Association Between Pak1 Expression and Subcellular Localization and Tamoxifen Resistance in Breast Cancer Patients

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Background: **p21-activated kinase 1 (Pak1) phosphorylates many proteins in both normal and transformed cells. Its ability to phosphorylate and thereby activate the estrogen receptor α (ERα) potentially limits the effectiveness of antiestrogen treatment in breast cancer. Here we studied associations between Pak1 expression and subcellular localization in tumor cells and tamoxifen resistance.** ***Methods:*** **Pak1 protein expression was evaluated in 403 primary breast tumors from premenopausal patients who had been randomly assigned to 2 years of adjuvant tamoxifen or no treatment. Tamoxifen response was evaluated by comparing recurrence-free survival in relation to Pak1 and ERα expression in untreated versus tamoxifen-treated patients. Tamoxifen responsiveness of human MCF-7 breast cancer cells that inducibly expressed constitutively active Pak1 or that transiently overexpressed wild-type Pak1 (Wt-Pak1) or Pak1 that lacked functional nuclear localization signals (Pak1ΔNLS) was evaluated by analyzing cyclin D1 promoter activation and protein levels as markers for ERα activation. The response to tamoxifen in relation to Pak1 expression was analyzed in naturally tamoxifen-resistant Ishikawa human endometrial cancer cells. All statistical tests were two-sided.** ***Results:*** **Among patients who had ERα–positive tumors with low Pak1 expression, those treated with tamoxifen had better recurrence-free survival than those who received no treatment (hazard ratio [HR] = 0.502, 95% confidence interval [CI] = 0.331 to 0.762; *P* = .001) whereas there was no difference in recurrence-free survival between treatment groups for patients whose tumors had high cytoplasmic (HR = 0.893, 95% CI = 0.420 to 1.901; *P* = .769) or any nuclear Pak1 expression (HR = 0.955, 95% CI = 0.405 to 2.250; *P* = .916). In MCF-7 cells, overexpression of Wt-Pak1, but not of Pak1ΔNLS, compromised tamoxifen response by stimulating cyclin D1 expression. Treatment of Ishikawa cells with tamoxifen led to an increase in the amount of nuclear Pak1 and Pak1 kinase activity, suggesting that tamoxifen, to some extent, regulates Pak1 expression.** ***Conclusions:*** **Our data support a role for Pak1, particular Pak1 localized to the nucleus, in ERα signaling and in tamoxifen resistance. [J Natl Cancer Inst 2006;98:671–80]**

Members of the family of serine/threonine kinases known as p21-activated kinases (Paks) are involved in a range of essential cellular functions, including cell motility, gene expression, apoptosis, angiogenesis, and mitogen-activated protein (MAP) kinase signaling *(1–3)*. Because of their extensive roles in these pathways, Paks have also been suggested to be involved in the progression of human cancer *(4,5)*. Pak1, the first member of the Pak family to be identified, is activated by several growth factors, tyrosine kinases, G proteins, and estrogen *(6)*. Most of our current understanding of the role of Pak1 in human cancer is derived from studies in breast cancer and mammary epithelial cells. Overexpression of constitutively active Pak1 mutants in these cells causes the accumulation of F-actin and the formation of lamellipodia and filopodia *(7)*. In addition to its cytoskeletal effects, Pak1 also activates the JNK/SAPK and p38MAPK kinase pathways and thus influences nuclear signaling *(8,9)*.

Several studies have suggested that Pak1 is involved in breast cancer, apart from its effects on cell motility. For example, Adam et al. *(10)* have shown that Pak1 activation has a mechanistic role in the increased invasiveness of breast cancer cells treated with growth factors. Furthermore, expression of a Pak1 mutant with an inactivated kinase domain in highly invasive breast cancer cell lines led to stabilization of stress fibers, enhanced cell spreading, reduced JNK–AP1 pathway activity, and reduced invasiveness *(11)*. Conversely, conditional expression of constitutively active Pak1 in the noninvasive breast cancer cell line MCF-7 promoted anchorage-independent growth *(12)*. Emerging data also suggest that Pak1 is overexpressed in human cancer. For example, PAK1 gene amplification and increased Pak1 protein levels have been reported in ovarian *(13)* and breast *(14,15)* cancer. The PAK1 gene is located at a 11q13, a chromosomal region that is that is frequently amplified in breast cancer, together with CCND1, the gene that encodes cyclin D1.

Recent data from transgenic mice expressing constitutively active Pak1 have established a role for Pak1 signaling in the development of hyperplasia in mammary epithelium and in the stimulation of estrogen-inducible genes. The underlying mechanism of Pak1 regulation of the estrogen pathway involves direct phosphorylation by Pak1 of estrogen receptor α (ERα) at Ser305, leading to ligand-independent stimulation of its activation function-2 domain *(16)*. For example, increased Pak1 expression in breast cancer cells is known to stimulate expression of cyclin D1 *(15)*, a well-known estrogen target gene, via Ser305

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See "Notes" following "References."

DOI: 10.1093/jnci/djj185

phosphorylation of ERα *(17)*. In addition to its cytoplasmic functions, Pak1 has also been found in the nuclear compartment of a subset of interphase breast cancer cells *(18)*. This localization has been attributed to nuclear localization signals that were recently identified in the Pak1 protein *(19)*.

These findings indicate that Pak1 may affect estrogen action by phosphorylation of the ERα. If so, the levels and subcellular localization of Pak1 could potentially affect the action of antiestrogen therapies, such as tamoxifen. Therefore, we investigated possible associations between tamoxifen response and the level and subcellular localization of Pak1 protein in human breast cancer. The availability of primary tumors from premenopausal patients participating in a randomized trial with long-term follow-up gave us the unique opportunity to examine treatment responses in subgroups defined by Pak1 status. Experimental models were further used to test the effect of tamoxifen in relation to Pak1 expression.

Materials and Methods

Patients

From January 1, 1986, to September 30, 1991, a total of 564 premenopausal breast cancer patients with invasive stage II disease were enrolled in SBII:2a *(20)*, a Swedish clinical trial in which patients were randomly assigned to receive 2 years of adjuvant tamoxifen (n = 276) or no treatment (control) (n = 288) and followed up for recurrence-free and overall survival. (When the study was initiated, the evidence indicating that 5 years of tamoxifen treatment was more effective than 2 years of treatment was not available.) Recurrence-free survival was defined as time from date of surgery to clinically defined recurrence including local, regional, and distant recurrences and breast cancer–specific death, but not contralateral breast cancer, as the primary event. All patients received surgery (either radical mastectomy or breast-conserving surgery) followed by radiotherapy, and fewer than 2% of patients were given adjuvant polychemotherapy. The median follow-up time for patients who had no breast cancer event was 13.9 years. A detailed description of the study has been reported previously *(20)*.

Tissue Microarrays and Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor material was available from 500 of the 564 patients in the trial. Areas representative of invasive cancer were selected and assembled in a tissue microarray. In brief, two 0.6-mm tissue cores from each donor block were placed in a recipient paraffin block (each of which consisted of approximately 200 cores) by using an automated tissue arrayer (Beecher Instruments Microarray Technology, Woodland, MD). Sections (4 μm thick) from the recipient blocks were mounted onto slides and deparaffinized with xylene and rehydrated in graded ethanol series. The slides were then microwave-treated in target retrieval solution (Dako, Glostrup, Denmark), pH 9.9, at 900 W, 750 W, 650 W, 450 W, and 300 W for 2 minutes each and processed for immunohistochemical staining with a rabbit polyclonal antibody against human Pak1 (1 : 25 dilution; Cell Signaling, Beverly, MA) in a Techmate 500 automated immunostainer using the Envision software (Dako). Bound Pak1 antibody was detected with diaminobenzidine. Pak1 cytoplasmic staining intensity and nuclear reactivity were initially evaluated by one observer (CH). An additional evaluation was then performed together with a second observer (GL). In general, there was a low interrater variability, with approximately 5% disagreement for Pak1 cytoplasmic staining as well as for nuclear reactivity. The few tumors with a disagreement in the grading were reexamined, followed by a conclusive decision. All immunohistochemical evaluations were performed without knowledge of the tumor characteristics. We were able to assess Pak1 staining in 403 tumors. Inability to assess staining was due either to loss of tumor cores or to nonrepresentative cores, i.e., cores that did not contain invasive tumor cells, on the tissue microarrays. In two tumors, the two cores from the same tumor gave discrepant results, and in three cases there was slight variability in Pak1 staining within one core, indicating that there was little heterogeneity of Pak1 staining. In total, 97 tumors could not be evaluated for Pak1 staining and were not included in the analysis; those tumors were somewhat smaller than the tumors that were included in the analysis and were more likely to be of low grade and low proliferation rate and were more likely to be progesterone receptor positive. One possible explanation for this finding could be that smaller tumors are more difficult to target for tissue microarray construction than larger tumors.

Estrogen receptor and progesterone receptor expression in the tumors had already been assessed either at the time of diagnosis or in a previous study *(20)* using enzyme immunoassay and immunohistochemical analysis. For correlation studies, we used the immunohistochemical data and 10% positively stained nuclei as a cutoff for positivity, which is a clinically established cutoff used for hormone receptor assessment in Sweden *(20)*. For survival analysis, to include as many patients as possible, we used tumors that were positive for ERα in either the enzyme immunoassay or by immunohistochemistry. Of the 403 patients whose tumors could be assessed for Pak1 staining, ER status was available for 383 patients. Of these, 285 patients were positive for ERα expression (151 in the control group and 134 in the treatment group). A total of 284 patients were included in the survival analysis (follow-up data were missing for one patient).

Amplification status of the cyclin D1 gene, CCND1, determined by fluorescence in situ hybridization analysis, and the intensity of the cyclin D1 protein, were already available for the tumor samples in this study *(21)*. The CCND1 gene was considered amplified when the ratio of intensity of the CCND1 probe to the centromere probe was greater than 1 in at least 20% of tumor cells. Expression of the proliferation marker Ki-67 in relation to tamoxifen response has been evaluated in a previous study, where tumors have been categorized according to the percentage of Ki-67–positive cells (0%–10%, 11%–25%, >25%) *(22)*.

Cell Lines and Western Blot Analysis

Six human breast cancer cell lines (American Type Culture Collection [ATCC], Manassas, VA) were used to verify the reactivity of the Pak1 antibody by immunohistochemistry and western blot analysis. MDA-MB-468, MDA-MB-231, BT-549, CAMA-1, and MCF-7 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1 mM sodium pyruvate, with the addition of 0.01 mg/mL insulin to MCF-7 cells. T-47D cells were grown in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FBS, 10 mM HEPES, and 0.02 U/mL insulin. Endometrial Ishikawa cells

(ATCC) were cultured in DMEM supplemented with 10% FBS. MCF-7/DA-Pak1 cells, a human breast cancer cell line that expresses catalytically active Pak1 under the control of an inducible promoter, was maintained in DMEM supplemented with 5% tetracycline-free serum (Sigma, St. Louis, MO).

MCF-7 cells were transiently transfected with 5 μg of myc-tagged Pak1 expression vector (provided by J. Chernoff, Fox Chase Cancer Center, Philadelphia, PA) or 5 μg of control vector (i.e., vector without insert) by using a Nucleofector kit (Amaxa Biosystem, Cologne, Germany) according to the manufacturer's instructions.

For immunohistochemistry, cells were harvested by trypsin treatment, washed with phosphate-buffered saline (PBS), fixed in 1 mL of 4% paraformaldehyde for 25 minutes, and stained with Mayers hematoxylin for 5 minutes. Cells were centrifuged at 2000*g* for 30 seconds, and the cell pellet was resuspended in ice-cold 70% ethanol, dehydrated in graded ethanol series, and embedded in paraffin.

For western blot analysis, cells were washed and scraped into ice-cold PBS, centrifuged at 300*g* for 5 minutes at 4 °C, and lysed on ice in 50 mM Tris–HCl pH 7, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM EDTA pH 8, 1 mM NaF and 0.1 mg/mL phenylmethylsulphonyl fluoride supplemented with Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany) for a total of 30 minutes with vortexing every 10 minutes. The protein lysates were centrifuged at 14 000*g* for 30 minutes and the supernatants were collected. Protein content was measured with a BCA Protein Assay kit (Pierce, Rockford, IL), and 30 μg of each protein sample was resolved on SDS–polyacrylamide gels and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were incubated overnight at 4 °C with the Pak1 antibody (1 : 1000 dilution) and a polyclonal goat anti–human beta-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with horseradish peroxidase–conjugated anti-rabbit (Amersham Life Science, Alesbury, UK) and anti-goat (Sigma) antibodies. Membrane-bound antibody was detected by using the ECL$^+$ system (Amersham).

Reporter Assay

MCF-7 cells were cultured for 24 hours in minimal essential medium without phenol red (Invitrogen, Carlsbad, CA) supplemented with 5% Dextran charcoal-stripped serum (Sigma) as previously described *(16)*. The cells were transiently transfected with a cyclin D1-luciferase reporter construct and either an empty cytomegalovirus (CMV) expression vector or CMV vector that expressed wild-type Pak1 (Wt-Pak1) or Pak1 bearing mutant nuclear localization signals (Pak1ΔNLS) *(15,19)* for 24 hours in the presence of FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. The total amount of DNA the cells were exposed to was kept constant by adding additional empty CMV vector. The cells were then treated with 1 μM tamoxifen (Sigma) for 16 hours at 37 °C, lysed with reporter-lysis buffer (Promega, Madison, WI), and centrifuged at 8000*g* for 5 minutes. The resulting supernatant was subjected to a luciferase assay using a luciferase reporter assay kit (Promega), and cyclin D1 reporter activity was measured in a luminometer as a measure of ERα activation. Transfections were carried out in six-well plates in triplicate.

Cell Growth Assay

Ishikawa cells were grown in DMEM supplemented with 5% charcoal-stripped serum for 48 hours and treated with tamoxifen (10^{-8} M) for 5 days and then counted in a Coulter counter.

Nuclear and Cytoplasmic Extract Preparation

Nuclear and cytoplasmic protein extracts were prepared from Ishikawa cells treated with tamoxifen (as above) by using a nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's instructions. Pak1 was detected in the extracts by western blot analysis, as described above.

Pak1 Kinase Assay

Pak1 kinase activity was assayed in Ishikawa cells by using myelin basic protein (MBP; Upstate Cell Signaling Solutions, Charlottesville, VA) as a substrate for phosphorylation, as previously described *(23)*. In brief, whole-cell lysates were prepared by using NP-40 lysis buffer (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 0.5% NP-40, 1× protease inhibitor cocktail, and 1 mM sodium vanadate) and immunoprecipitated with the Pak1 antibody (Cell Signaling). Immunocomplexes were recovered with Protein A beads (Amersham Biosciences) and washed three times with NP-40 lysis buffer and two times with kinase buffer (20 mM HEPES [pH 7.4], 1 mM dithiothreitol, 10 mM $MnCl_2$, 10 mM $MgCl_2$). The immunocomplexes were resuspended in 30 μL of kinase buffer containing 20 ng of MBP and 10 μCi of [γ^{32}P]ATP and incubated for 30 minutes at 30 °C. The kinase reaction was terminated by adding 10 μL of 4× SDS–polyacrylamide gel electrophoresis sample buffer (250 mM Tris–HCl [pH 6.8], 40% glycerol, 0.04% bromophenol blue). Samples were then resolved on SDS–12% polyacrylamide gels and transferred to membranes. Phosphorylated MBP was detected by Phosphoimager using ImageQuant software.

Confocal Immunofluorescence Microscopy Studies

We used indirect immunofluorescence to examine the subcellular distribution of Pak1 in MCF-7/DA-Pak1 cells, as previously described *(24)*. In brief, cells grown on glass cover slips were fixed in 4% paraformaldehyde for 20 minutes at room temperature, incubated with the Pak1 antibody (1 : 200 dilution) for 2 hours at room temperature, washed three times with PBS, and incubated with Alexa Fluor 488–labeled secondary antibody (Molecular Probes, Eugene, OR). We used Topro-3 (Molecular Probes) to stain the DNA. Confocal microscopy was performed with the use of a Zeiss laser-scanning confocal microscope.

Statistical Methods

We used Spearman's rank-order correlation coefficient (ρ) and Pearson's chi-square test to examine the statistical significance of associations between Pak1 expression levels and other categorical variables. The Kruskal–Wallis test was used to compare medians for continuous variables. The Kaplan–Meier method was used to estimate recurrence-free survival, and the log-rank test was used to compare recurrence-free survival among different treatment groups. A Cox proportional hazards regression model was used for multivariable analysis, and proportional hazards assumptions were verified graphically. By using the conservative Bonferroni

adjustment in the correlation analyses, a P value less than .0025 would be considered statistically significant. The P values presented here have not been adjusted for multiple testing. All P values corresponded to two-sided tests, and P less than .05 was considered statistically significant. Statistical analyses were performed using SPSS software (version 11.0; SPSS, Chicago, IL).

Results

Specificity of Pak1 Immunohistochemical Staining

The immunohistochemical reactivity of the Pak1 antibody was analyzed in six human breast cancer cell lines in parallel with western blot analysis of protein extracts from the cell lines (Fig. 1, A). A band corresponding to a 68-kDa protein (the predicted molecular mass of Pak1) was observed on the Pak1 western blot. In general, the intensity of Pak1 antibody staining detected by immunohistochemistry corresponded with the level of Pak1 detected by western blot analysis. MCF-7 cells transiently transfected with a myc-tagged Pak1 expression vector displayed stronger Pak1 antibody reactivity than MCF-7 cells transfected with empty vector, both in western blots and by immunohistochemistry (Fig. 1, B), confirming the specificity of the Pak1 antibody. A careful examination of MCF-7 cells overexpressing Pak1 revealed Pak1 antibody staining of the nuclear compartment (Fig. 1, B).

Pak1 Expression in Human Breast Tumors

During our initial examination of the Pak1 antibody–stained tissue microarrays, cytoplasmic Pak1 staining intensity varied among the tumor cores, ranging from no or low staining to very strong staining. This difference in staining intensity formed the basis for the definition of low versus high cytoplasmic Pak1 staining used below. Similar variations in Pak1 staining patterns were observed when analyzing other tissue microarrays from patients not included in the study (data not shown), providing more

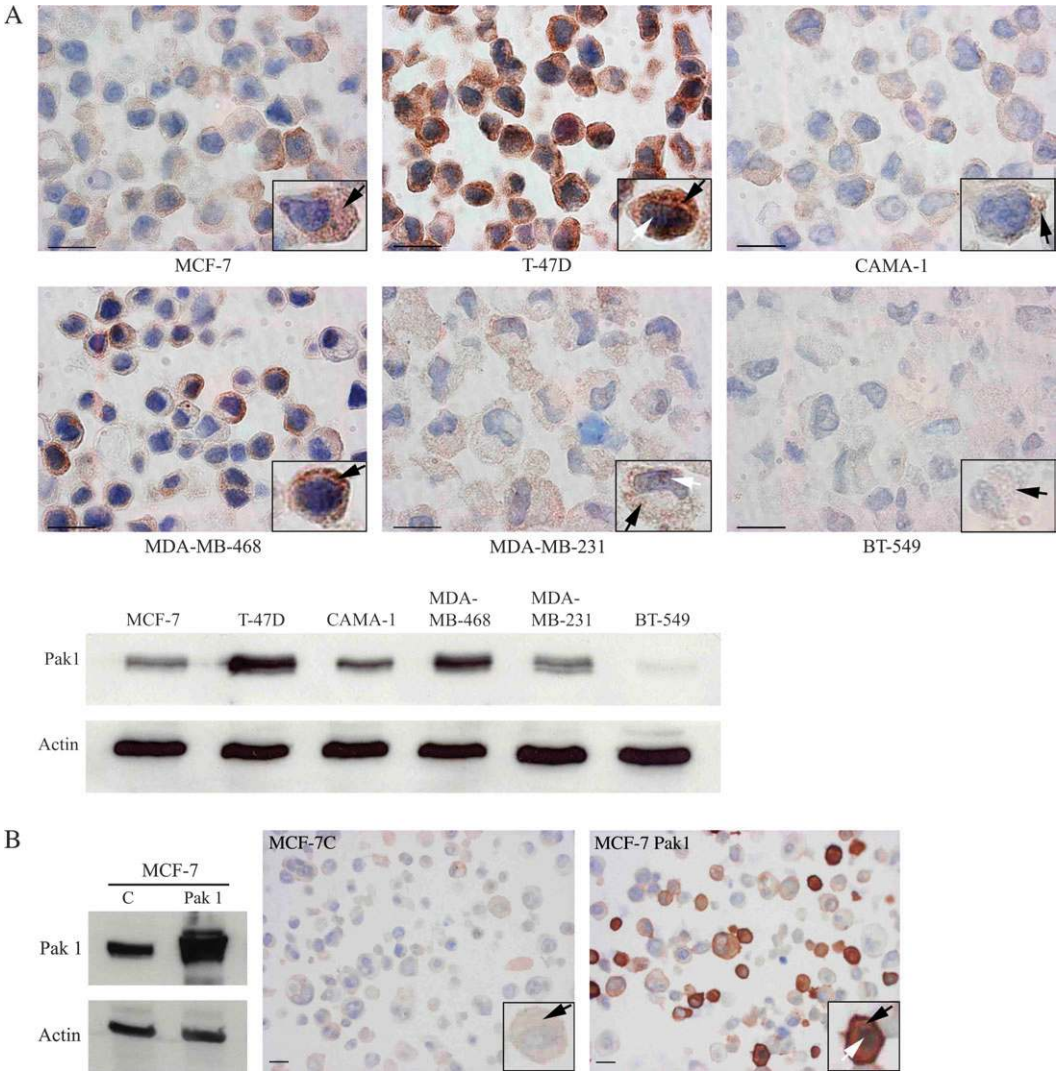

Fig. 1. Immunohistochemical and western blot analyses of Pak1 expression. **A**) Six human breast cancer cell lines were analyzed by immunohistochemistry (**upper panels**) and by western blotting (**bottom panels**) for the expression of Pak1 by using an antibody against human Pak1. **Arrows** in the magnified boxes indicate the presence of cytoplasmic (**black arrows**) and nuclear (**white arrows**) localization of Pak1 (**brown**) in the cell lines. **Scale bar** = 20 μm. Thirty micrograms of protein was resolved on sodium dodecyl sulfate–polyacrylamide gel, and actin was used as loading control. One band corresponding to 68 kDa was observed for the Pak1 antibody. **B**) Pak1 antibody staining of MCF-7 cells transiently transfected with myc-tagged Pak1 expression vector (MCF-7 Pak1) or empty expression vector (MCF-7 C). Both cytoplasmic (**black arrow**) and nuclear (**white arrow**) localization of overexpressed Pak1 (**arrows in the magnified boxes**) is evident.

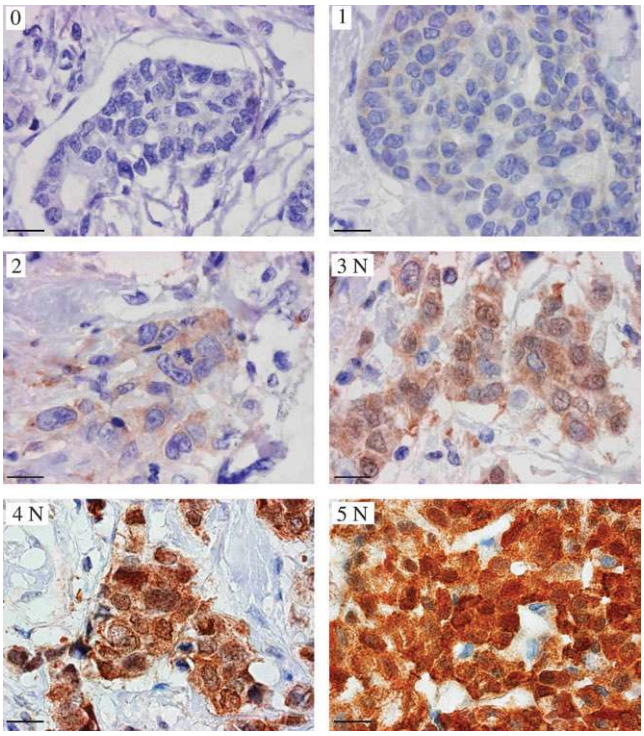

Fig. 2. Pak1 staining of primary breast cancer samples. Breast cancer tissue microarrays were stained with the Pak1 antibody (**brown**) and the staining was scored from 0 (**no staining**) to 5 (**strongest staining**) on the basis of a qualitative assessment of staining intensity. Tumors were also assessed for the presence of any nuclear (N) staining in combination with cytoplasmic staining. **Scale bar** = 20 μm.

support for classifying the tumors on the basis of Pak1 staining intensity. We also noted a more modest but consistent variation in staining intensity within both the low- and high-staining tumors; therefore, we subdivided the tumors with low and high cytoplasmic Pak1 staining into six subcategories of staining intensity (groups 0, 1, 2, 3, 4, and 5) on the basis of these subtle (i.e., qualitative) differences (Fig. 2). In the following statistical analyses, we combined tumors in group 0 with those in group 1 and the tumors in group 4 with those group 5 because of the small number of tumors in each group. We also observed that the presence of nuclear Pak1 staining varied among the samples; thus, all tumor cores were also categorized by the presence or absence of nuclear Pak1 staining. Among the 403 primary breast tumors analyzed, 76 tumors (19%) were categorized as having high cytoplasmic Pak1 expression (groups 3–5) and 52 tumors (13%) were positive for nuclear Pak1 staining. (Tumors with highest cytoplasmic staining, i.e., group 5, also had high nuclear staining.) Nuclear Pak1 staining was statistically significantly correlated with cytoplasmic Pak1 staining (Spearman's $\rho = .571$; $P<.001$; Table 1), and all tumors with highest intensity of cytoplasmic Pak1 staining (groups 4–5) also had nuclear Pak1 staining, whereas tumors in group 2 and 3 regarding cytoplasmic Pak1 staining intensity varied with respect to nuclear Pak1 positivity.

Pak1 Staining Intensity and Subcellular Localization and Clinicopathologic Tumor Parameters

We next analyzed associations between Pak1 staining intensity and subcellular localization and clinicopathologic parameters of the breast tumors (Table 2). There was a statistically significant association between cytoplasmic Pak1 staining and tumor

Table 1. Correlation between cytoplasmic and nuclear Pak1 staining*

	Cytoplasmic Pak1 staining intensity group			
Nuclear Pak1 staining	0–1 (N = 208)	2 (N = 119)	3 (N = 52)	4–5 (N = 24)
–	208	112	31	0
+	0	7	21	24

*– = negative; + = positive. Correlation was calculated using Spearman correlation test. Spearman's $\rho = .571$; $P<.001$.

type ($P = .006$, Pearson's chi-square test); a higher percentage of lobular breast cancers were Pak1 negative (78%) than ductal (50%) or medullary (30%) breast cancers. Cytoplasmic Pak1 staining intensity was also strongly correlated with histologic grade (Spearman's $\rho = .211$; $P<.001$) and with the level of tumor cell proliferation, defined as the percentage of tumor cells positive for Ki-67 (Spearman's $\rho = .266$; $P<.001$), suggesting that tumor with a more malignant phenotype, which are often less differentiated and highly proliferative, have higher Pak1 expression than those with a less malignant phenotype. We also found that positive nuclear Pak1 staining was correlated with tumor cell proliferation (Spearman's $\rho = .145$; $P = .006$). The relationship between Pak1 expression and hormone receptor status was complex. In general, Pak1 staining intensity was inversely correlated with ERα positivity (Spearman's $\rho = -.136$; $P = .007$); however, ERα positivity was positively correlated with strong Pak1 staining (groups 4–5); (Spearman's $\rho = .207$; $P = .001$). Pak1 staining intensity was not associated with patient age at surgery, tumor size, or lymph node status.

Pak1 Staining Intensity, CCND1 Gene Amplification, and Cyclin D1 Staining Intensity

Pak1 is involved in the regulation of cyclin D1 *(15,17)*, and both PAK1 *(13,25)* and CCND1 *(14,25)* have been localized to the same chromosomal region, 11q13. Consequently, we examined whether Pak1 protein expression was correlated with expression of cyclin D1 and/or with CCND1 gene amplification in the breast tumors. For this analysis, we used cyclin D1 protein expression and CCND1 gene copy number data, which were obtained for these same tumors in a previous study *(21)*. As summarized in Table 2, CCND1 gene amplification was correlated with high Pak1 expression in the cytoplasm (Spearman's $\rho = .135$; $P = .039$) and with the presence of nuclear Pak1 ($P = .008$, Fisher's exact test). Cytoplasmic Pak1 staining intensity was also positively correlated with cyclin D1 staining intensity (Spearman's $\rho = .109$; $P = .033$), as has been found in other recent studies *(15)*.

Pak1 Expression and Subcellular Localization and Tamoxifen Response in Patients

Several recent studies *(16,17)* have shown that Pak1 interacts with and phosphorylates ERα, leading to activation and potential modulation of the receptor and its response to estrogens and antiestrogens. This finding raises the possibility that high expression of Pak1 in breast cancer cells induces tamoxifen resistance in vivo, i.e., disease relapse despite tamoxifen treatment. We therefore analyzed the tumor-specific expression of Pak1 in breast cancer patients who had been randomly assigned to tamoxifen or no treatment as part of a clinical trial to examine whether the

Table 2. Distribution of Pak1 staining category in breast tumors according to clinico-pathologic and molecular parameters*

Variable	Pak1 cytoplasmic staining category					Pak1 nuclear staining category		
	0–1 N = 208	2 N = 119	3 N = 52	4–5 N = 24	P†	Negative N = 351	Positive N = 52	P†
Median age at surgery,y (range)	44 (27–57)	43 (25–56)	44 (31–52)	45 (37–54)	.293‡	44 (25–57)	44 (33–54)	.713‡
Tumor size, mm								
≤20	78	36	16	9	.265	122	17	.876§
≥21	129	83	36	15		228	35	
Missing cases: 1								
Tumor type								
Ductal	167	101	44	22	.006‖	291	43	.156‖
Lobular	25	4	1	2		30	2	
Medullary	7	10	6	0		17	6	
Missing cases: 14								
Lymph node status (no. involved nodes)								
Negative (0)	56	37	19	5	.701	101	16	.579
Positive (1–3)	107	62	18	13		179	21	
Positive ≥4	43	20	15	6		69	15	
Missing cases: 2								
NHG								
I	31	5	1	1	<.001	36	2	.243
II	89	44	13	14		139	21	
III	80	67	37	9		165	28	
Missing cases: 12								
Ki-67–positive, %								
0–10	100	34	10	5	<.001	139	10	.006
11–25	49	30	14	11		87	17	
>25	36	40	22	4		84	18	
Missing cases: 48								
ER–positive, %								
<10	52	50	28	1	.007	113	18	.752§
>10	151	66	23	22		229	33	
Missing cases: 68								
PR–positive, %								
<10	53	48	31	1	.003	115	18	1.00§
>10	147	62	20	22		218	33	
Missing cases: 71								
CCND1 gene amplified								
No	107	57	26	5	.039	174	21	.008§
Yes	16	11	3	8		27	11	
Missing cases: 170								
Cyclin D1 staining intensity								
Low/Moderate	181	103	47	17	.033	305	43	.126§
High	13	14	4	6		29	8	
Missing cases: 18								
Treatment group								
Control	113	56	29	8	.163§	183	23	.302§
Tamoxifen 2 years	95	63	23	16		168	29	
Missing cases: 0								

*NHG = Nottingham histologic grade; ER = estrogen receptor; PR = progesterone receptor.

†Correlations were calculated using Spearman's ρ unless otherwise specified. *P* values were not adjusted for multiple testing.

‡Kruskal–Wallis test (two-sided).

§Fisher's exact test (two-sided).

‖Pearson's chi-square test (two-sided).

level of Pak1 expression was associated with tamoxifen resistance. These analyses were restricted to patients with ERα-positive breast cancer, i.e., patients with the potential to respond to an ERα-targeting therapy such as tamoxifen.

The association of tamoxifen treatment with recurrence-free survival was evaluated separately in strata defined by Pak1 staining intensity (Fig. 3). Among the patients whose tumors had the lowest expression of Pak1 (groups 0–1 and 2), those who were treated with tamoxifen had better recurrence-free survival than those who were not treated with tamoxifen (HR = 0.568, 95% CI = 0.344 to 0.940; *P* = .026 and HR = 0.392, 95% CI = 0.185 to 0.828; *P* = .011, respectively) (Fig. 3, A). However, among the two groups of patients whose tumors had the highest expression of Pak1 (groups 3 and 4–5), those who were treated with tamoxifen did not have better recurrence-free survival than those who were not treated with tamoxifen (HR = 0.897, 95% CI = 0.318 to 2.529; *P* = .838 and HR = 0.984, 95% CI = 0.296 to 3.275; *P* = .980, respectively). When recurrence-free survival was analyzed in relation to tamoxifen treatment for the two main groups of cytoplasmic Pak1 staining intensities (i.e., groups 0–2 and groups 3–5), the low-expression group showed a difference, whereas the high-expression group did not (HR = 0.502, 95% CI = 0.331 to 0.762; *P* = .001 and HR = 0.893, 95% CI = 0.420 to 1.901; *P* = .769, respectively) (Fig. 3, B). The same pattern was observed after stratification for nuclear staining of Pak1, with a positive effect of tamoxifen on recurrence-free

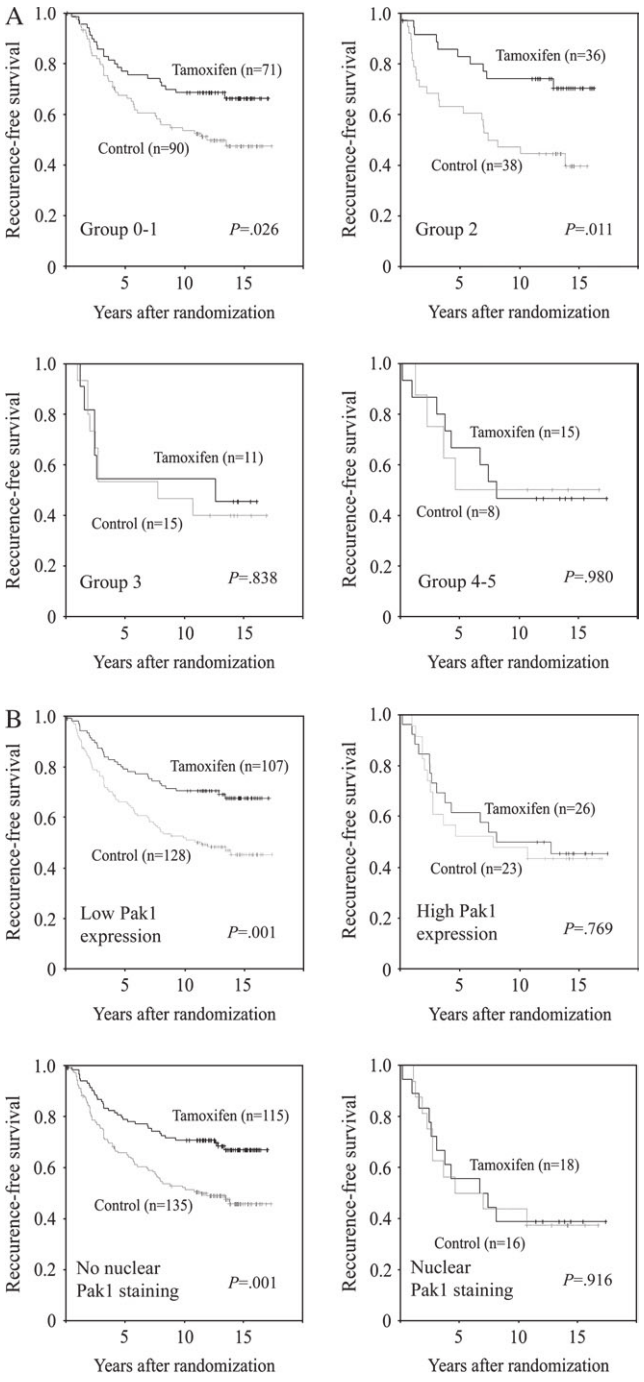

Fig. 3. Recurrence-free survival among breast cancer patients with estrogen receptor α–positive tumors randomly assigned to tamoxifen treatment or control according to Pak1 staining status. **A)** Patients were separated into four groups according to Pak1 cytoplasmic staining intensity and recurrence-free survival was determined. Number of censored patients: groups 0–1: control n = 45, tamoxifen n = 48; group 2: control n = 16, tamoxifen n = 26; group 3: control n = 6, tamoxifen n = 5; groups 4–5: control n = 4, tamoxifen n = 7. **B)** Recurrence-free survival determined for patients separated into low expression (groups 0–1 and group 2), high expression (groups 3 and 4–5), no nuclear, or nuclear staining of Pak1 in tumors. Number of censored patients: low expression: control n = 61, tamoxifen n = 74; high expression: control n = 10, tamoxifen n = 12; no nuclear staining: control n = 65, tamoxifen n = 79; nuclear staining: control n = 6, tamoxifen n = 7.

survival in the subgroup with no nuclear staining but not in the subgroup with nuclear Pak1 expression (HR = 0.515, 95% CI = 0.344 to 0.770; $P = .001$ and HR = 0.955, 95% CI = 0.405 to 2.250; $P = .916$, respectively) (Fig. 3, B).

To test whether the effect of tamoxifen treatment on recurrence-free survival was statistically significantly different between subgroups defined by Pak1 expression, a Cox model with main effects for Pak1 and tamoxifen treatment and an interaction term (Pak1 treatment) was fitted. There was no statistically significant difference in tamoxifen treatment effect on recurrence-free survival between low and high Pak1 cytoplasmic staining groups ($P = .137$, Table 3). However, the effect of tamoxifen treatment was statistically significantly different between the two subgroups defined by nuclear staining of Pak1 ($P = .044$, Table 3).

Effect of Tamoxifen on Pak1 Expression In Vitro

We next used MCF-7/DA-Pak1 cells, a human breast cancer cell line that expresses constitutively active Pak1 under the control of an inducible promoter *(12,16)*, to examine the effects of tamoxifen on the subcellular distribution of Pak1. In MCF-7/DA-Pak1 cells in which the inducible promoter is active, tamoxifen cannot exert its antiestrogenic effects after estrogen stimulation *(23)*. In MCF-7/DA-Pak1 cells grown in the presence of inducer, Pak1 was localized primarily in the cytoplasmic compartment when cells were cultured in the absence of tamoxifen. However, when cultured in the presence of tamoxifen these cells displayed increased accumulation of Pak1 in the nuclear compartment (Fig. 4, A). Tamoxifen had no effect on the subcellular distribution of Pak1 in MCF-7/DA-Pak1 cells with the inducible promoter inactive (data not shown). These findings are consistent with our findings in the human tumor specimens and suggest a potential role of nuclear Pak1 in conferring tamoxifen resistance. In support of this hypothesis, we found that tamoxifen induced expression of the estrogen target gene, cyclin D1, in MCF-7/DA-Pak1 cells with the inducible promoter on (Fig. 4, B).

We next investigated whether nuclear localization of Pak1 was necessary to suppress the antiestrogenic effect of tamoxifen. We found that overexpression of wild-type Pak1 promoted ERα-transactivating functions in response to tamoxifen treatment, as measured by an increase in cyclin D1 promoter–driven luciferase activity, whereas overexpression of Pak1 that lacked nuclear localization signals (Pak1ΔNLS) did not (Fig. 5, A). Furthermore, cells that overexpressed Pak1ΔNLS showed decreased luciferase activity in response to tamoxifen treatment. These findings are consistent with the notion that nuclear localization of Pak1 is intimately linked to tamoxifen resistance.

To further examine this possibility, we examined the potential relationship between Pak1 localization and activity and tamoxifen resistance in Ishikawa endometrial cancer cells, which are naturally resistant to tamoxifen (Fig. 5, B). We found that tamoxifen treatment of Ishikawa cells was accompanied by increased Pak1 kinase activity (Fig. 5, C) as well as increased accumulation of Pak1 in the nuclear fraction (Fig. 5, D), indicating that tamoxifen may, to some extent, regulate the localization and the kinase activity of Pak1, which in turn seems to be closely linked to the sensitivity of breast tumor cells to the antiestrogenic effect of tamoxifen.

Prognostic Value of Pak1 in Breast Cancer

To assess whether Pak1 expression had prognostic value for patients with breast cancer, we examined recurrence-free survival among patients in the untreated control group (which included both ER-negative and ER-positive patients) according to Pak1 staining intensity in their breast tumors (Fig. 6). Neither

Table 3. Two multivariable Cox proportional hazards models for Pak1 cytoplasmic and nuclear staining status and treatment interaction based on ERα-positive breast cancer patients (n = 284)*

Variable		Recurrence-free survival		
		HR	95% CI	*P*
Pak1 cytoplasmic staining	high versus low	1.09†	0.55 to 2.16	.814
Treatment	tamoxifen versus control	0.46‡	0.29 to 0.72	.001
Interaction variable§	tamoxifen × Pak1 cytoplasmic staining	2.09	0.79 to 5.52	.137
Pak1 nuclear staining	positive versus negative	1.09‖	0.49 to 2.41	.829
Treatment	tamoxifen versus control	0.45¶	0.29 to 0.70	<.001
Interaction variable§	tamoxifen × Pak1 nuclear staining	3.05	1.03 to 9.02	.044

*Other factors included in the multivariable analysis: age (continuous), tumor grade (NHG I+II vs. III), proliferation (Ki67 ≤25% vs. >25%), and nodal status (negative versus positive). ER = estrogen receptor; NHG = Nottingham Histologic Grade; HR = hazard ratio; CI = confidence interval.

†Pak1 cytoplasmic staining hazard ratio for the control group.

‡Treatment hazard ratio for low cytoplasmic Pak1 staining group.

§Interaction variable states whether there is a difference in the treatment response in relation to Pak1 status.

‖Pak1 nuclear staining hazard ratio for the control group.

¶Treatment hazard ratio for the Pak1 nuclear staining-negative group.

cytoplasmic Pak1 staining intensity nor the presence of nuclear Pak1 staining showed any association with recurrence-free survival for this group of patients. Similar findings were obtained when we analyzed the entire cohort of tamoxifen-treated and untreated patients (data not shown).

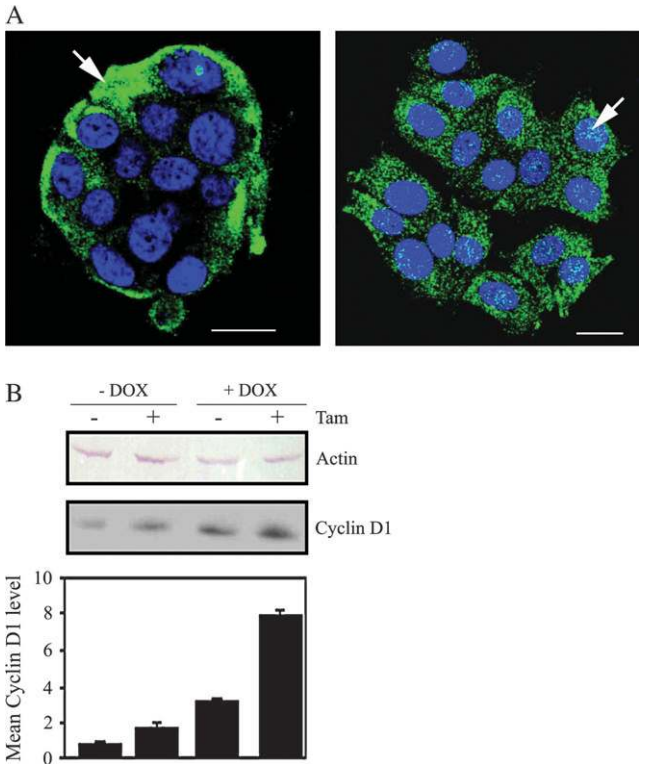

Fig. 4. Effect of tamoxifen on subcellular localization of Pak1 and cyclin D1 expression in MCF-7/DA-Pak1 cells. **A**) MCF-7/DA-Pak1 cells, a human breast cancer cell line that expresses catalytically active Pak1 under the control of an inducible promoter, were cultured in the presence of inducer (doxycycline) with (**right panel**) or without (**left panel**) tamoxifen for 1 hour and subjected to immunofluorescence localization of Pak 1 (cytoplasmic [**left panel**] and nuclear [**right panel**] localization of Pak1 (**green**) is denoted with **arrows**). Topro-3 (**blue**) was used to stain DNA. **Scale bar** = 10 μm. **B**) MCF-7/DA-Pak1 cells grown in the presence (+) or absence (–) of doxycycline (Dox) and tamoxifen (Tam) and subjected to western blot analysis of expression of the ERα target gene, CCND1. **Lower panel** shows quantitation of cyclin D1 levels from three independent experiments using an ImageQuant 5.1 software. **Error bars** correspond to 95% confidence intervals.

Discussion

Results of several recent studies *(10–12,15–17,23)* have indicated that Pak1 might be involved in the progression of breast cancer and in the regulation of estrogen response through its interaction with ERα. Our analysis of primary breast cancer samples from participants in a randomized treatment trial, combined with our studies in experimental model systems, revealed that Pak1 expression, specifically nuclear localization of Pak1, is intimately linked to tamoxifen resistance in breast cancer.

ERα is a nuclear protein that regulates gene transcription in response to the hormone estrogen. Approximately 70% of all breast cancers express ERα, making antiestrogen-based therapies particular useful for this subgroup of tumors. The most widely used antiestrogen is tamoxifen, which is a selective estrogen receptor modulator that functions as an agonist or antagonist in a tissue-specific manner *(26)*. An overview of randomized trials *(27)* reported that tamoxifen as an adjuvant treatment for early breast cancer reduces the risk of recurrence and improves survival in patients with ERα-positive tumors. However, many patients either do not respond to tamoxifen treatment or develop resistance to tamoxifen. Because of the complexity of ERα regulation, different mechanisms that contribute to tamoxifen resistance have been suggested, including growth factor stimulation of ERα by phosphorylation, overexpression of HER-2/neu, overexpression of coactivators, and decreased expression of corepressors *(28,29)*.

Key regulators of central biologic processes such as proliferation, differentiation, or motility are often deregulated in cancer. Pak1 has been shown to be involved in the regulation of invasion and migration of breast cancer cells *(10,11)*, and overexpression of constitutively active Pak1 in mouse mammary gland has been shown to induce hyperplasia in the mammary epithelium through activation of MAP kinase pathways and ERα target genes *(16)*. In the same study, it was shown that Pak1 phosphorylates the activation function-2 domain of ERα at Ser305. More recently, Balasenthil et al. *(15)* observed high expression of Pak1 in 34 of 60 breast tumor specimens and demonstrated that Pak1 signaling regulates the expression of cyclin D1 in mammary epithelial and breast cancer cell lines.

In this study, we observed a paradoxical association between the level of Pak1 expression and ERα positivity. That is, several tumors that were ERα positive had low or no expression of Pak1,

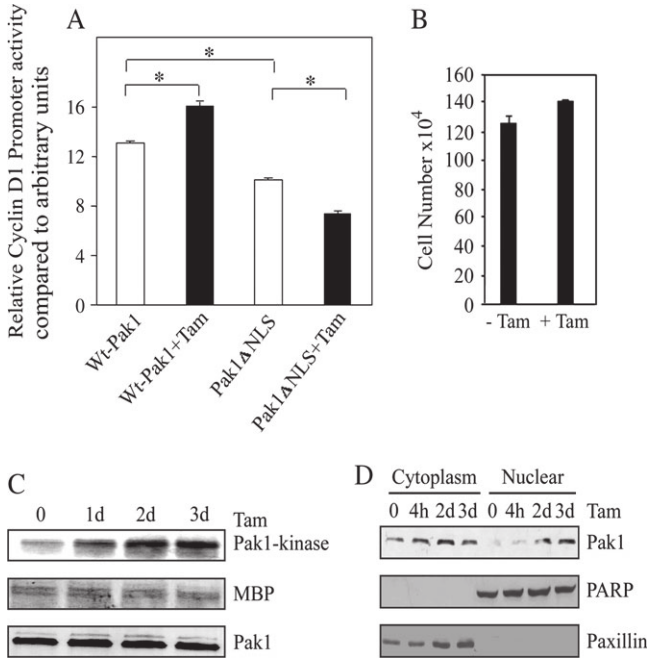

Fig. 5. Nuclear localization of Pak1 and prevention of antiestrogen action of tamoxifen. **A**) Cyclin D1 promoter luciferase assay of MCF-7 cells transiently transfected with vector that expressed wild-type Pak1 (Wt-Pak1) or Pak1 that lacked nuclear localization signals (Pak1ΔNLS) in response to tamoxifen (Tam). **Error bars** correspond to 95% confidence intervals from three independent experiments. *, $P<.001$. **B**) Ishikawa human endometrial cancer cells, which are naturally resistant to tamoxifen, were cultured with 5% charcoal-stripped serum for 48 hours and treated with (+) or without (–) tamoxifen (10^{-8} M) for 5 days. Cell were then counted in a Coulter counter. **Error bars** correspond to 95% confidence intervals from three independent experiments. **C**) Ishikawa cells were treated with tamoxifen for the indicated days and Pak1 kinase assay was performed. **D**) Ishikawa cells were treated with tamoxifen for the indicated days and cytoplasmic and nuclear extracts from tamoxifen (Tam)-treated Ishikawa cells were subjected to western blot analysis of Pak1. Purity of cytoplasmic and nuclear fractions was confirmed by immunoblotting the same blot with antibodies against paxillin, a marker for cytoplasmic fraction, and poly-ADP–ribose polymerase (PARP), a marker for nuclear fraction.

whereas almost all tumors with the highest Pak1expression were ERα positive (Table 2). The reason for this discrepancy is unclear, but it may have been influenced by the fact that several different pathways modulate the expression of Pak1, which in turn may be coupled to ERα positivity. Pak1 expression was also associated with several aggressive features in breast cancer such as tumor type, histologic grade, and proliferation level. We observed low Pak1 expression among the lobular breast cancers, most of which are ERα positive and therefore potentially also responsive to tamoxifen.

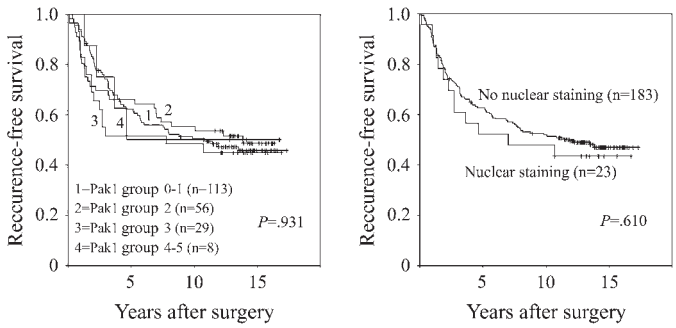

Fig. 6. Recurrence-free survival among untreated breast cancer patients according to Pak1 cytoplasmic staining intensity (group 0–1, 2, 3, and 4–5) (**left panel**) and nuclear staining category (**right panel**).

By analyzing tumors from patients who were randomly assigned to tamoxifen versus no tamoxifen and comparing their recurrence-free survival, we observed that Pak1 expression was associated with tamoxifen resistance; patients who had high cytoplasmic Pak1 expression or nuclear Pak1 expression showed no response to tamoxifen. Further testing of this observation in multivariable analyses revealed that, despite minor differences between the recurrence-free survival regarding Pak1 low or high expression or Pak1 nuclear staining, only nuclear Pak1 showed a statistically significant interaction with tamoxifen treatment (Table 3).

Certain chromosomal regions are often mutually amplified in breast cancer, creating sets of overexpressed gene products. PAK1 and CCND1 colocalize at chromosomal region 11q13, which is amplified in up to 15% of primary breast cancers *(25)*. Our finding that Pak1 expression was statistically significantly associated with cyclin D1 protein expression as well as with CCND1 gene amplification suggests that PAK1 and CCND1 are coamplified. Despite intensive analysis of the 11q13 region, only a few oncogenes connected to breast cancer development have been found at 11q13, and CCND1 is the major candidate *(25)*. In an analysis of the same tumor material used in this study, Jirstrom et al. *(21)* observed that tamoxifen-treated patients with CCND1-amplified tumors had worse recurrence-free survival than untreated patients with CCND1-amplified tumors, even when CCND1 amplification was not accompanied by overexpression. This agonist effect could perhaps be due to another gene product; Pak1 would certainly be a suitable candidate for this product because it interacts with and phosphorylates ERα, which could potentially induce an agonist effect *(30)*. However, we did not observe an agonist effect of tamoxifen in patients whose tumors had high cytoplasmic Pak1 expression or nuclear Pak1 expression. Instead, we merely observed no difference in recurrence-free survival between treated and untreated patients, which might suggest the existence of another ER-regulating gene product at the 11q13 chromosomal locus.

In parallel with the studies of clinical breast cancer samples, we further studied how Pak1 influenced the ERα signaling in breast cancer cell lines. We used MCF-7 cells that expressed catalytically active Pak1 under the control of a tetracycline-inducible promoter. When the cells were grown in the presence of doxycycline, the overexpressed Pak1 was mostly cytoplasmic; however, when the induced cells were treated with tamoxifen, the overexpressed Pak1 accumulated in the nucleus. Also, upon tamoxifen treatment, the expression of cyclin D1 increased mostly in cells that overexpressed Pak1, suggesting that nuclear Pak1 is important for mediating tamoxifen resistance by increasing cyclin D1 expression. Overexpression of wild-type Pak1 in the presence of tamoxifen resulted in increased ERα transactivation, as measured by cyclin D1 promoter activity, compared with overexpression of Pak1 in the absence of tamoxifen. This finding might be interpreted as an agonist response. Conversely, overexpression of Pak1ΔNLS could not activate the cyclin D1 promoter in response to tamoxifen. We then analyzed Ishikawa cells, an endometrial cancer cell line that is naturally resistant to tamoxifen, and found that tamoxifen treatment further increased both nuclear Pak1 expression and nuclear Pak1 kinase activity. Taken together, these data support an intimate link between Pak1 expression and ERα signaling. That is, Pak1 expression appears to influence the response to tamoxifen, as demonstrated in the tumor samples, and

tamoxifen seems to modulate the expression, localization, and activity of Pak1, as shown by our experimental models.

One limitation of this study is the subjective categorization and immunohistochemistry-based analyses of Pak1 expression and localization in the breast cancer samples. However, there was a low interrater variability in the immunohistochemical evaluation and the cell line data are also in accordance with the clinical breast cancer data supporting the validity of the results presented. *P* values in the correlation studies have not been adjusted for multiple testing and should therefore be interpreted with caution. Nevertheless, with a more stringent definition of the statistical significance level based on Bonferroni correction, Pak1 cytoplasmic staining was still associated with tumor grade and proliferation.

To our knowledge, this is the first study delineating the prognostic and predictive value of Pak1 expression and subcellular localization in breast cancer. Overall, our observations suggest that Pak1 activation and nuclear localization contribute to the reduced tamoxifen sensitivity that has been observed in some breast tumor cells. Therapies that target Pak1 expression or activity may therefore represent a strategy to increase the endocrine treatment response in breast cancer. However, this signaling pathway requires further delineation in additional primary breast cancers and experimental models. Larger clinical studies are also required to examine the effect of antiestrogen treatment in patients with tumors of different Pak1 status that are negative for nuclear ERα, because the components of ER pathways have been shown to be functional in the cytoplasmic compartment *(31)*. Nevertheless, our results raise the possibility that premenopausal breast cancer patients whose tumors overexpress Pak1 most likely will not respond to tamoxifen and may need to be offered alternative endocrine treatment.

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Notes

We thank the South Swedish and South-East Swedish Breast Cancer Groups. We are grateful for excellent technical assistance from Elise Nilsson and statistical expertise from Pär-Ola Bendahl, and Seethraman Balasenthil for assistance in microscopy. We also thank J. Chernoff for kindly providing a Wt-Pak1 expression vector.

Supported by grants from the Swedish Cancer Society, Lund University Research Funds, Malmö University Hospital Research and Cancer Funds, Swegene/Wallenberg Consortium North (WCN), and National Institutes of Health grants CA 65746 and CA 90970 (to R. Kumar). None of the funding agencies had a role in the design, conduct, analysis, or reporting of the results.

Manuscript received August 8, 2005; revised March 13, 2006; accepted March 30, 2006.