Estrogen Receptor-alpha Phosphorylation at Serine 305, Nuclear p21-Activated Kinase 1 Expression, and Response to Tamoxifen in Postmenopausal Breast Cancer

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Estrogen receptor-α phosphorylation at serine 305, nuclear Pak1 expression and response to tamoxifen in postmenopausal breast cancer

Running title: pER^{ser305}, Pak1 and tamoxifen in breast cancer

Key words: Pak1; estrogen receptor; serine 305; treatment prediction; immunohistochemistry

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Abstract

Purpose: *In vitro*, p21-activated kinase 1 (Pak1) phosphorylates the serine 305 residue of the estrogen receptor α (ERα) and influences the response of breast cancer cells to tamoxifen. We investigated the influence of Pak1 and pERα^{ser305} on breast cancer prognosis and results of tamoxifen therapy.

Experimental Design: We examined Pak1 and pERα^{ser305} protein by immunohistochemistry in a series of 912 tumors from node-negative breast cancer patients randomized to tamoxifen or no adjuvant endocrine treatment.

Results: Cytoplasmic Pak1 correlated to large tumors and ER negativity, while nuclear Pak1 and pERα^{ser305} correlated to small tumors and ER positivity. Nuclear expression of Pak1 and pERα^{ser305} predicted reduced response to tamoxifen in patients with ERα-positive tumors (tamoxifen versus no tamoxifen, HR=1.33; 95% CI, 0.42-4.2, p=0.63), whereas patients lacking this combination benefitted significantly from tamoxifen (HR=0.43; 95% CI, 0.30-0.62; p<0.0001). Similar non-significant trends were detected in analyses of the proteins separately. Pak1 in the cytoplasm was an independent prognostic marker, indicating increased recurrence rate (HR=1.79; 95% CI, 1.17-2.74, p=0.0068) and breast cancer mortality (HR=1.98; 95% CI, 1.14-3.46, p=0.016) for patients randomized to no adjuvant treatment.

Conclusions: Our results suggest that patients with tumors expressing Pak1 and pERα^{ser305} in combination are a group in which tamoxifen treatment is insufficient. In addition, the pathway may be of interest as drug target in breast cancer. Furthermore, the findings support previous studies showing that Pak1 has differential roles in the cytoplasm and the nucleus.

Translational Relevance

Adjuvant endocrine treatment with tamoxifen or aromatase inhibitor significantly improves the survival of women with estrogen receptor (ER) positive breast cancer, but resistance to treatment is a huge clinical problem. Modifications of the ER by phosphorylation of several serine residues, leading to increased transcriptional activation, are part of the crosstalk between ER and growth factor receptors that when perturbed may cause resistance. We investigated the expression of pERα^{ser305} and p21-activated kinase 1 (Pak1), which may phosphorylate ER at serine 305, in a large tumor material originating from a randomized tamoxifen trial with postmenopausal patients. Patients with tumors positive for pERα^{ser305} and nuclear Pak1 showed decreased benefit from tamoxifen suggesting that these proteins might become biomarkers for tamoxifen resistance. In addition, cytoplasmic Pak1 was found to be an independent prognostic factor among systemically untreated patients. Those dual roles of Pak1 may make it an interesting target in cancer therapy.

Introduction

Breast cancer is the most common type of cancer worldwide in the female population (1). Mammography screening and improved surgery, radiotherapy and adjuvant systemic treatments correlate with decreased mortality during the last decades.

Tamoxifen is an estrogen competitor, commonly used as adjuvant therapy in estrogen receptor-positive breast cancer. Nonetheless, many tumors relapse due to *de novo* resistance, or over time, *acquired* resistance (2). Tamoxifen response seems to be affected by estrogen receptor α (ERα) modifications, including phosphorylation of the ERα and its coregulators as well as other alterations affecting coregulator dynamics (3). Phosphorylation of ERα serine, threonine and tyrosine residues is a posttranslational event changing the secondary structure of the highly flexible receptor and is caused by growth-factor regulated kinases. This influences the receptor in several aspects, such as subcellular localization, dimerization, DNA binding, coregulator interaction, and transcriptional activity (4). There are several known phosphorylation sites in ERα, where serines 118, 167 and 305, controlled by MAPK-, Akt-, PKA- and Pak1-signaling pathways, seem to be the most interesting in relation to endocrine therapy (5, 6). These sites have been shown to be involved in estrogen-independent transcriptional activation of some ER-target genes, such as CCND1 (7, 8).

Tharakan and colleges recently showed that an ERα^{S305E} mutant, mimicking a constitutively phosphorylated state, increased receptor dimerization in the presence of estrogen compared to wild-type ERα. The mutant receptor also increased ERα-target gene binding compared to wild-type in the absence of ligand (9). p21-activated kinase (Pak1) activates the ERα by phosphorylation of the serine residue at position 305, leading to ERα-transcriptional activation of ER-target genes (7). Additionally, Pak1 phosphorylates the ERα-coactivator

NRIF3, leading to a potentiation of the ERα- transactivation (10). Various combinations of phospho-modifications of the serines 118, 236 and 305 led to different ERα-conformational changes upon anti-estrogen treatment, seen by FRET structural analysis (11). Within the cytoplasm of breast epithelial cells active Pak1 interferes with numerous cellular events, of which several influence tumor progression (12, 13).

This study was based on pERα^{ser305} and Pak1 immunohistochemical analysis of a large series of tumors from node negative postmenopausal breast cancer patients, who participated in a randomized tamoxifen trial. We report that phosphorylated ERα^{ser305}, along with detection of Pak1 protein in the nucleus, correlate with reduced response to tamoxifen in ERα-positive breast cancer. Also, Pak1 localization influences prognosis and tamoxifen treatment prediction in this set of patients.

Material and methods

Patients and tumors

During the years of 1976 through 1990, a cohort of Swedish postmenopausal breast cancer patients were included in a controlled trial to evaluate tamoxifen as adjuvant treatment (14). Patients with low-risk tumors, defined as node negative and $\leq$30 mm in diameter, were included in the present study. Patients were treated either with modified radical mastectomy or with breast conserving therapy and radiation therapy to the breast with a total dose of 50 Gy with 2 Gy per fraction, 5 days a week, for about 5 weeks. All patients were randomized to either 2 y of tamoxifen (40 mg daily) or no adjuvant endocrine therapy. In 1983 a new trial was initiated, recurrence-free patients were after 2 y again randomized to either tamoxifen for 3 more y or no further therapy. The standard procedure for tissue collection was fixation at room temperature in 4% phosphate buffered formalin. Estrogen receptor status was determined by isoelectric focusing and the cut-off level was set to 0.05 fmol/µg DNA (14). Follow-up data was collected through regional population registers and the Swedish Cause of Death Registry. The median follow-up period for recurrence-free patients in the present study was 17.8 y. The present study was approved by the Stockholm regional Ethics board.

Tissue micro array

Archived tumor tissues were collected from 912 of the 1780 patients included in the original study. Representative tumor tissues were selected as donor blocks for the tissue micro array (TMA). Sections were cut from each donor block and stained with hematoxylin/eosin. From these slides three morphologically representative regions were chosen in each sample. Three cylindrical core tissue specimens with a diameter of 0.8 mm were taken from these areas in each case and mounted in a recipient block. Sixteen tissue array blocks were constructed using a manual arrayer (Beecher Inc, WI, USA). TMA blocks were cut with a microtome into

4 μm sections and mounted on frost coated glass slides. The subset in the present study did not significantly differ from the original study including 1780 patients, with respect to a tumor size of 20 mm or less (79% versus 81%), positive ER-status (78% versus 80%) or tamoxifen treatment (52% versus 50%) (Supplementary table).

Hormone receptor status

Retrospectively, additional ER and PR status of the tumors was evaluated with immunohistochemistry using the Ventana® automated slide stainer (Ventana Medical Systems, S.A., Cedex, France). The antibodies used were the monoclonal Ventana® Medical Systems' CONFIRM mouse anti-Estrogen Receptor primary antibody (clone 6F11) and the monoclonal Ventana® Medical Systems' CONFIRM mouse anti-Progesterone Receptor primary antibody (clone 16). Cut-off level was set to 10% positively stained tumor cell nuclei. In this study, these data were used for ER and PR status. However, when immunohistochemical data on ER status was missing, results from the cytosol assay were used. In this way ER status could be defined for 886 of the 912 tumors.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor TMA slides were deparaffinized with xylen and rehydrated in a graded ethanol series. The slides later stained with Pak1 antibody were boiled in target retrieval solution (pH 9.9) (Dako, Glostrup, Denmark) in microwave oven for 4 x 5 min, at 750 W up to boiling point, and thereafter switching between 160 W and 350 W to control boiling. The slides later stained with $pER\alpha^{ser305}$ antibody were boiled in citrate buffer (pH 6.0) in pressure-cooker at 125^{o}C for 30 sec and withdrawn when the temperature reached 95^{o}C to expose antigenic epitopes. The sections were cooled in room temperature for 20 min, placed in 3% H_2O_2 in methanol for 5 min to inactivate endogenous peroxidase, incubated with

serum free protein block (Dako, Carpinteria, CA, USA) for 10 min and incubated with rabbit polyclonal Pak1 primary antibody (Cell Signaling Technology, MA, USA) diluted 1:25 at 4°C in a moisturized chamber for 20 h or rabbit polyclonal pERα^{ser305} primary antibody (Bethyl Laboratories, TX, USA) diluted 1:300 at 4°C in a moisturized chamber for 17 h. All slides were washed, incubated with the anti-rabbit Dakocytomation Envision+ system labeled with horse radish peroxidase antibody (Dako, USA) for 30 min at 4°C, visualized with 3.3-diaminobenzidin hydrochloride in phosphate buffer and 0.03% H_2O_2 and counterstained with hematoxylin. All washing steps were performed in phosphate buffer solution with 0.5% bovine serum albumin. Staining intensity was evaluated on three separate biopsies for each tumor. Pak1 nuclear reactivity was graded as positive when >10% of the tumor cells showed staining. Pak1 cytoplasmic protein expression was graded as negative, weak, moderate and strong, where moderate and strong staining was considered as abnormal expression of the protein in subsequent statistical analysis. Fourteen percent of the patients (n=126) were excluded in the Pak1-assay due to non-representative or missing tissues. pERα^{ser305} nuclear staining was graded with respect to percent positively stained nuclei in four steps; negative, 1-24%, 25-74% and ≥75%. As the proportion of positive staining was moderate, cut-off point for positive staining was set to the ≥1% level in the statistical analysis. pERα^{ser305} was frequently visible in the cytoplasm, but only nuclear staining was graded. Eight percent of the patients (n=71) in the evaluation of pERα^{ser305} were excluded due to non-representative or missing tissues. Only minor differences were detected between subsets evaluated for protein staining and excluded samples compared to the original study cohort (Supplementary table). For each antibody, all tumors were stained in one batch and the whole range of staining intensities was represented on each slide, due to the large number of tumors. In addition, normal liver samples on all slides served as control. All tumor samples were evaluated by two independent observers, blinded to pathological and clinical data, and scored under a Leica

DM LS (Leica Microsystems) light microscope. Where discordance was found, cases were re-evaluated to reach consensus. Pictures were generated using an Olympus SC20 camera with a Leica 40 X objective.

Antibody validation

Validation of the pERα^{ser305} antibody included blocking with immunizing peptide according to manufacturers instruction (Bethyl Laboratories, TX, USA) and dephosphorylation of proteins in acetone-fixed T47D breast cancer cells by λ-phosphatase (λ-ppase) (New England Biolabs, MA, USA). In the λ-ppase assay slides were treated with 1000 U λ-ppase or water (control) for 2 h at 37^{o}C followed by immunohistochemical staining according to protocol used for the pERα^{ser305} primary antibody. One concern in the detection of phospho-proteins regards tissue collection routines. Validation of several ER phospho-antibodies did not show a significant decreased detection of phospho-ERs in relation to increased time of collection (15). The specificity of the Pak1 antibody was previously confirmed by Western blot and immunohistochemistry (16).

Statistical analysis

To compare protein expression data with prognostic and clinical characteristics, the Pearson χ^2 test was applied. Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated using Cox proportional hazards model. Recurrence-free survival time was calculated as the time between diagnosis and any of the events: locoregional recurrence, distant metastasis or breast cancer death. Recurrence-free survival time distributions were compared with the log-rank test and plots were drawn using the Kaplan-Meier technique. Multivariate analysis of recurrence rates and breast cancer mortality rates were performed with Cox proportional hazard regression, a method also used for the interaction analysis of

different factors and treatment by including the variables: potential predictive factor (PF), tamoxifen treatment, interaction variable (tamoxifen x PF). Tumor size was also included in the model. Analysis of prognosis was restricted to patients randomized to no tamoxifen treatment and treatment prediction of tamoxifen was restricted to patients with ERα-positive tumors. P-values ≤ 0.05 were considered significant, with the exception of the associations presented in Table 1, where p-values ≤ 0.01 were considered significant to compensate for the effect of multiple comparisons. All statistical data was analyzed using the Statistica 8.0 software program.

Results

Expression analysis of Pak1 and phosphorylated ERα^{ser305} proteins with immunohistochemistry was successful with 786 and 841 tumors, respectively. Pak1 cytoplasmic overexpression was observed in 57.6%, Pak1 nuclear expression was observed in 21.8% and pERα^{ser305} nuclear expression was detected in 36.3% of the tumors (Fig 1 A-F). As Pak1 previously has been shown to increase the transcriptional activity of ERα through phosphorylation of its amino acid residue serine 305, we investigated whether the expression of the two proteins in the nuclear compartment was correlated, but they were not when all tumors were considered (Table 1). However, restricting the analysis to Pak1 positive tumors, independent of localization, rendered a significant correlation between Pak1 nuclear expression and pERα^{ser305} nuclear expression (p=0.042). Pak1 cytoplasmic staining correlated significantly to a tumor size larger than 20 mm and a negative ER status, while nuclear staining of Pak1 as well as pERα^{ser305} correlated to small size and ER positivity. The expression of pERα^{ser305} correlated with ER status. Twenty-eight percent (51/182) of the tumors defined as ER negative showed some staining for the pERα^{ser305}. However, the cut-off level for ER status was set to 10% positive nuclei, whereas cut-off for pERα^{ser305} was set to $\geq$1% positive nuclei. The correlation was stronger when the cut-off level for pERα^{ser305}-positivity was raised, and also when analyzing the pERα^{ser305} staining in four categories with increasing percentage of positive cells (p=0.0026). Tumors defined as ER positive were also positive for the pERα^{ser305} in 39% (246/636).

Antibody specificity

The pERα^{ser305} antibody specificity was determined using the immunizing peptide used for antibody production, including 12-15 amino acids surrounding the phospho-site and a phosphorylated serine at the position corresponding to serine 305 of ERα. Incubation of the

antibody with the peptide prior to immunoassay resulted in loss of staining (Fig 1 G-H). For further evaluation of the phospho-specificity of the antibody, T47D breast cancer cells on glass slides were treated with λ-protein phosphatase (λ-ppase), resulting in staining of control cells and no visible staining of λ-ppase treated cells (Fig 1 I-J). Previously, the pERα^{ser305} antibody used in this study was validated through lack of detection of the mutated pERα^{S305A} compared to the wild type pERα on Western blot (17). Taken together, data indicate that the pERα^{ser305} antibody specifically recognizes ERα phosphorylated at serine 305.

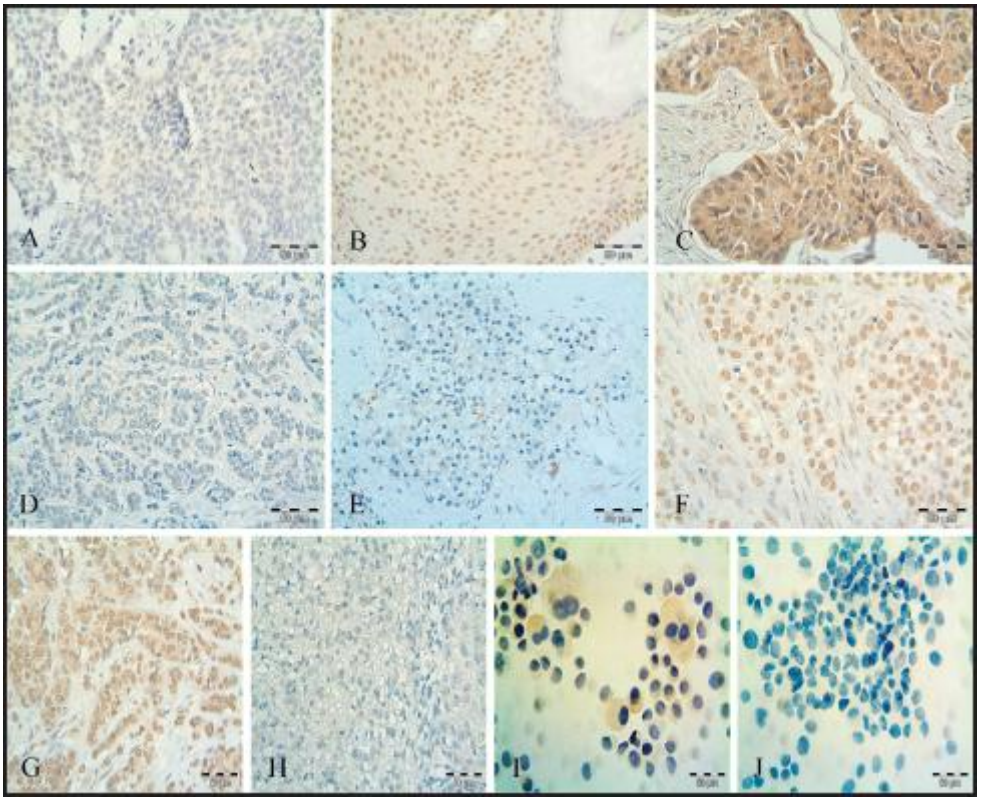

Figure 1 Immunohistochemical detection of Pak1 and pERα^{ser305} in human breast cancer sections and validation of ERα phospho-antibody with blocking peptide in breast-tumor tissue and with λ-phosphatase-treated T47D breast cancer cells. Magnification, x 40. Pak1 negative cytoplasmic staining and negative nuclei (**A**), Pak1 positive cytoplasm and positive nuclei (**B**), Pak1 positive cytoplasm and negative nuclei (**C**)**,** pERα^{ser305} negative nuclear staining (**D**), pERα^{ser305} positive staining in ≥1-25% of nuclei (**E**), pERα^{ser305} positive staining in ≥75% of nuclei (**F**), pERα^{ser305} without blocking peptide (**G**), pERα^{ser305} with blocking peptide (**H**), pERα^{ser305} without λ-phosphatase (**I**), pERα^{ser305} with λ-phosphatase (**J**).

Tamoxifen treatment prediction

Recurrence-free survival in tamoxifen treated versus non-tamoxifen treated groups in relation to protein expression levels was analyzed to estimate the benefit from adjuvant tamoxifen treatment in subsets of ERα-positive breast cancer patients. Our data show that patients with no nuclear expression of Pak1 in the tumor were highly sensitive to tamoxifen treatment (p=0.00003) (Fig 2A), whereas expression of Pak1 in the nucleus was associated with less marked benefit from the treatment (p=0.15) (Fig 2B). Pak1 protein expression in the cytoplasm did not influence the efficacy of tamoxifen in this series of patients. Similarly, lack of pERα^{ser305} staining resulted in good response to treatment (p=0.0003) (Fig 2C), whereas for positive staining the benefit was reduced (p=0.13) (Fig 2D). However, when tested for interaction neither Pak1 nor pERα^{ser305} nuclear staining were significantly correlated with reduced effect of tamoxifen (Table 2). Pak1 is phosphorylating serine 305 on the ERα and this may result in tamoxifen resistance in breast tumors. Analyzing Pak1 and pERα^{ser305} nuclear expression together could give a further hint on how tamoxifen response is affected by this pathway. The results showed tamoxifen benefit in patients with normal or no expression of any of the two proteins (p<0.00001) (Fig 2E). Patients with tumors expressing the two proteins simultaneously within the nucleus did not appear to respond to treatment (p=0.63) (Fig 2F), and we found a significant interaction with tamoxifen benefit (Table 2).

In this study we detected nuclear staining of pERα^{ser305} in a group of patients previously defined as ERα negative by standard methods. Adding these pERα^{ser305} positive tumors to the ERα-positive group when analyzing tamoxifen response reinforced the results for both Pak1 nuclear expression (test for interaction: p=0.15), pERα^{ser305} nuclear expression (p=0.098) as well as for positive staining of the two proteins in combination (p=0.013), showing reduced benefit from tamoxifen treatment when these proteins were expressed.

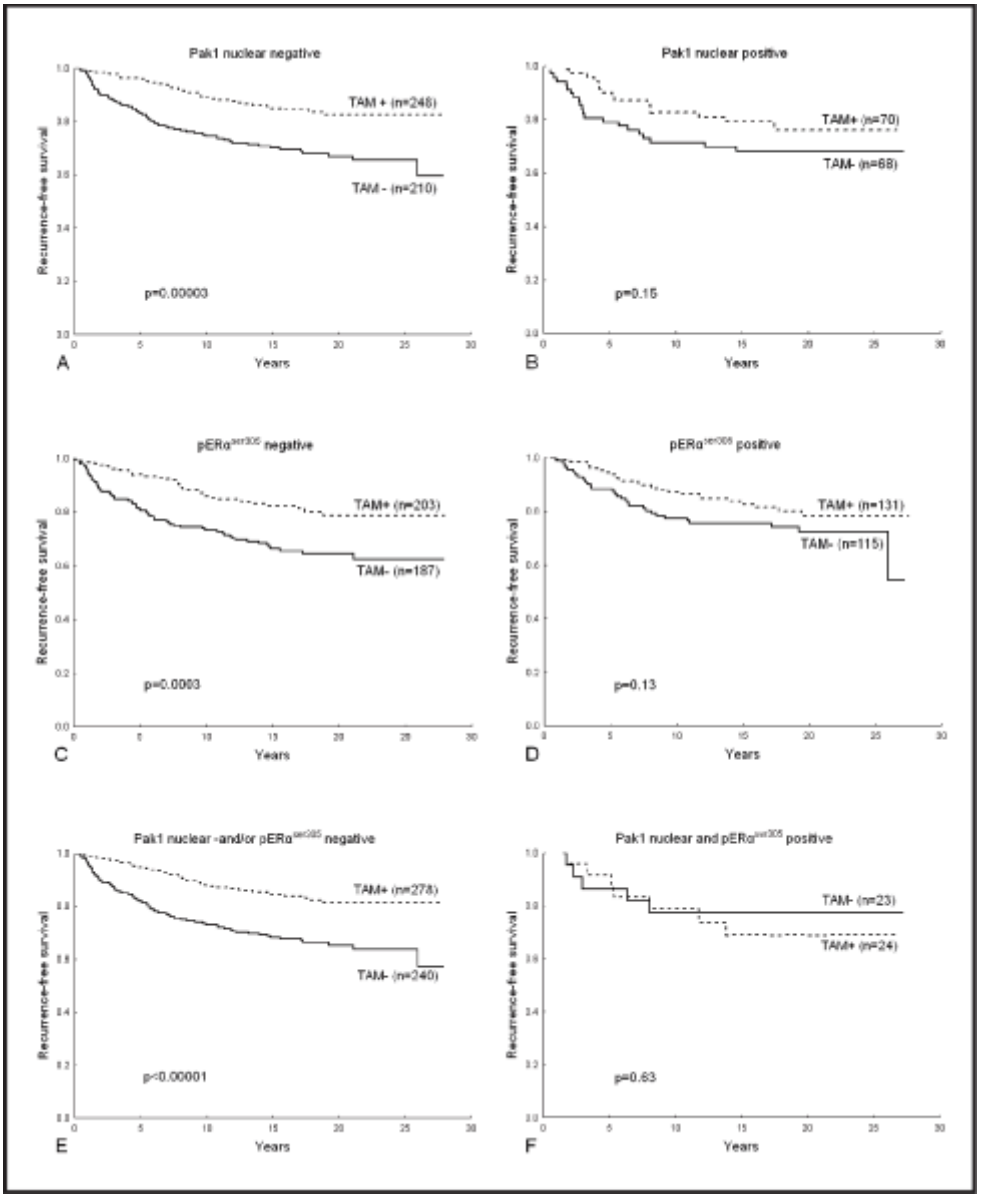

Figure 2 Tamoxifen response among ER-positive patients in relation to nuclear protein expression of Pak1 negative (A), Pak1 positive (B), pERα^{ser305} negative (C), pERα^{ser305} positive (D), Pak1 and/or pERα^{ser305} negative (E), and Pak1 and pERα^{ser305} positive (F).

Prognosis

We used patients randomized to no adjuvant tamoxifen treatment to evaluate prognosis. Cytoplasmic expression of the Pak1 protein correlated with decreased recurrence-free survival (overexpressed versus normal; HR=1.80; 95% CI, 1.21-2.67, p=0.0027) (Fig 3A). This was true also when restricting the analysis to ER positive patients (HR=2.00; 95% CI, 1.26-3.17, p=0.0023), but not in the ER negative group (p=0.53). Cytoplasmic Pak1 also correlated with

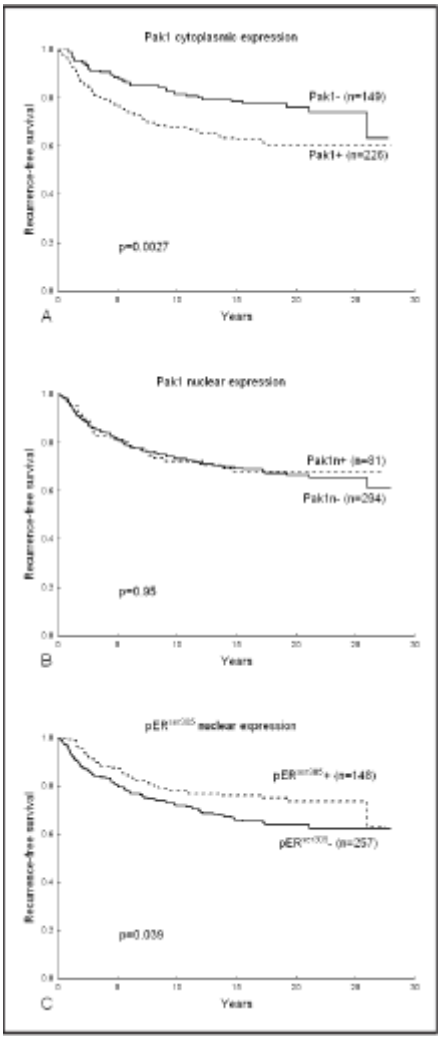

Figure 3 Recurrence-free survival for all patients given no adjuvant systemic treatment in relation to protein expression detected by immunohistochemistry. Pak1 cytoplasmic expression (A), Pak1 nuclear expression (B) and pERα^{ser305} nuclear expression (C).

decreased breast-cancer specific survival (HR=1.95; 95% CI, 1.17-3.26, p=0.0086). In multivariate analysis cytoplasmic Pak1 protein was significantly related to increased recurrence rate and increased breast cancer mortality (Table 3). Pak1 nuclear expression was not prognostic (HR=0.99; 95% CI, 0.63-1.53, p=0.95) (Fig 3B). pER^{ser305}-positive expression in the nucleus indicated better prognosis than no expression (HR=0.67; 95% CI, 0.46-0.99, p=0.039) (Fig 3C), but this relationship did not remain significant in multivariate analysis (Table 3).

Discussion

Pak1 may contribute to tamoxifen resistance in breast cancer patients by phosphorylation of the serine 305 within the activating function-2 region of the ERα-ligand binding domain. Here, we report nuclear and cytoplasmic overexpression of Pak1 in 22% and 58%, respectively. Nuclear expression of the phosphorylated ERα^{ser305} was detected in 36%.

Recently, we found that amplification of the gene encoding Pak1 predicts poor response to tamoxifen (18). Other studies showed that Pak1 is a kinase that interacts with ERα and phosphorylates the serine 305 residue, and this modulation triggers activation of the receptor (7, 8). Therefore we investigated the possible predictive value of this signaling pathway in this series of breast tumors stained for Pak1 and pERα^{ser305}. Interestingly, in Pak1 positive tumors nuclear localization of Pak1 was correlated to pERα^{ser305}, but we did not find a significant correlation between Pak1 and pERα^{ser305} expression overall. An explanation to this result could be another protein, PKA, and possibly other kinases also phosphorylating the ERα at the same position (6). Hypothetically, PKA may be the dominant kinase and comparatively high levels of nuclear active Pak1 could be needed to compete with the action of PKA. A cooperative role of the two proteins may also be considered. Hence, our result does not exclude the importance of Pak1 phosphorylation of the ERα in subgroups of breast tumors. Worth noticing is that the Pak1 antibody used in this study was not selective for kinase-active Pak1. However, among relapsing tamoxifen treated patients the nuclear coexpression of Pak1/pERα^{ser305} correlated significantly ($p=0.023$, data not shown). This may indicate that Pak1 phosphorylated ERα in these patients and counteracted tamoxifen. Pak1 in the nucleus and pERα^{ser305} correlated to similar clinicopathologic factors, seen in Table 1, whereas Pak1 in the cytoplasm correlated inversely to these factors, indicating nuclear interaction of ERα and Pak1.

In line with our previous findings and other experimental studies, we found that Pak1 located to the nucleus indicated reduced response to tamoxifen treatment (16, 18, 19). A similar result was observed for pERα^{ser305} staining in the nucleus, which has been suggested in a recent study of premenopausal breast cancer (20). Expression of these two proteins together suggested a further reduction of tamoxifen response, indicating importance of this signaling pathway for treatment prediction. However, the group with simultaneous expression, includes less than 10% of the ER-positive patients and the data must be interpreted with caution.

Activation of the ERα is a highly regulated, not yet fully elucidated process, involving phosphorylation of several amino acid residues and coregulator recruitment in a dynamic and possibly cyclic system (21). An imbalance in this process may cause changes in the gene-expression pattern, regulated by the receptor, and influence receptor dependent malignant cells to develop drug resistance mechanisms. Other ERα phospho-sites have previously been studied in breast cancer. In contrast to results seen for the phosphorylation of the serine 305 site, detection of pERα^{ser118} in the ligand-independent domain of the ERα was shown to associate with a differentiated phenotype, improved survival, and maintenance of tamoxifen sensitivity (22-24). ERK1/2 in the MAPK signaling pathway phosphorylates ERα^{ser118} leading to dissociation of the receptor with its coactivator AIB1 in the presence of tamoxifen (25). Phosphorylation of this site has been found to recruit both coactivators and corepressors, possibly in a dynamic time-dependent fashion (26, 27). Interestingly, phosphorylation of serine 118 is suggested to enhance the estrogen driven activity of the ER, driving the oncogenesis forward (28). Ligand-dependent transcription of the ERα-target genes; *pS2*, *CCND1*, *c-myc* and *PR* has been detected in a separate study, reinforcing the hypothesis that detection of pERα^{ser118} in breast tumor is a marker for tamoxifen sensitivity (29). Expression

of pERα^{ser167} increased survival and tamoxifen response after relapse, in a small study based on tamoxifen treated breast cancer patients (30). In contrast, other results have indicated that activation of Akt, which may be responsible for the phosphorylation of ERα^{ser167} in breast cancer, may be linked to reduced tamoxifen sensitivity (17, 31). From these studies, no consensus regarding the role of the different phospho-sites in the ERα can be established.

Apart from modulating the ERα in the nucleus, Pak1 plays multiple roles in the cytoplasmic compartment. Pak1 is a serine/threonine kinase involved in several signaling pathways in the normal breast epithelial cell, as well as in oncogenic regulation in the neoplastic cell. Cytoskeletal organization, anchorage-independent growth, cell invasion, migration and anti-apoptosis are examples of cellular functions where Pak1 has been shown to be important (32-35). Recent studies have examined the role of Pak1 in several different malignancies, such as glioblastoma, hepatocellular carcinoma, renal cell carcinoma and uveal carcinoma (36-39). They all support involvement of cytoplasmic Pak1 in invasion, migration and metastasis. Activation of the Rho family GTPases Cdc42 and Rac1, regulators of Pak1 kinase activity, was suggested to induce antiestrogen resistance in breast cancer cell lines through a Pak1 dependent pathway (40). However, the Pak1 target ERα is thought to localize to the cytoplasm under certain conditions in the breast cancer cell, binding to membrane-associated proteins as for example growth-factor receptors (41, 42). Whether Pak1 influences ERα in this setting is yet to be elucidated.

We observed that cytoplasmic Pak1 overexpression indicated worse outcome in the tamoxifen untreated group. Functional cell experiments have given Pak1 several roles in tumorigenesis, strengthening its prognostic role (32-35). These results are not fully in line with previous smaller studies where prognosis did not seem to be related to cytoplasmic Pak1 (16, 18). Shou

et al suggest that tamoxifen may also be an agonist on the ERα, when located to the cell membrane, where ERα signaling is thought to cross-talk with growth-factor signaling pathways (41). We did not observe membrane-associated staining by the pERα^{ser305} antibody. This may be explained by that all tumors analyzed were primary tumors, where acquired resistance not yet has occurred. This study is limited to postmenopausal patients with an early breast cancer. To confirm the observations made in the present study, analyses of activated Pak1 in both primary tumors and metastases might be of importance. Also the role of pERα^{ser305} in combination with other ER phospho-sites in treatment prediction needs further inquiry.

In conclusion, nuclear Pak1 and pERα^{ser305} may be involved in resistance to tamoxifen treatment in postmenopausal breast cancer and could be of interest as drug targets for patients receiving tamoxifen treatment.

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Table 1 │ Correlations between Pak1 cytoplasmic expression, Pak1 nuclear expression, pER^{ser305} and clinicopathological parameters in postmenopausal breast carcinomas

	Pak1 nuclear expression *n* (%)			*Pak1 cytoplasmic expression* *n* (%)			*pER^{ser305} nuclear expression* *n* (%)		
	-	+	P-value	-	+	P-value	-	+	P-value
Total	615 (78)	171 (22)		333 (42)	453 (58)		536 (64)	305 (36)	
Tamoxifen									
-	294 (78)	81 (22)	0.92	149 (40)	226 (60)	0.15	257 (63)	148 (37)	0.87
+	321 (78)	90 (22)		184 (45)	227 (55)		279 (64)	157 (36)	
Tumor size									
≤ 20mm	451 (75)	149 (25)	<0.001	277 (46)	323 (54)	<0.001	389 (61)	253 (39)	<0.001
> 20mm	149 (90)	17 (10)		46 (28)	120 (72)		134 (75)	45 (25)	
ER status									
-	144 (86)	24 (14)	0.01	57 (34)	111 (66)	0.009	131 (72)	51 (28)	0.008
+	458 (77)	138 (23)		270 (45)	326 (55)		390 (61)	246 (39)	
PR status									
-	279 (83)	56 (17)	0.02	139 (41)	196 (59)	0.59	243 (68)	113 (32)	0.07
+	281 (76)	87 (24)		160 (43)	208 (57)		240 (62)	148 (38)	
pER^{ser305}									
-	384 (80)	99 (20)	0.46	178 (37)	305 (63)	0.001			
+	203 (77)	60 (23)		129 (49)	134 (51)				

Table 2 │ Cox proportional hazard models for Pak1 nuclear expression, pER^{ser305} nuclear expression, Pak1 and pER^{ser305} in combination and the benefit from tamoxifen in patients with ER-positive tumors

	Recurrence *Tamoxifen versus no tamoxifen*		
	HR (95% CI)	*P-value*	*P-value in test for interaction* [a]
Pak1 nuclear expression			
-	0.43 (0.29-0.64)	<0.001	0.32
+	0.62 (0.32-1.20)	0.15	
pER^{ser305} nuclear expression			
-	0.48 (0.32-0.72)	<0.001	0.24
+	0.66 (0.39-1.13)	0.13	
Pak1 and pER^{ser305} nuclear expression			
-	0.43 (0.30-0.62)	<0.001	0.029
+	1.33 (0.42-4.19)	0.63	

Abbreviations: CI, confidence interval; ER, estrogen receptor; HR, hazard ratio.

[a] For details see material and methods.

Table 3 | Multivariate analysis of patients randomized to no adjuvant tamoxifen treatment using Cox proportional hazard regression

	Recurrence		Breast cancer death	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Tumor size				
>20mm versus ≤20mm	1.88 (1.24-2.85)	0.0029	2.57 (1.57-4.22)	0.0002
ERα-status				
Positive versus negative	0.64 (0.38-1.11)	0.11	0.68 (0.36-1.30)	0.25
PR-status				
Positive versus negative	1.69 (1.05-2.71)	0.031	1.18 (0.65-2.12)	0.58
Pak1 cytoplasmic expression				
Overexpressed versus normal	1.79 (1.17-2.74)	0.0068	1.98 (1.14-3.46)	0.016
Pak1 nuclear expression				
Positive versus negative	1.05 (0.64-1.73)	0.84	0.76 (0.39-1.51)	0.44
pER^{ser305} nuclear expression				
Positive versus negative	0.81 (0.53-1.24)	0.32	0.95 (0.56-1.61)	0.84

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