Loss of p27KIP1 Expression in Fully-staged Node-negative Breast Cancer: Association with Lack of Hormone Receptors in T1a/b, but not T1c Infiltrative Ductal Carcinoma

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Abstract. *Nuclear expression of the cell cycle inhibitor p27KIP1 is reduced in a variety of human malignancies, including breast cancer. Loss of nuclear p27KIP1 during tumor progression, documented by immunohistochemistry (IHC), has been studied for its potential prognostic implication. We examined by IHC the association between nuclear p27KIP1 expression and hormone receptor status in T1N0M0 breast cancer. Patients and Methods: The correlation between nuclear p27KIP1 expression and estrogen (ER) and progesterone (PR) hormone receptor status was analyzed in 122 human T1N0M0 (68 T1a/b, 54 T1c) breast cancer specimens. All patients were staged as N0 by axillary node dissection. Results: A statistically significant reduction in p27KIP1 expression was observed as tumor size increased from T1a/b (7%) to T1c (22%). The proportion of tumors with low nuclear p27KIP1 expression was higher in the ER-negative/PR-negative group compared to the ER-positive/PR-positive group, but this difference was only statistically significant in the T1a/b subgroup (p=0.0007). Conclusion: Further investigations into causes of p27KIP1 deregulation and their relationship to hormone receptor expression in T1N0M0 breast ductal carcinomas are warranted. Such studies may help identify prognostic, as well as predictive, markers of therapy resistance.*

The tumor-node-metastasis (TNM) staging system used for breast cancer and other solid tumors has served for decades as an indicator of the primary cancer's capability to metastasize. Increasingly, however, these traditional prognostic parameters are being integrated with intrinsic molecular subtyping (1) that has identified the luminal A subgroup as the most common and prognostically favorable category. While patients with luminal A cancer clearly benefit from inhibition of estrogen-driven pathways, because of their frequency and persistent tendency to relapse, they still account for the majority of deaths among breast cancer patients across all ethnicities (2). Therefore, a focus on molecular events in small breast carcinomas (≤1 cm in size for T1a/b and >1 cm but ≤2 cm for T1c), that are predominantly of the luminal A subtype may be of interest. We sought to determine whether the $p27^{KIP1}$ cell cycle inhibitor was increasingly deregulated with enlarging tumor size, and how it related to estrogen receptor (ER) and progesterone receptor (PR) in small surgically staged tumors.

Interest in nuclear $p27^{KIP1}$ expression arises from its possible relationship to proliferation. Constitutive activation of oncogenic pathways, frequently accompanied by loss of cell cycle regulatory checkpoints, drives tumor proliferation. Orderly progression through the cell cycle requires sequential activation and inactivation of cyclin-dependent kinases (CDK). The restriction point at the G_1 to S transition, mediated by the CDK2/cyclin E complex, is critical for cells in determining whether or not they continue to proliferate. The CDK inhibitor $p27^{KIP1}$ binds to and inhibits CDK2/cyclin E, preventing G_1-S phase transition (3). Progressive decrease in the level of nuclear $p27^{KIP1}$ during G1, due to up-regulated Rous Sarcoma subfamily (RAS) pathways and Schmidt-Ruppin A-2 viral oncogene homolog (SRC)/Abelson murine leukemia viral oncogene homolog (ABL)-mediated tyrosine phosphorylation of $p27^{KIP1}$ to promote S-phase kinase-associated protein 1, Cullin, F-box containing complex, S-phase kinase-associated protein 2 $(SCF)^{SKP2}$-mediated $p27^{KIP1}$ proteolysis, allows for cell cycle progression (4). Whereas nuclear $p27^{KIP1}$ inhibits CDK2 to stop cell cycle progression, excessive cytoplasmic $p27^{KIP1}$ appears to have a pro-oncogenic effect, promoting

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cancer invasion and metastasis (5). Oncogenic signal transduction *via* phosphoinositide 3-kinase (PI3K)/v-akt thymoma viral oncogene homolog 1 (AKT) or RAS/mitogen-activated protein kinase (MAPK) pathways correlates with increased cytoplasmic $p27^{KIP1}$ localization (5).

Recent studies have further elucidated the central role of $p27^{KIP1}$ in cellular proliferation and migration (6). In various human malignancies, reduced or mislocalized $p27^{KIP1}$, documented by immunohistochemistry (IHC), is associated with poor clinical outcome (3). Progressive nuclear $p27^{KIP1}$ loss is observed during the histologic progression of neoplasia from benign to *in situ*, and to invasive breast cancers (7). In this initial report, we investigate nuclear $p27^{KIP1}$ staining using IHC in breast cancer specimens among patients diagnosed by wide excision (lumpectomy) or mastectomy and axillary dissection with pathologic stage T1ab/N0M0 and T1cN0M0. Our goal is to determine how nuclear $p27^{KIP1}$ levels vary with tumor size and further define the association of stage with low nuclear $p27^{KIP1}$ levels and hormone receptor status. Its relationship to outcome is being explored in a larger cohort of patients that include patients also staged by sentinel node sampling.

Patients and Methods

Samples. Breast cancer specimens were obtained from 122 patients operated on by two breast surgeons (co-authors DFR, AG). Histological types were determined according to the WHO criteria (8). Samples were obtained between January 1, 1997 and April 1, 2000 from patients in the New York University and the Bellevue Hospital tumor registry following an approved Institutional Review Board protocol. All patients had undergone axillary dissection and deemed N0 without subjecting any samples to additional IHC for cytokeratins. Tumor size was defined as the largest diameter of the largest invasive foci of infiltrating ductal carcinoma. Sixty-eight tumors were T1a/bN0M0 (≤1 cm) and 54 were T1cN0M0 (1.1-2 cm). Age at diagnosis ranged from 29 to 86 years (median age 60 years).

Immunohistochemistry. IHC was routinely performed (as part of the initial diagnosis) on paraffin-embedded tissue samples using specific monoclonal antibodies against ER, PR, and HER2/neu. To be deemed positive for ER and PR required staining at least 10% of tumor cells; HER 2 determination preceded use of U.S. Food and Drug Administration (FDA) approved antibodies and fluorescence *in situ* hybridization (FISH), and positive testing included only those with 3+ immonostaining (not subjected to further analysis). IHC for $p27^{KIP1}$ was routinely carried out in our Molecular Pathology Laboratory (directed by co-author GI), and positive dense nuclear staining in >50% of cells was required to be considered positive; if not meeting this criterion, samples were classified as low or negative. Intensity of staining (0 to 2+) was also reported (but not shown) since the 0 to 1+ category applied only to low $p27^{KIP1}$ expression.

Statistical analysis. The two-sided Fisher's exact test was used to assess the association of low $p27^{KIP1}$ with the other molecular markers studied.

Results

There was a higher proportion of low $p27^{KIP1}$ expressing tumors in the >1 to ≤2 cm tumors (22%) than in the ≤1.0 cm tumors (7%) (Table I). This difference was statistically significant (Fisher's exact test $p=0.03$). The proportions of tumors ER-/PR- and HER2/neu3+ tumors were slightly higher in the >1 to ≤2 cm than in the ≤1.0 cm tumor group, but these differences were not significant (Fisher's exact test $p=0.33$ and 0.77, respectively) (Table I). HER2/neu 3+ staining occurred in two instances, each with low nuclear $p27^{KIP1}$ among the 5 T1a/b and 12 T1c tumors, respectively.

There was a higher proportion of low $p27^{KIP1}$ expressing tumors in ER-/PR- group than in ER+ or PR+ groups for both small and larger tumors, but this difference was statistically significant only in the group of small tumors (Table II).

Finally, the proportions of low $p27^{KIP1}$ expressing tumors in the ER-/PR- group did not differ between T1a/b and T1c tumors (44% and 36%, respectively; Fisher's exact test $p=0.99$). However, the proportion of low $p27^{KIP1}$ expressing tumors in the ER^+, and PR^+ groups was considerably smaller in the ≤1.0 cm tumor group (2%) than in the >1.0 cm group (19%; Fisher's exact test $p=0.004$).

Discussion

The prognostic significance of the CDK inhibitor $p27^{KIP1}$ in breast cancer has received wide attention (7, 9-12). Consistent with previous reports, we confirm that patients with smaller breast carcinomas that are predominantly categorized as luminal A (1) have a correspondingly high nuclear $p27^{KIP1}$ expression, while reduced $p27^{KIP1}$ expression may identify patients at increased risk for recurrence and metastasis (outcome data not pursued in our limited sample). Our study was initially conceived to extend the observations of Tan *et al*. (10) on nuclear $p27^{KIP1}$ expression in tumors from patients mostly not receiving adjuvant therapy who had T1a/b breast carcinomas: the median survival was 174 months in patients whose tumors displayed high $p27^{KIP1}$ expression (≥50% $p27^{KIP1}$-positive cells) compared to 139 months in those whose tumors had low $p27^{KIP1}$ expression (<50% $p27^{KIP1}$-positive cells; $p=0.0042$). This lower survival was independent of other parameters, such as grade, microvessel density and hormone receptor status. Only the presence of nodal metastases, associated with a 4.9-fold relative risk of death, surpassed the ability of $p27^{KIP1}$ to significantly predict poor prognosis and outcome.

Other studies subsequently demonstrated high expression of nuclear $p27^{KIP1}$ in low-grade ductal carcinoma *in situ*, whereas it was reduced in high-grade ductal carcinoma *in situ* (7). In invasive carcinomas, the level of $p27^{KIP1}$

Table I. *Distribution of markers of poor prognosis [low nuclear p27^{KIP1}), negative estrogen (ER) and progesterone (PR) hormone receptors, HER2/neu score 2-3+] in T1N0M0 ductal breast carcinomas.*

Tumor size	Sample size	Low p27^{KIP1}	ER$^-$/PR$^-$	HER2/Neu
≤1.0 cm	68	5 (7%)	9 (13%)	6 (9%)
>1 cm - ≤2 cm	54	12 (22%)	11 (20%)	6 (11%)

Table II. *Inverse relationship between low nuclear p27^{KIP1} expression and presence of hormone receptors.*

Tumor size	p27^{KIP1} status	ER$^-$/PR$^-$	ER$^+$ or PR$^+$	*P*-value*
≤1.0 cm	Low	4 (44%)	1 (2%)	0.0007
	Normal	5 (56%)	58 (98%)	
>1.0 cm	Low	4 (36%)	8 (19%)	0.23
	Normal	7 (64%)	35 (81%)	

ER: ≥10%; PR ≥10%; normal p27^{KIP1}=>50% nuclear staining
*Fisher's exact test.

expression was closely related to the degree of cellular differentiation: high p27^{KIP1} was noted in 45 out of 56 (80%) well-differentiated tumors, in 56 out of 84 (67%) moderately differentiated, and only in 1 out of 28 poorly differentiated invasive tumors. In another study (9), expression of p27^{KIP1} was significantly lower in tumors with high nuclear grade (p=0.028), confirming the relationship between p27^{KIP1} expression and differentiation; moreover, p27^{KIP1} expression significantly decreased in advanced stages (p=0.001). Coupling low p27^{KIP1} with high Ki67 expression imparted a particularly unfavorable prognostic signature in T1 and T2 invasive carcinomas of the breast (11). However, in a detailed study of 830 patients and a median follow-up of 104 months, Barnes *et al*. concluded that "the inverse relationship between p27^{KIP1} levels and histological grade and individual grade components suggests a role for p27^{KIP1} in both cell proliferation and differentiation, but is not clinically useful" (12).

More recently, attention has shifted from the prognostic value of a low p27^{KIP1} expression to its possible therapeutic implications. Arteaga, reflecting on the requirement of a threshold level of p27^{KIP1} for response of hormone receptor positive tumors to antiestrogens, predicted failure of these drugs when the biomarker is low (13). In fact, a low p27^{KIP1} expression may be a promising marker of antiestrogen resistance (14, 15). Less developed is the suggestion that nuclear p27^{KIP1} loss may be involved in trastuzumab resistance (16) and epidermal growth factor receptor signaling (17). Finally, retrospective evaluation of tumor samples from clinical trials (14, 18) and a meta-analysis (19) have provided renewed confirmation of the adverse prognostic and predictive aspects of low or absent p27^{KIP1} expression in breast cancer: the Austrian Breast and Colorectal Cancer Study Group in their adjuvant trial 06 studied 483 patients and concluded 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" (14). The Southwest Oncology Group Intergroup Trial S-9313, including 3122 patients with moderate-risk primary breast cancer treated with adjuvant doxorubicin and cyclophosphamide concurrently or sequentially, found that a low p27^{KIP1} expression was associated with a worse overall survival (hazard ratio=1.42, 95% confidence interval=1.05 to 1.94) than those with higher expression levels among patients with hormone-receptor positive tumors, after adjustment for treatment, menopausal status, tumor size and number of positive lymph nodes. By contrast, in patients with hormone receptor-negative tumors, the benefit of chemotherapy as reflected by 5-year survival was similar in the high and low p27^{KIP1} expression subsets (18).

In line with other reports, we found that a positive hormone receptor status was associated with high nuclear p27^{KIP1} expression. In our study, loss of nuclear p27^{KIP1} staining was observed to be associated with the absence of hormone receptors, but it was statistically significant (p=0.0007) only in T1a/b not in T1c tumors. The proportion of hormone-positive tumors with low p27^{KIP1} expression was smaller in the ≤1.0 cm group (2%) compared to the >1.0 cm group (19%; p=0.004), indicating that absence of ER is not involved in lowering p27^{KIP1} expression. However, the relationship between these signaling pathways is complex, since estrogen binding to the ER promotes p27^{KIP1} proteolysis and cyclin E-CDK2 activation, driving cell cycle progression and breast

carcinoma proliferation (20). Preclinical studies and the analysis of $p27^{KIP1}$ expression in clinical trials indicate that high nuclear $p27^{KIP1}$ may segregate with responsiveness to hormonal therapy, and therefore, with the common luminal A subtype (18-24). In addition, nuclear $p27^{KIP1}$ proteolysis has been shown to be reversed by the combined use of tamoxifen and rapalogs (25) and by concomitant inhibition of epidermal growth factor receptor (EGFR)/ERBB2 pathways with lapatinib (26) or trastuzumab (27).

In summary, while in the smaller tumors, loss of nuclear $p27^{KIP1}$ rarely occurs in tumor that are ER^+, with increasing tumor size, the percentage of tumors with low levels of nuclear $p27^{KIP1}$ expression increases in both hormone receptor-positive and -negative tumors. While our initial interest in $p27^{KIP1}$ as a prognostic marker (10, 28) was met with skepticism, recent data indicate that loss of nuclear $p27^{KIP1}$ may in fact predict for failure of tamoxifen therapy in patients with ER^+ tumors (14) and may help define patients at great risk for dissemination (29). Our study also provides a framework for using the induction of nuclear $p27^{KIP1}$ expression (in initially low $p27^{KIP1}$ expressing cells) in studying responses to 'targeted' drugs in 'window of opportunity' pre- and post intervention studies. In the future, evaluation of such post-treatment reappearance of baseline absence of nuclear $p27^{KIP1}$ expression could prove to be a valuable predictor of response to novel biologic agents, particularly in ER+ tumors.

Conflict of Interest

There is no conflict of interest for any of the Authors in any aspects of the article.

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