that p27 is not a surrogate marker but is mechanistically a true biological variable for response to tamoxifen.

Immunohistochemistry is a simple and appropriate detection method that can reliably be done on formalin-fixed, paraffin-embedded tumor specimens and has been widely used to assess p27 protein expression in tumor specimens. However, in previous studies, different cutoff points, depending on the percentage of positive tumor cells ranging from 10% to 50% (32, 43, 47–49) or different scores consisting of staining intensity and percentage of positive tumor cells (18, 32, 33, 41, 44, 50), have been applied. In the present study, we explored two different cutoff points (50% and 70%) and obtained similar results. According to the results of the spline fitting for p27, we suggest a cutoff point of 70% but the 50% cutoff could also be used. Thus, there is clearly a need to standardize the p27 detection assays and scoring systems to ensure that determination of p27 levels is comparable between laboratories before p27 can become part of the routine histopathologic procedure for breast carcinoma specimens and even used as a new biomarker for specific treatments.

This study was conducted according to a detailed working plan. All of the patients had been included in a randomized clinical trial and received an identical adjuvant hormonal therapy—tamoxifen for 5 years. Adjusting on standard prognostic variables and specifying an objective cutoff point for defining positivity strengthen the reported results.

In conclusion, women with hormone receptor–positive, early-stage breast cancer and low p27 expression have a higher probability of early relapse and death after 5 years of tamoxifen therapy, as compared with women with high p27 expression. Whether p27 expression may help to select patients who will benefit from tamoxifen has to be determined in additional prospective studies using standardized methods for analysis.

Appendix

The following investigators and pathologists also participated in this study:

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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