Phosphorylation/Cytoplasmic Localization of p21$^{Cip1/WAF1}$ Is Associated with HER2/*neu* Overexpression and Provides a Novel Combination Predictor for Poor Prognosis in Breast Cancer Patients

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ABSTRACT

Purpose: **The diversity of biological functions makes p21$^{Cip1/WAF1}$ (p21) a controversial marker in predicting the prognosis of breast cancer patients. Recent laboratory studies revealed that the regulation of p21 function could be related to different subcellular localizations of p21 by Akt-induced phosphorylation at threonine 145 in HER2/*neu*-overexpressing breast cancer cells. The purpose of this study was to verify these findings in clinical settings.**

Experimental Design: **The expression status of the key biological markers in the HER2/*neu*-Akt-p21 pathway in 130 breast cancer specimens was evaluated by immunohistochemical staining and correlated with patients' clinical parameters and survival. In addition, an antibody against phospho-p21 at threonine 145 [phospho-p21 (T145)] was also used for better validation of these findings.**

Results: **Cytoplasmic localization of p21 is highly correlated with overexpression of phospho-p21 (T145). Both cytoplasmic p21 and overexpression of phospho-p21 (T145) are associated with high expression of HER2/*neu* and phospho-Akt. Cytoplasmic localization of p21 and overexpression of phospho-p21 (T145), HER2/*neu*, and phospho-Akt are all associated with worse overall survival. Multivariate analysis of the Cox proportional hazard regression model revealed that cytoplasmic p21 and overexpression of HER2/*neu* are independently associated with increased risk of death. Combining these two factors stratified patients' survival into four distinct groups, with a 5-year survival rate of 79% in low HER2/*neu* and negative/nuclear p21 patients, 60% in high HER2/*neu* and negative/nuclear p21 patients, 29% in low HER2/neu and cytoplasmic p21 patients, and 16% in high HER2/*neu* and cytoplasmic p21 patients.**

Conclusions: **The present study, in addition to supporting the mechanisms of p21 regulation derived from laboratory investigation, demonstrates the prognostic importance of phospho-p21 (T145) for the first time and also provides a novel combination of p21 and HER2/*neu* for better stratification of patients' survival than any single clinicopathological or biological marker that may play important diagnostic and therapeutic roles for breast cancer patients.**

INTRODUCTION

Overexpression or amplification of the receptor tyrosine kinase HER2/*neu* (also known as ErbB2) has been noted in ~30% of breast cancer patients and is frequently associated with shorter survival and poor prognostic features, including earlier relapse and increased number of lymph node metastases (1). The underlying mechanisms by which HER2/*neu* promotes tumorigenesis, invasiveness, and metastasis of cancer cells have been extensively studied (2). The phosphatidylinositol-3-OH kinase/Akt pathway is an important HER2/*neu* downstream cascade in preventing cells from undergoing apoptosis and contributing to cell proliferation (3–5). For example, after phosphorylation, the activated Akt can phosphorylate various substrates, such as Bad (6), caspase-9 (7), Forkhead family transcription factors (8, 9), MDM2 (10, 11), and p21$^{Cip1/WAF1}$ (p21; Ref. 12), resulting in either suppression of apoptosis or promotion of cell proliferation. Activation of Akt has also been correlated with poor outcome in breast cancer patients (13).

Among the downstream substrates of Akt, p21 is a critical modulator of cell cycle and cell survival, although its regulation and function have largely remained unclear. p21 was initially considered to be an inhibitor of cell cycle progression and has been shown to suppress tumor formation in xenograft models (14, 15). However, several recent studies have suggested that this protein can also promote cell survival and cell cycle progression (16–18). In addition, elevated p21 protein levels have been observed in various aggressive malignancies, such as glioma and leukemia, and may contribute to chemoresistance (19, 20). The role of p21 in breast cancers has also been controversial in laboratory and clinical studies. It has been shown that HER2/*neu*-overexpressing breast cancer cells can induce chemoresistance through increased expression of p21 (21) and that p21

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overexpression is associated with poor prognosis in breast cancer patients (22). On the other hand, several studies indicated that p21 expression provides no prognostic information for patients with breast cancer (23, 24). The contradictory effects on tumorigenesis, as well as inconsistent reports about clinical outcomes of p21, could be related to the subcellular localization of this special protein because recent studies have revealed that the cell growth-inhibitory activity of p21 is strongly correlated with its nuclear localization. However, p21 can also localize in the cytoplasm, where it plays an important role in protecting cells from apoptosis (16, 18) and was associated with poor prognosis in breast cancer patients (25).

The cellular localization of p21 has been proposed to be critical for the regulation of p21 function (26), and we recently identified the mechanism by which p21 is phosphorylated by Akt at a consensus threonine residue (threonine 145), which results in cytoplasmic localization and suppression of growth-inhibiting activity (12). Because our previous findings clearly demonstrated the regulation of p21 localization and function of the HER2/*neu*-Akt pathway in a laboratory setting, to further address how this signaling pathway is related to survival and other clinical parameters of breast cancer patients, we analyzed the expression status of p21, HER2/*neu*, and phospho-Akt by immunohistochemical (IHC) staining in 130 breast cancer specimens and compared their expression levels and subcellular localization with clinical outcome. In addition, we used a newly developed antibody against phospho-p21 at threonine 145 [phospho-p21 (T145)] to further validate the results.

MATERIALS AND METHODS

Patients and Tumor Specimens. We obtained 130 archived blocks containing formalin-fixed, paraffin-embedded infiltrating breast carcinoma from the Department of Pathology, Shanghai East Breast Disease Hospital, People's Republic of China. All of the patients were women with nonmetastatic disease who had undergone mastectomy and axillary lymph node dissection between 1988 and 1994. After surgical treatment, the patients were offered adjuvant chemotherapy and/or radiotherapy and hormone therapy, depending on the number of lymph node metastases, status of menopause, and estrogen and/or progesterone receptor positivity. The clinicopathological characteristics of the study population, including age, tumor size, lymph node status, tumor grade, and estrogen receptor/progesterone receptor positivity, were obtained from medical records. The estrogen and progesterone receptor status was unavailable for 20 and 19 tumor specimens, respectively. The stage was assessed by the TNM clinical staging system of the American Joint Committee on Cancer (27). Patients were followed 4–72 months, with a median follow-up of 48 months.

Generation of Anti-Phospho-p21 (T145) Antibody. The polyclonal antibody against phosphorylated human p21 protein was generated by immunization of rabbits with a carrier protein, keyhole limpet hemocyanin, in conjunction with a phosphorylated 11-mer peptide [KRRQT-(PO_3)-SMTDFY] at the terminal region of the p21 sequence encompassing the Akt phosphorylation site (threonine 145). Peptides were synthesized for antibody production by SynPeptide, Inc. (Dubin, CA) and were checked under stringent analytical specifications, which included high performance liquid chromatography, mass spectrometry, and UV analysis. The polyclonal antibody was also generated and affinity-purified by SynPeptide, Inc.

Immunoprecipitation and Immunoblotting. 293T cells were transiently transfected by use of SN liposome (28). After transfection for 36 h, 293T cells were washed with PBS and scraped into RIPA-B buffer. After brief sonication, the cell lysates were centrifuged at 14,300 $\times$ *g* for 30 min at 4°C to remove insoluble cell debris. The supernatant was preincubated with protein G-agarose (Roche) for 1 h at 4°C. Flag-tagged p21 was immunoprecipitated overnight with anti-Flag (M2) antibody (Sigma) and protein G-agarose. The immunocomplex was washed four times with RIPA-B buffer, dissolved in sampling buffer, and subjected to SDS-PAGE; the proteins were then transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% BSA in Tris-buffered saline and were incubated with anti-phospho-p21 (T145) antibody (1:1000 diluted in Tris-buffered saline-Tween containing 3% BSA) and then with horseradish peroxidase-conjugated antirabbit secondary antibodies. The immunoblots were visualized by use of the ECL kit (Amersham Pharmacia Biotech).

IHC Staining for p21, Phospho-p21 (T145), HER2/*neu*, and Phospho-Akt. The immunoperoxidase staining method was modified from the avidin-biotin complex technique as described previously (29). In brief, slides (5 μm) were deparaffinized. After antigen retrieval, the slides were digested in 0.05% trypsin. The endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide, and the slides were then treated with 10% normal goat or horse serum for 30 min. After overnight incubation with primary antibodies, including

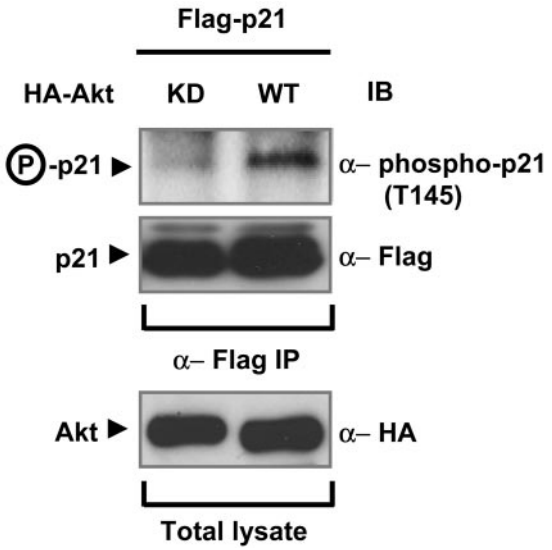

Fig. 1 Detection of p21 (T145) phosphorylation by anti-phospho-p21 (T145) antibody. Flag-tagged p21 and HA-tagged wild-type (*WT*) or kinase-dead Akt (*KD*) were cotransfected transiently into 293T cells. After 36 h of transfection, lysates of cells were subjected to p21 immunoprecipitation (*IP*) with anti-Flag antibody. p21 (T145) phosphorylation by Akt was detected by an anti-phospho-p21 (T145)-specific antibody. Expression levels of Akt (WT) and Akt (KD) were assessed by immunoblotting (*IB*).

(*a*) rabbit polyclonal anti-p21 (c-19; 1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA); (*b*) rabbit polyclonal anti-phospho-p21 (T145) (1:15 dilution; generated by SynPeptide Inc); (*c*) rabbit polyclonal anti-HER2/*neu* (anti-c-erbB-2, 1:300 dilution; DAKO, Carpinteria, CA); and (*d*) rabbit polyclonal anti-phospho-Akt (T308) (1:80 dilution; New England Biolabs Inc.), the slides were incubated with biotinylated secondary antibodies and subsequently incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). Antibody detection was performed with the 0.125% aminoethylcarbazole chromogen substrate solution (AEC substrate) from Sigma Chemical Co. After counterstaining with Mayer's hematoxylin (Sigma), the sides were mounted. For negative control, all incubation steps were identical except that PBS was used rather than primary antibody. A previously identified strongly staining tumor tissue section was used as a positive control. The prepared slides were examined by light microscopy.

Fluorescent *in Situ* Hybridization (FISH). The paraffin-embedded tissue sections were baked at 65°C for 2 h and deparaffinized as described previously (29). The FISH assay was performed with the PathVysion HER2 DNA probe Kit (Vysis Inc) according to the manufacturer's recommendation.

Scoring of Immunoreactivity. The immunoreactivity of these antibodies was scored according to the subcellular localization (membrane, nuclear, and/or cytoplasmic), staining intensity (strong, moderate, weak, and faint or slightly above background), and fraction of positive staining. The mean fraction of positive tumor cells was determined in at least nine areas at ×100 or ×200 magnification. p21 immunoreactivity was determined by the percentage of positively stained tumor cells as well as the subcellular localization of staining and was categorized as negative, nuclear, and cytoplasmic. Negative was defined as undetectable cytoplasmic or nuclear staining. Nuclear p21 was defined as the fraction of tumor cells with positive nuclear staining greater than or equal to that of positive cytoplasmic staining. Cytoplasmic p21 was defined as the fraction of cytoplasmic staining greater than that of nuclear staining. HER2/*neu* immunoreactivity was determined by membrane staining and categorized as 0 to 3+. A score of 0 was defined as undetectable staining or membrane staining in <10% of tumor cells, a score of 1+ was defined as faint membrane staining in >10% of tumor cells, a score of 2+ was defined as weak to moderate membrane staining in >10% of tumor cells, and a score of 3+ was defined as intense membrane staining in >10% of tumor cells. The immunoreactivities of phospho-Akt and phospho-p21 (T145) were also ranked into four groups according to the percentage of positively stained tumor cells, including cytoplasmic and nuclear staining: 0, no staining; 1+, <20% cells stained; 2+, 20–49% cells stained; and 3+, >50% cells stained. For analysis, HER2/*neu*, phospho-Akt, and phospho-p21 (T145) expressions was further classified as low (score 0 and 1+) or high (scores 2+ and 3+). The slides were read independently by two investigators without knowledge of the clinical data. If differences between observers occurred, both investigators used a multiheaded microscope to reexamine the slides.

Statistical Analysis. Data on eligible patients were summarized by use of standard descriptive statistics and frequency tabulation. The associations between expression of biomarkers (p21, phospho-p21, HER2/*neu*, and phospho-Akt) and various clinicopathological parameters were assessed by cross-tabulation χ^2 tests. All correlations between each biomarker were analyzed by Kendall's τ-b analysis. The overall survival after surgery was plotted by use of the Kaplan–Meier method. The

Table 1 Clinicopathological and immunohistochemical data for the patients

Characteristic	Patients	
	n	%
Total	130	100
Age (yrs)		
≤48	66	51
>48	64	49
Tumor status		
T_1	66	51
T_2	53	41
T_3	11	8
Lymph node status		
N_0	68	52
N_1	25	19
N_2	32	25
N_3	5	4
Stage		
I	45	35
II	46	35
III	39	30
Tumor grade[a]		
1	28	22
2	43	33
3	59	45
Estrogen receptor		
Negative	68	52
Positive	42	32
Unknown	20	15
Progesterone receptor		
Negative	57	44
Positive	54	41
Unknown	19	15
p21[b]		
Negative	53	41
Nuclear	34	26
Cytoplasmic	43	33
Phospho-p21 (T145)		
Low		
0	64	49
1+	26	20
High		
2+	27	21
3+	13	10
HER2/*neu*		
Low		
0	42	32
1+	42	32
High		
2+	21	16
3+	25	19
Phospho-Akt		
Low		
0	73	56
1+	23	18
High		
2+	14	11
3+	20	15

[a] Tumor grade was classified by WHO criteria (grade 1, well differentiated; grade 2, moderately differentiated; grade 3, poorly differentiated).
[b] Nuclear p21, fraction of nuclear staining greater than or equal to fraction of cytoplasmic staining; cytoplasmic p21, fraction of cytoplasmic staining greater than fraction of nuclear staining.

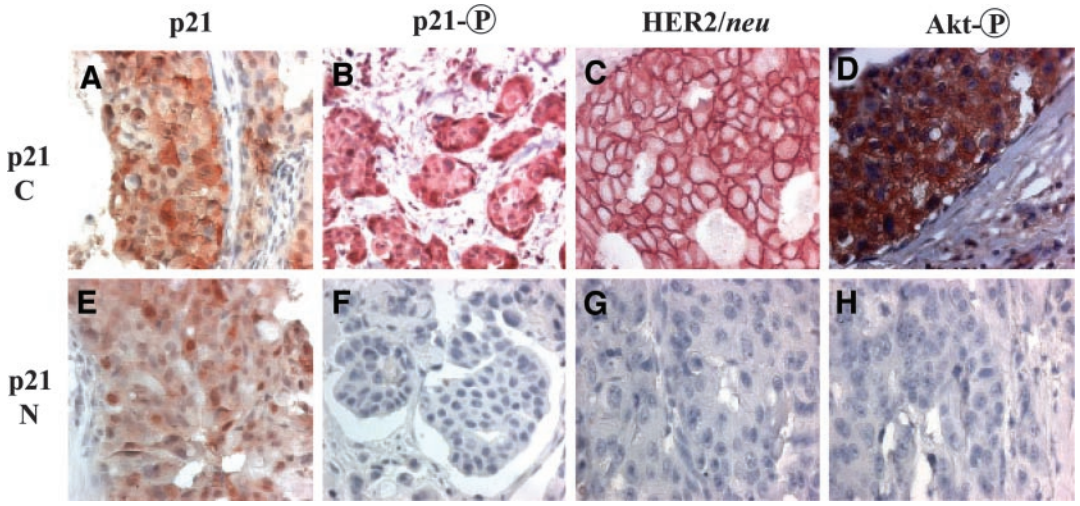

Fig. 2 Representative immunohistochemical pictures of phospho-p21 (T145), HER2/*neu*, and phospho-Akt in cytoplasmic p21 (*C*) or nuclear p21 (*N*) specimens. Tissue sections from patients with cytoplasmic p21 (*A–D*) and nuclear p21 (*E–H*) were stained with specific antibodies against p21 (*A*, cytoplasmic; *E*, nuclear), phospho-p21 (T145) (*B*, overexpression; *F*, negative), HER2/*neu* (*C*, overexpression; *G*, negative), and phsopho-Akt (*D*, overexpression; *H*, negative).

log-rank test was used to analyze differences in survival time. The Cox proportional hazard regression model was used to assess effect of patients' prognostic factors on overall survival. Statistical analysis was performed with SAS 8.0 and S-plus 2000 software. All tests were two-sided, and the level of significance was set at 0.05.

RESULTS

Characterization of Anti-Phospho-p21 (T145) Antibody. Our previous study demonstrated that activated Akt can phosphorylate p21 at threonine 145 and lead to cytoplasmic localization of p21 in HER2/*neu*-overexpressing breast cancer cells (12). To further validate that p21 is phosphorylated on threonine 145 in cancer cells, we developed an anti-phospho-p21 (T145) antibody to recognize this phosphorylation site (see "Materials and Methods"). To characterize the specificity of this antibody, we cotransfected p21 with either wild-type or kinase-dead Akt into 293T cells to determine the specificity of this antibody by a biochemical method. As shown in Fig. 1, phosphorylation of p21 at threonine 145 was detected by this antibody, suggesting that this antibody could recognize phopho-p21 (T145) specifically.

Clinicopathological and IHC Profiles of the Study Population. Patient and tumor characteristics for the entire study population are shown in Table 1. For the 130 patients, the median age was 48 years (range, 26–87 years). No patients had T4 disease or detectable distant metastasis at the time of the surgery. Pathology examination revealed that 118 (91%) of the tumors were infiltrating ductal carcinomas; the remaining 12 (9%) were infiltrating lobular carcinomas. Representative IHC photographs of p21, phospho-p21 (T145), HER2/*neu*, and phospho-Akt are shown in Fig. 2. Positive p21 expression was found in 59% of patients, and the immunoreactivity was in both the cytoplasm and nucleus for the majority of these patients, with 26% mainly in the nucleus and 33% mainly in the cytoplasm. Phospho-p21 (T145) levels were high in 40 (31%) patients, with staining predominantly in the cytoplasm. Phospho-Akt levels were high in 34 (26%) patients, with staining located mainly in the cytoplasm and nucleus. HER2/*neu* levels were high in 46 (35%) patients, with staining mainly in the cell membrane and cytoplasm. To validate the results from the IHC staining, we used FISH to examine randomly selected IHC breast tumors from this cohort. Among HER2/*neu* 3+ and 2+ tissues, we found high degree of correlation, nearly 2+ (87.5%), 3+ (100%), and 0 (100%). Thus, the IHC result is highly consistent with the result from FISH (Table 2 and Fig. 3).

The relationship between various clinicopathological parameters and the biological markers are shown in Table 3. No significant association was noted between the subcellular localization of p21 and age, tumor size, lymph node status, stage, tumor grade, or estrogen and progesterone receptor status. When compared with low expression of phospho-p21, HER2/*neu*, and phospho-Akt, overexpression of these biological markers was also not associated with any clinicopathological parameter of breast cancer patients.

Cytoplasmic p21 Is Highly Correlated with Overexpression of Phospho-p21 (T145), Which Is Located Primarily in the Cytoplasm. To further study the correlation between phospho-p21 (T145) and overall survival of breast cancer patients,

Table 2 Comparison of immunohistochemical staining and fluorescence *in situ* hybridization

	HER2/*neu* IHC			
HER2/*neu* probe[a] FISH[b]	3+	1+	0	Total
Amplified	5	7	0	12
Nonamplified	0	1	5	6
Total	5	8	5	18

[a] Pearson's χ^2 test (SPSS): $P = 0.95$ ($P > 0.05$).
[b] FISH, fluorescence *in situ* hybridization; IHC, immunohistochemical staining.

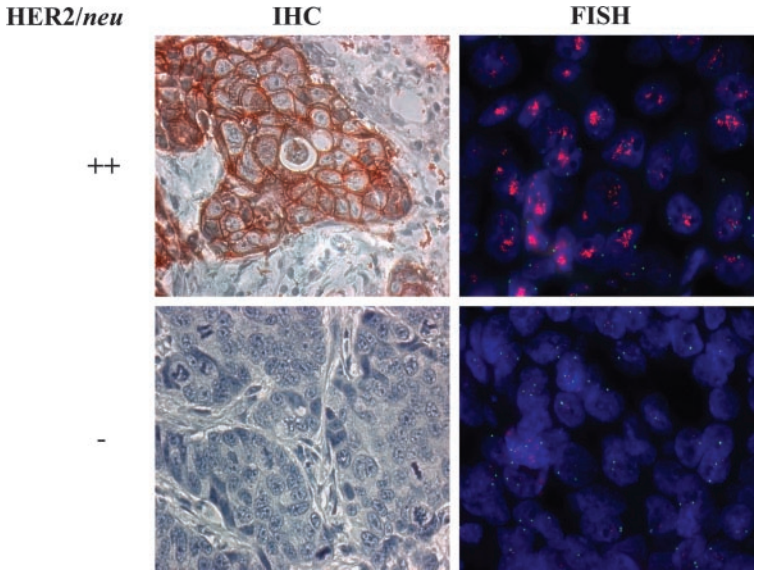

Fig. 3 HER2/*neu* expression and amplification detected by immunohistochemical staining (*IHC*) and fluorescence *in situ* hybridization (*FISH*), respectively. IHC negative (−) is related to FISH nonamplified (−); IHC high positive (++) is correlated to FISH amplified (++). For the FISH assay, *green* is chromosome 17 centromere; and *red* is HER2/*neu*.

we examined surgical specimens from breast cancer patients immunohistochemically, using this antibody. The subcellular location of positive staining was predominantly in the cytoplasm (Fig. 2*B*). Nuclear staining could be detected in only 2 of the 66 specimens with cytoplasmic staining and was very weak. In addition, the expression of phospho-p21 (T145) was highly correlated with the subcellular localization of p21 (Table 4). When p21 was localized mainly in the cytoplasm, phospho-p21 (T145) was likely to be highly expressed (see also Fig. 2, *A* and *B*). In contrast, when p21 was located mainly in the nucleus, phospho-p21 (T145) expression tended to be low or negative [see Fig. 2, *E* and *F*; correlation coefficient (r) = 0.33; P = 0.001].

Table 3 Association of p21, phospho-p21, HER2/*neu*, and phospho-Akt with clinicopathological parameters in 130 breast cancer patients
$P > 0.05$ for all associations between biomarkers and clinicopathological variables analyzed by cross-tabulation (χ^2 test).

	No. of patients (%)			
Parameters	Cytoplasmic p21[a]	High phospho-p21 (2+/3+)	High HER2/*neu* (2+/3+)	High phospho-Akt (2+/3+)
Total (*n*)	43	40	46	34
Age (yrs)				
≤48	20 (15.4%)	18 (13.8%)	25 (19.2%)	16 (12.3%)
>48	23 (17.7%)	22 (16.9%)	21 (16.2%)	18 (13.8%)
Tumor size				
T_1	17 (13.1%)	17 (13.1%)	19 (14.6%)	18 (13.8%)
T_2 and T_3	26 (20.0%)	23 (17.7%)	27 (20.8%)	16 (12.3%)
Lymph node				
Negative	18 (13.8%)	17 (13.1%)	21 (16.2%)	16 (12.3%)
Positive	25 (19.2%)	23 (17.7%)	25 (19.2%)	18 (13.8%)
Stage				
I	9 (6.9%)	12 (9.2%)	14 (10.8%)	14 (10.8%)
II	17 (13.1%)	12 (9.2%)	13 (10.0%)	6 (4.6%)
III	17 (13.1%)	16 (12.3%)	19 (14.6%)	14 (10.8%)
Tumor grade				
1	6 (4.6%)	7 (5.4%)	6 (4.6%)	6 (4.6%)
2	12 (9.2%)	13 (10.0%)	14 (10.8%)	13 (10.0%)
3	25 (19.2%)	20 (15.4%)	26 (20.0%)	15 (11.5%)
Estrogen receptor[b]				
Negative	16 (14.5%)	18 (16.4%)	19 (17.3%)	20 (18.2%)
Positive	15 (13.6%)	15 (13.6%)	17 (15.5%)	9 (8.2%)
Progesterone receptor[c]				
Negative	15 (13.5%)	20 (18.2%)	15 (13.5%)	13 (11.7%)
Positive	16 (14.4%)	9 (8.2%)	21 (18.9%)	16 (14.4%)

[a] p21 status was divided into three groups: negative, nuclear, and cytoplasmic.
[b] Data from 20 patients are missing.
[c] Data from 19 patients are missing.

Table 4 Correlation between subcellular localization of p21 and immunoreactivity of phospho-p21 (T145)
$r = 0.33$; $P = 0.001$ (Kendall's τ -b analysis).

Phospho-p21 staining	p21 subcellular localization, *n* (%)		
	Nuclear ($n = 34$)	Cytoplasmic ($n = 43$)	Total[a] ($n = 77$)
0	16 (21%)	6 (8%)	22 (29%)
1+	8 (10%)	10 (13%)	18 (23%)
2+	6 (8%)	18 (23%)	24 (31%)
3+	4 (5%)	9 (12%)	13 (17%)

[a] Fifty-three patients with negative staining for p21 were excluded.

Table 5 Correlation between immunoreactivity of HER2/*neu* and phospho-Akt
$r = 0.45$; $P < 0.0001$.

Phospho-Akt staining	HER2/*neu* staining, *n* (%)				
	0 ($n = 42$)	1+ ($n = 42$)	2+ ($n = 21$)	3+ ($n = 25$)	Total ($n = 130$)
0	38 (29%)	20 (15%)	8 (6%)	7 (5%)	73 (56%)
1+	3 (2%)	10 (8%)	6 (4%)	4 (3%)	23 (18%)
2+	1 (1%)	7 (5%)	2 (2%)	4 (3%)	14 (11%)
3+	0 (0%)	5 (4%)	5 (4%)	10 (8%)	20 (15%)

Cytoplasmic Localization of p21 and Overexpression of Phospho-p21 (T145) Are Associated with Overexpression of HER2/*neu* and Phospho-Akt. Because we previously demonstrated that activated Akt phosphorylates p21 and determined its subcellular localization in HER2/*neu*-overexpressing cells (12), we examined the correlations among these molecules in this pathway. As shown in Table 5, the level of phospho-Akt expression was strongly correlated with the level of HER/*neu* expression, supporting the previous report that HER2/*neu* overexpression activates Akt by phosphorylation of Akt (12). The association between the levels of HER2/*neu* or phospho-Akt expression and the cellular localization of p21 was also significant (Tables 6 and 7), indicating that when HER2/*neu* or phospho-Akt was highly expressed, p21 was localized mainly in the cytoplasm (see Fig. 2, *A*, *C*, and *D*). In contrast, when the levels of HER2/*neu* or phospho-Akt expression were low or negative, p21 tended to be negative or localized mainly in the nucleus (see Fig. 2, *E*, *G*, and *H*). Furthermore, the levels of HER2/*neu* and phospho-Akt expression were positively associated with the levels of phospho-p21 (T145) expression ($r = 0.49$, $P < 0.001$ for HER2/*neu*; $r = 0.52$, $P < 0.001$ for phospho-Akt). All of these data consistently support the hypothesis that overexpression of HER2/*neu* can activate/phosphorylate Akt, which in turn phosphorylates p21 at threonine 145 and leads to cytoplasmic localization of p21.

Cytoplasmic Localization of p21 and Overexpression of Phospho-p21 (T145) Are Associated with Poor Patient Survival. We next sought to clinically determine whether subcellular localization of p21 and the levels of phospho-p21 (T145)

Table 6 Correlation between subcellular localization of p21 and HER2/*neu* immunoreactivity
$r = 0.37$; $P = 0.0003$.

HER2/*neu* staining	p21 subcellular localization, *n* (%)			
	Negative ($n = 53$)	Nuclear ($n = 34$)	Cytoplasmic ($n = 43$)	Total ($n = 130$)
0	28 (22%)	10 (8%)	4 (3%)	42 (32%)
1+	15 (12%)	13 (10%)	14 (11%)	42 (32%)
2+	5 (4%)	5 (4%)	11 (9%)	21 (16%)
3+	5 (4%)	6 (5%)	14 (11%)	25 (19%)

Table 7 Correlation between subcellular localization of p21 and immunoreactivity of phospho-Akt
$r = 0.32$; $P = 0.0011$.

Phospho-Akt staining	p21 subcellular localization, *n* (%)			
	Negative ($n = 53$)	Nuclear ($n = 34$)	Cytoplasmic ($n = 43$)	Total ($n = 130$)
0	38 (29%)	21 (16%)	14 (11%)	73 (56%)
1+	10 (8%)	3 (2%)	10 (8%)	23 (18%)
2+	4 (3%)	3 (2%)	7 (5%)	14 (11%)
3+	1 (1%)	7 (5%)	12 (9%)	20 (15%)

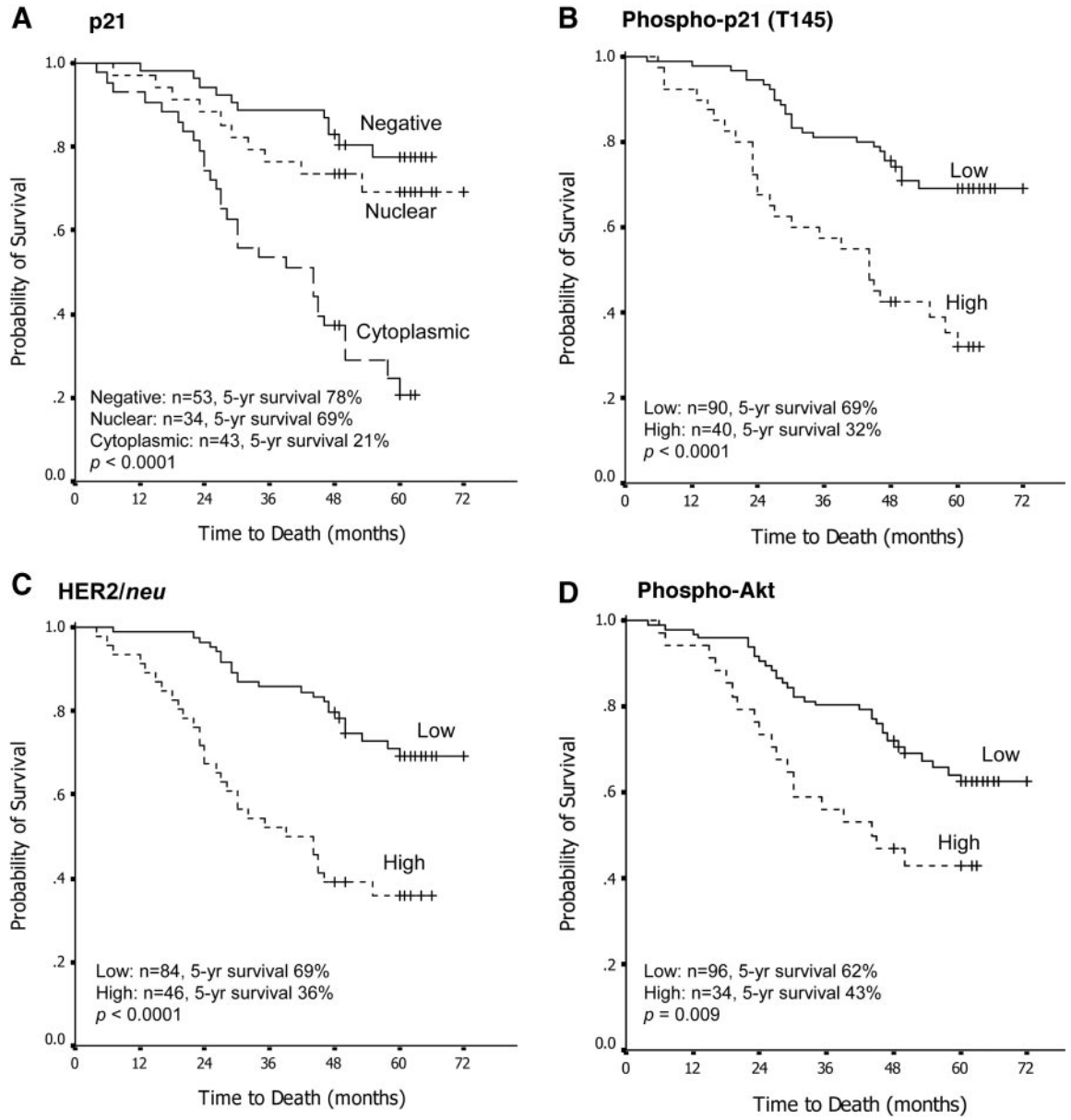

Fig. 4 Kaplan-Meier analyses of overall survival of breast cancer patients. Survival curves are stratified by expression status of p21 (*A*), phospho-p21 (T145) (*B*), HER2/*neu* (*C*), and phospho-Akt (*D*). Differences in survival distributions were evaluated by log-rank tests.

expression affected patient survival. We found that the overall survival of patients with p21 localized in the cytoplasm was much worse than that of patients in whom p21 was negative or localized in the nucleus ($P < 0.0001$; Fig. 4*A*). However, the difference in overall survival between patients negative for p21 and for nuclear p21 was not significant ($P = 0.32$). Consistent with cytoplasmic localization of p21, high levels of phospho-p21 (T145) were also associated with poor patient survival compared with low phospho-p21 (T145) levels (Fig. 4*B*). In addition, poor overall survival was noted in patients with overexpression of HER2/*neu* and phospho-Akt (Fig. 4, *C* and *D*). The effects of p21 subcellular localization and phospho-p21 (T145), HER2/*neu*, and phospho-Akt status on disease-free survival revealed trends similar to those for overall survival (data not shown).

Combined Subcellular Localization of p21 and Expression Status of HER2/*neu* Provide a Better Prognostic Factor. To determine the prognostic importance of clinicopathological parameters and the HER2/*neu*-Akt-p21 pathway members in breast cancer, we performed Cox proportional hazard regression analyses on 130 breast cancer patients. Univariate analysis revealed that large tumor size; advanced nodal status; advanced TNM stage; overexpression of HER2/*neu*, phospho-Akt, and phospho-p21 (T145); and cytoplasmic localization of p21 were associated with a poor outcome (Table 8*A*). In multivariate analysis, only large tumor size, advanced nodal status, overex-

Table 8 Cox's proportional hazard model analysis of prognostic factors in breast cancer patients

Variable	Parameter estimate	SE	*P*	Relative risk
A. Univariate analysis				
Age (> 48 *vs.* ≤48 yrs)	0.41	0.28	0.14	1.50
Tumor (II *vs.* I)	0.91	0.32	0.004	2.49
Tumor (III *vs.* I)	1.95	0.42	<0.0001	7.03
Node (N_1 *vs.* N_0)	0.68	0.40	0.09	1.97
Node (N_2 or N_3 *vs.* N_0)	0.80	0.37	0.033	2.22
Stage (II or III *vs.* I)	1.17	0.37	0.001	3.23
Grade (2 or 3 *vs.* 1)	0.69	0.41	0.09	1.98
HER2/*neu* (high *vs.* low)	1.31	0.28	<0.0001	3.72
Phospho-Akt (high *vs.* low)	0.77	0.29	0.008	2.16
Phospho-p21 (T145) (high *vs.* low)	1.22	0.28	<0.0001	3.39
p21 (cytoplasmic *vs.* others)	1.55	0.29	<0.0001	4.73
HER2/*neu* (low) + p21 (cytoplasmic)[a]	1.35	0.43	0.0018	3.85
HER2/*neu* (high) + p21 (neg.[b]/nuclear)[a]	1.04	0.44	0.018	2.83
HER2/*neu* (high) + p21 (cytoplasmic)[a]	2.32	0.37	<0.0001	10.18
B. Multivariate analysis				
Tumor status (T_2 *vs.* T_1)	0.42	0.33	0.21	1.52
Tumor status (T_3 *vs.* T_1)	2.17	0.47	<0.0001	8.77
Node (N_1 *vs.* N_0)	0.56	0.41	0.17	1.75
Node (N_2 or N_3 *vs.* N_0)	0.70	0.39	0.071	2.01
HER2/*neu* (high *vs.* low)	1.09	0.31	0.001	2.97
p21 (cytoplasmic *vs.* others)	1.31	0.31	<0.0001	3.71
HER2/*neu* (low) + p21 (cytoplasmic)[a]	1.37	0.44	0.002	3.92
HER2/*neu* (high) + p21 (neg./nuclear)[a]	1.15	0.45	0.011	3.15
	2.41	0.39	<0.0001	11.08

[a] The combination of HER2/*neu* (low) and p21 (negative/nuclear) is used as the baseline. The relative risk of the combined HER2/*neu* (low) and p21 (cytoplasmic) against baseline is 3.85 by univariate analysis, 3.92 by multivariate analysis.
[b] neg., negative.

pression of HER2/*neu*, and cytoplasmic localization of p21 were significant and independent prognostic factors in this study (Table 8*B*). To better predict the prognosis of breast cancer patients, we combined the expression status of HER2/*neu* and subcellular localization of p21, creating a new prognostic factor. To simplify the classification, patients negative for p21 and nuclear p21 were combined into one group (negative/nuclear p21) because their survival rates were not significantly different. When patients' overall survival was stratified by this new combined factor, the 5-year survival rates were, respectively, 79, 60, 29, and 16% for the four groups: (*a*) patients with low HER2/*neu* and negative/nuclear p21; (*b*) patients with high HER2/*neu* and negative/nuclear p21; (*c*) patients with low HER2/*neu* and cytoplasmic p21; and (*d*) patients with high HER2/*neu* and cytoplasmic p21 ($P < 0.001$; Fig. 5*A*). This new factor was found to be a more powerful predictor of patient survival than any individual clinicopathological or biological marker in this study by Cox regression model and Kaplan-Meier survival analyses (see also Table 8 and Fig. 4), with a relative risk by multivariate analysis of 11.08 ($P < 0.0001$) for patients with overexpressed HER2/*neu* and cytoplasmic p21. Furthermore, the predictive power and accuracy of this new prognostic factor could be comparable to TNM staging, the "gold standard" prognostic determinant of breast cancer (Fig. 5*B*).

DISCUSSION

The present study examined the expression status of the key members of the HER2/*neu*-Akt-p21 pathway in breast cancers as well as their relationship with clinicopathological parameters and patient survival. We demonstrated that the cytoplasmic localization of p21 is highly correlated with overexpression of phospho-p21 (T145). Both cytoplasmic p21 and overexpression of phospho-p21 (T145) are associated with high expression levels of HER2/*neu* and phospho-Akt, and all of these biological conditions are associated with poor survival of breast cancer patients. In addition, by the multivariate Cox proportional hazard model, overexpression of HER2/*neu* and cytoplasmic p21 were found to be significant and independent factors in predicting outcome. Further stratification of patients' survival by combined HER2/*neu* and p21 status provides a more accurate prediction than any individual clinicopathological and biological factor. The predictive power of this new combined factor could be comparable to that of TNM staging.

The role of p21 in the prognosis of breast cancer patients has been controversial. Several studies demonstrated that by using a monoclonal antibody (clone 4D10, mouse IgG1 subtype; Novocastra, Newcastle upon Tyne, United Kingdom), the expression of p21 was predominantly in the nucleus and provided no prognostic information (23, 24). However, using another monoclonal antibody (Ab-1; Calbiochem, Cambridge, MA), one study revealed that p21 immunoreactivity was both cytoplasmic and nuclear in the majority of breast cancers and that cytoplasmic p21, rather than the total p21 level, was associated with poor survival (25). In our study, two antibodies that recognized different forms of p21 were used. With anti-p21 (c-19; Santa Cruz Biotechnology), the immunoreactivity was both cytoplas-

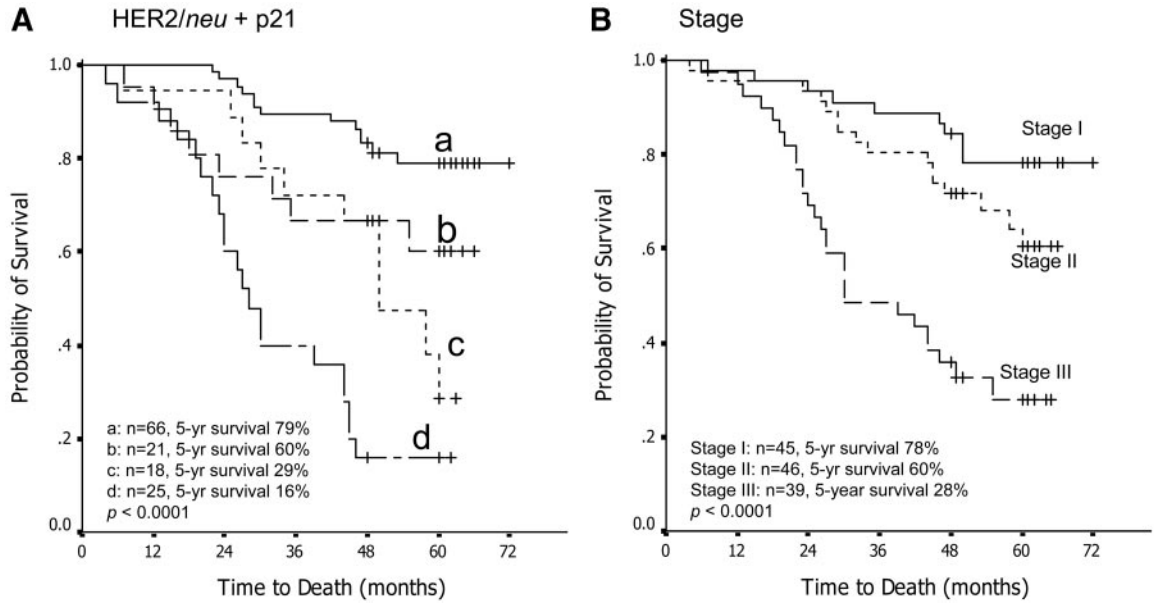

Fig. 5 Kaplan-Meier analyses of overall survival of breast cancer patients. Survival curves are stratified by a combination of expression status of HER2/*neu* and subcellular localization of p21 (*A*; *line a*, low HER2/*neu* and negative/nuclear p21; *line b*, high HER2/*neu* and negative/nuclear p21; *line c*, low HER2/*neu* and cytoplasmic p21; *line d*, high HER2/*neu* and cytoplasmic p21) and TNM stage (*B*). Differences in survival distributions were evaluated by log-rank tests.

mic and nuclear. With anti-phospho-p21 (T145), the immunoreactivity was primarily in the cytoplasm. Both antibodies consistently showed that p21, particularly the phosphorylated form, can be localized in the cytoplasm and that cytoplasmic localization of p21 was associated with a poor outcome for breast cancer patients. On the other hand, nuclear p21 was more closely associated with low levels of HER2/*neu* and phospho-Akt, and the overall survival of patients with nuclear p21 was not significantly different from that of those negative for p21. Our results suggest that the previous controversy over clinical results might be caused by the different antibodies used in IHC staining and different interpretation of subcellular localization of p21.

Many laboratory findings suggest that p21 might play dual roles in regulating cell cycle progression and apoptosis by changing its subcellular localization. Our data demonstrate that cytoplasmic p21 has a prognostic implication different from that of nuclear p21 in breast cancer patients. However, the molecular mechanisms by which p21 changes its subcellular localization remained elusive until we reported that Akt may play a pivotal role in p21 localization (12). In that study, we showed that activated Akt physically associates with and phosphorylates p21 at threonine 145, resulting in cytoplasmic localization of p21. Blocking of the Akt pathway with an Akt (KD) mutant restores the nuclear localization and cell-growth inhibition of p21. In the present study, we consolidate these findings in a clinical setting by showing that overexpression of activated/phosphorylated Akt is highly correlated with overexpression of phospho-p21 (T145) and cytoplasmic localization of p21 and that phospho-p21 (T145), when detected, is primarily localized in the cytoplasm. Our results also indicate that the Akt-p21 pathway plays an important role in the prognosis of breast cancer patients because high levels of phospho-Akt and phospho-p21 (T145) and cytoplasmic localization of p21 are associated with poor patient survival.

Although we clearly showed that activated Akt induces cytoplasmic localization of p21 and correlates with shorter survival of patients, the clinical sequence by which cytoplasmic p21 leads to poor prognosis remains unclear. Using a multivariate Cox proportional hazard model and analyses of the correlation with clinicopathological parameters, we found that cytoplasmic p21 is an independent prognostic factor that correlated with no clinicopathological parameters, such as large tumor size, advanced lymph node metastases, or poor tumor grade in this study. However, recent studies revealed that breast cancer cells and leukemia cells can induce resistance to chemotherapeutic drugs through overexpression of p21 (20, 21) and that activation of Akt can lead to cytoplasmic localization and stabilization of p21 (12, 30). We therefore hypothesize that after phosphorylation by activated Akt, p21 can increase its expression levels, translocate into the cytoplasm, induce the phenotype of chemoresistance, and finally lead to poor survival of breast cancer patients irrespective of tumor size, grade, and lymph node status.

The relationship between HER2/*neu* and the Akt-p21 pathway has rarely been addressed. In this study, we found that cytoplasmic localization of p21 and overexpression of phospho-p21 (T145) and phospho-Akt were highly associated with overexpression of HER2/*neu*, in concordance with our previous finding that the Akt pathway is required for HER2/*neu*-mediated cell proliferation (12). However, our patients' data also implied that overexpression of HER2/*neu* may not be the only mechanism to induce Akt activation and p21 phosphorylation/cytoplasmic localization in breast cancer patients because the mul-

tivariate Cox proportional hazard model revealed that HER2/*neu* overexpression and p21 cytoplasmic localization are significant but independent prognostic factors, suggesting that, in addition to HER2/*neu*, other pathways could activate Akt or phosphorylate p21 and cause p21 to localize in the cytoplasm. Furthermore, overexpression of HER2/*neu* can reduce patient survival through many pathways other than Akt-p21.

The advance of laboratory studies has provided many biological markers to predict the prognosis of patients and provide targets for therapy. The accumulated results of studies also indicate that, with appropriate selection, multiple markers might be more informative than any single marker for the prediction of clinical outcome in breast cancer patients (31). In this study, in addition to consolidating the mechanisms of p21 regulation through the activation of the HER2/Akt pathway, we also demonstrated that cytoplasmic p21 was an independent prognostic factor in breast cancer patients. A novel combination of subcellular localization of p21 and expression status of HER2/*neu* clearly stratified the patients into four distinctly different survival groups. Among them, the 5-year survival rate of patients with low HER2/*neu* and negative/nuclear p21 was 79%, in contrast to only 16% in those patients with high HER2/*neu* and cytoplasmic p21. This novel combination not only provides a better prognostic prediction than any individual clinicopathological or biological marker, it also indicates that targeting only one molecule, such as HER2/*neu*, could be insufficient. Novel therapeutic agents that target phosphorylation/cytoplasmic localization of p21 may also contribute to optimal treatment of breast cancer patients.

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Phosphorylation/Cytoplasmic Localization of p21$^{Cip1/WAF1}$ Is Associated with HER2/ *neu* Overexpression and Provides a Novel Combination Predictor for Poor Prognosis in Breast Cancer Patients

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