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PKA-induced phosphorylation of ERα at serine 305 and high PAK1 levels are associated with sensitivity to tamoxifen in ER positive breast cancer.

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Running title Sensitivity to tamoxifen is influenced by PKA/ERαS305P and PAK1

Disclaimers None

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ABSTRACT

Purpose

Phosphorylation of estrogen receptor α at serine 305 (ERαS305-P) by protein kinase A (PKA) or p21-activated kinase 1 (PAK1) has experimentally been associated with tamoxifen sensitivity. Here, we investigated the clinical application of this knowledge to predict tamoxifen resistance in ER-positive breast cancer patients.

Methods

Using immunohistochemistry, a score including PAK1 and co-expression of PKA and ERαS305-P (PKA/ ERαS305-P) was developed on a training set consisting of 103 patients treated with tamoxifen for metastatic disease, and validated on 231 patients randomized between adjuvant tamoxifen or no treatment.

Results

In the training set, PAK1 levels were associated with tumor progression after tamoxifen (HR 1.57, 95% CI 0.99-2.48), as was co-expression of PKA and ERαS305-P (HR 2.00, 95% CI 1.14-3.52). In the validation set, a significant tamoxifen benefit was found among the 73% patients negative for PAK1 and PKA/ERαS305-P (HR 0.54, 95% CI 0.34-0.87), while others (27%) were likely to have no benefit from tamoxifen (HR 0.88, 95% 0.42-1.82). The test for interaction showed a significant difference in recurrence-free survival between groups defined by PAK1 and PKA/ERαS305-P (p=0.037). Elevated PAK1 and PKA/ ERαS305-P appeared to influence tamoxifen sensitivity.

Conclusion

Both PAK1 and PKA/ERαS305-P levels were associated with sensitivity to tamoxifen in breast tumors and the combination of these variables should be considered in predicting tamoxifen benefit.

Keywords PKA, PAK1, phosphorylation of ER, tamoxifen sensitivity, breast cancer

INTRODUCTION

Resistance to anti-estrogens is one of the major challenges in breast cancer treatment. For more than 25 years, the golden standard for endocrine treatment of breast cancer has been tamoxifen. However, approximately half

of the patients with estrogen receptor alpha (ERα)-positive tumors do not respond to tamoxifen [1]. More recently, novel classes of endocrine agents have been introduced, including aromatase inhibitors and fulvestrant [2]. These drugs have a different mechanism of action as compared to tamoxifen [3, 4], and consequently, a different spectrum of patients responds to these novel agents [5-7]. To select an optimal adjuvant treatment [8], the identification of additional biomarkers is essential to select patients who will have no benefit from tamoxifen and should thereby be treated with alternative anti-estrogens.

Various mechanisms can contribute to tamoxifen resistance [9], including kinase activity that results in phosphorylation of ERα [10]. Phosphorylation of ERα at serine 305 (ERαS305-P) by protein kinase A (PKA) leads to an activation of ERα and to transcription of ERα-responsive genes in response to tamoxifen treatment [11, 12], thus inducing resistance. In addition, modification of ERα has been associated with p21-activated kinase 1 (PAK1) [13-15], and nuclear expression and amplification of the PAK1 gene correlates with tamoxifen resistance as well [16, 17].

Recently, we have presented the first clinical study showing that ERαS305-P may be a biomarker that, as suggested by the experimental studies, can identify patients unlikely to respond to tamoxifen [18]. In order to gain insights into the association between both PAK1 and active PKA with ERαS305-P and to determine their clinical relevance, we studied these three putative markers in a clinical setting (n=334). We developed and validated a score integrating PKA activity, PAK1 and, ERαS305-P that is associated with benefit from

tamoxifen benefit, enabling in more than 50% of the patients that will develop tamoxifen resistance.

MATERIALS AND METHODS

NKI-AVL, Lund and Linköping Universities ethical boards approved the studies. The data are presented according to the REMARK recommendations [19].

Patients training series

As previously described [18, 20], a consecutive series of breast cancer patients who had been treated at the Netherlands Cancer Institute-Antoni van Leeuwenhoekhuis hospital were selected according to the following criteria: 1) invasive ERα-positive breast carcinoma, 2) no adjuvant systemic treatment, 3) development of relapse before 2002, for which first-line tamoxifen mono-therapy had been given. Tamoxifen was administered according to national guidelines of that time. Twenty nine patients were excluded due to insufficient tumor material. Analyses presented here are based on 103 patients. The clinico-pathological properties of the 103 patients were similar to those of the whole group of patients (data not shown).

Patients validation series

As described before, 564 premenopausal breast cancer patients were randomized to either 2 years of tamoxifen (n=276) or no systemic treatment (control) (n=288) [17, 18, 21, 22]. Here, we analyzed ERα-positive tumors of 231 patients of this series for whom PAK1, pPKA and, ERαS305-P immunohistochemistry were available.

Clinical endpoints

In the training series, time to tumor progression (TTP) was considered the primary endpoint measured from the start of tamoxifen treatment until treatment was ended because of progression of disease. In the validation series, recurrence-free survival (RFS) was the primary endpoint measured from surgery to either local, regional, or distant recurrence or breast cancer specific death.

Immunohistochemistry (IHC)

Tissue microarrays (TMAs) were constructed using formalin-fixed paraffin-embedded (FFPE) tumor blocks [23]. From both patient series, primary breast tumors were examined by immunohistochemistry.

Details regarding IHC are presented in the **Supplementary Data (Table A1)**. ERα was considered positive when > 10% of invasive cells showed nuclear reactivity [22]. IHC and scoring with the monoclonal ERαS305-P antibody (Millipore # 124-9-4) has been described previously [18]. PAK1 staining was performed and scored as described before and nuclear expression was assessed [17]. For antigen retrieval of phosphorylated PKA-catalytic subunit (threonine 197) (pPKA) (Cell Signaling, #4781), citrate buffer (10 mM, pH 6.0) was pre-heated; slides were added for 15 minutes (microwave 300W), incubated overnight with the antibody (dilution 1:40) and detected using diaminobenzidine. pPKA cytoplasmatic intensity was evaluated. As this is the first report describing this antibody for IHC, we used a simple cut-off comparing no pPKA with any pPKA expression. Scoring of the TMAs was performed without any information on disease outcome of the patients. To

control phospho-specificity, a sample expressing pPKA was dephosphorylated with 1000 U lambda phosphatase (2 hours, 37° C)(Millipore). TMA images are available (http://telepathology.nki.nl login: ER305PAK1PKA, password:tamoxifen).

Cell culture, transfection, western blotting

MCF-7 cells were cultured in DMEM medium supplemented with 10% fetal calf serum and standard antibiotics. Two days before transfection, cells were kept in phenol red-free DMEM containing 5% charcoal-treated serum (Hyclone). 3x 10^6 Cells were transfected [12] with expression constructs encoding PAK1 K423E [24], and/or the catalytic subunit of PKA [25] and after two days prepared for western blotting [18]. To activate PKA, forskolin (Sigma) was added 15 min prior to lysis at a concentration of 10^{-5} M. Antibodies against ERα (Stressgen Biotechnologies Corp), ERαS305P (Millipore), PAK1 (Cell Signaling, #2602), PKA C-α (Cell Signaling, #4782), and, α-tubulin (Sigma) were used at the recommended concentrations.

Gene expression analysis

The gene expression dataset has been described previously and is part of the training series described in this paper [20]. Agilent 44K expression data are available at http://research.agendia.com.

Analyses were performed using BRB Array Tools (version 3.6). First, using the gene set expression comparison tool, 302 pathways as defined by Biocarta were analyzed. The evaluation of pathways that are differentially expressed between ER305-P positive and ER305-P negative samples was done using a functional class scoring analysis as described by Xu et al. [26]. Fisher's Least Square (LS) summary statistic (10,000 permutations) was used to test which pathways were differentially expressed in ERαS305-P-positive tumors. First, a p-value is computed for each gene in a pathway. Then the set of p-values for a pathway is summarized by the LS summary statistics. For a set of N genes, the LS summary statistic ($LS = \sum_{i=1}^{N}(-\log(p_i))/N$) is defined as the mean negative natural logarithm of the p-values of the appropriate single gene univariate test [27].

Second, we related the pathways to either PKA or PAK1 using the Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/Genes/GeneFinder) and 27 and 12 pathways out of the 302 were found to be related to PKA and PAK1, respectively. Next, we tested whether the list of significant pathways as defined by the LS statistic ($p<0.05$)(see above) was enriched for PKA-related pathways using Fisher's Exact test [28].

PAK1-PKA/ ERαS305-P Predictive Score

Tumors that express no PAK1 and show no co-expression of pPKA and ERαS305-P are classified as 'negative'. A negative PAK1-PKA/ ERαS305-P Score corresponds to a high likelihood of being tamoxifen sensitive. Tumors that express PAK1 or show co-expression of pPKA and ERαS305-P are

classified as ‘positive’. A positive PAK1-PKA/ ERαS305-P Score corresponds to a high likelihood of being tamoxifen resistant.

Statistics

Level of agreement between observers for pPKA staining was expressed by means of kappa. Clinicopathological characteristics according to pPKA were compared using Fisher’s exact or Mann-Whitney U tests. Hazard ratios (HRs) were calculated using Cox regression analysis. Variables were entered into the multivariable model in one single step and including the clinicopathological variables used in previous studies for comparison [17, 18]. To evaluate whether tamoxifen benefit was modified by marker level, we allowed the tamoxifen-related HR to vary by marker level while adjusting for the main effect of the marker. Homogeneity of the tamoxifen-related HRs across marker levels was assessed by adding an interaction term between tamoxifen and marker level to a model including main effects for both factors. Analyses were performed using SPSS (version 15.0.1).

RESULTS

In order to determine the clinical relevance of both PAK1, pPKA and phosphorylation of ERα at serine 305, we evaluated the expression of these three markers in a series of breast cancer patients (n=103) who had received tamoxifen for metastatic disease (training series, clinical characteristics are presented in **Table 1**).

Tests for PAK1 and ERαS305-P have been described before [17, 18]. Active PKA was determined using an antibody detecting PKA phosphorylated at threonine 197 of the catalytic subunit (pPKA) [29]. To ensure that the antibody detects the phosphorylated form of PKA in FFPE tissues, sections were dephosphorylated prior to IHC. After treatment with lambda-phosphatase, no pPKA signal was detected (**Supplementary Data Figure A1**). Scoring of the intensity of pPKA staining by two observers resulted in a kappa of 0·84 ($p<0·0001$). Clinico-pathological parameters according to pPKA are shown in the **Supplementary Data (Table A2), and in Figure 1, which** showed the proportion of tumors that express PAK1, pPKA or both. In addition, the co-expression of PAK1 and PKA with ERαS305-P was summarized.

Previously, we have shown that ERαS305-P status alters tamoxifen response in the adjuvant setting [18]. The association between ERαS305-P status and outcome after tamoxifen in the metastatic disease setting was less pronounced (**Figure 2A**) [18]. pPKA positivity alone was not associated with TTP after tamoxifen treatment (**Figure 2B**). However, patients with a tumor that co-expressed pPKA and ERαS305-P had a significantly worse outcome after tamoxifen treatment (**Figure 2C**, univariate HR=2.00, p=0.017). In

addition, PAK1 was related to tumor progression after tamoxifen (**Figure 2D**, univariate HR 1.57, p=0.055, which is in line with our previous results [17]. Although PAK1 has been linked to ERαS305-P *in vitro* [13], the tumors that co-expressed PAK1 and ERαS305-P did not show a significantly increased risk for tumor progression compared to tumors that expressed PAK1 alone (**Figure 2E**).

These data suggested that PKA activity is linked to ERαS305-P, since tumors expressing both pPKA and ERαS305-P have a poor outcome after tamoxifen, while activation of PAK1 seems to be related to reduced tamoxifen sensitivity independent of ERαS305-P. To verify the connection between pPKA and ERαS305-P in a direct manner, we activated PKA in the breast tumor cell line MCF7 by forskolin (an activator of PKA via cAMP induction) treatment. The levels of ERαS305-P were increased upon PKA activation (**Figure 3**, lane 2). Over-expression of the catalytic subunit of PKA also induced ERαS305-P (**Figure 3**, lane 5). Over-expressed PAK1 (PAK1 T423E, a constitutive kinase-active construct), however, did not affect ERαS305-P levels in MCF7 cells (**Figure 3**, lane 3). These *in vitro* results confirmed the clinical data indicating that ERα is phosphorylated by PKA and not by PAK-1.

To further test whether ERαS305-P is indeed associated with PKA in human breast tumors, we evaluated gene expression of 11 tumors known to have a phosphorylated ERα at serine 305 and 47 ERαS305-P-negative tumors. These 58 tumors were selected because for these both IHC and gene expression data were available. The ERαS305-P-positive tumors were not significantly different from the rest of the trainingset presented above with

respect to the proportion of PAK1 and pPKA expression (data not shown). Of the 302 pathways tested, 19 were differentially expressed in ERαS305-P-positive tumors (permutated $p<0.05$, **Table 2**). The enrichment for pathways (5/19=26%) that include one or more PKA subunits was significant ($p=0.019$), while none of the 12 PAK1-related pathways was involved.

The *in vitro* experiments, the gene expression analysis as well as the clinical data in the training series, indicated that ERαS305-P is associated with pPKA but not with PAK1, suggesting that PAK1 is associated with sensitivity to tamoxifen via a mechanism independent of ERαS305-P. Indeed, upon adjustment for PAK1, co-expression of pPKA and ERαS305-P (PKA/ERαS305-P) was still significantly associated with TTP (multivariable HR 1.37, 95% CI 1.05-1.79, $p=0.022$). In addition, we did not see a significant overlap in between the PAK1 positive and PKA/ERαS305-P positive tumors ($p=0.25$, Fisher's Exact test). In order to capture both resistance mechanisms for predicting tamoxifen sensitivity, we combined PAK1 with pPKA-associated ERαS305-P which classified 38% (39/103) of the patients in the training series (**Figure 4A and 4B**) with an increased risk for progression after tamoxifen (**Figure 2F**). In particular, the number of patients was increased by the combination of the two independent predictive markers. Multivariable Cox regression analysis revealed that the PAK1-PKA/ ERαS305-P Score identified a group of breast cancer patients who have a poor outcome after tamoxifen treatment independent of traditional factors (**Table 3A**).

Next, we validated the PAK1-PKA/ ERαS305-P Score in an independent series (n=231), which was a subgroup of patients from an adjuvant tamoxifen

trial for whom sufficient material was available in order to assess the markers. They were not significantly different from the remaining group of patients with an ERα-positive tumor (**Supplementary Data Figure A2, Table A3**). In addition, the benefit from tamoxifen in the subset of 231 cases was similar to the tamoxifen benefit in the entire trial (**Supplementary Data Figure A3**). The proportions of tumors expressing ERαS305-P, pPKA or PAK1 in the validation series were comparable to those found in the training series (**Table 1** and **Figure 1**). 27% of the tumors in the validation series had either PAK1 expression and/or pPKA-associated ERαS305-P (**Figure 4C**). These patients had no significant benefit from adjuvant tamoxifen (**Figure 5B**, HR=0.88, 95% CI 0.42-1.82). whereas patients who were negative according to the PAK1-PKA/ ERαS305-P Score did benefit from tamoxifen (**Figure 5A**, HR=0.54, 95% CI 0.34-0.87).

This difference was statistically significant in a multivariable analysis (interaction, p=0.037, **Table 3B**). Notably, the PAK1-PKA/ ERαS305-P Score was not significantly associated with RFS in patients not treated with tamoxifen (**Figure 5C**, HR=0.88, 95% CI 0.49-1.56) or in the tamoxifen treated subpopulation (**Figure 5D**, HR=1.41, 95% CI 0.74-2.70).. **Table A4** presents the distribution of prognostic factors in the subgroup of patients who were negative according to the PAK1-PKA/ ERαS305-P Score. No statistical differences in prognostic factors were seen between the treated arm versus the control group. The patients who were positive for the PAK1-PKA/ ERαS305-P Score had a poorer survival after tamoxifen than the Score-

negative group of patients (**Figure 5D**). This difference was, however, not statistically significant (p=0.30, HR=1.41, 95% CI 0.74-2.70).

DISCUSSION

Here, we developed a score for tamoxifen sensitivity in metastatic breast cancer patients based on a combination of PKA-induced phosphorylation of ERα at serine 305 and levels of PAK1 in the primary tumor of the patient. The score was validated in an independent series of patients who were randomized between tamoxifen and no systemic treatment and identified breast cancer patients who benefit less from tamoxifen and who could be offered alternative treatment options. Besides assessment of the modification of the drug target (ERα), the PAK1-PKA/ ERαS305-P Score incorporates information on kinase activities that can potentially modify the drug target. The PAK1-PKA/ ERαS305-P Score has several advantages over the use of a single marker: Firstly, a combination of markers may capture several resistance mechanisms, which is relevant for a heterogeneous disease such as breast cancer. Secondly, involvement of a particular signaling pathway is more reliably assessed by multiple measurements within that pathway, in particular in archived material using semi-quantitative assays. Our data validate this concept.

Although tamoxifen has reduced breast cancer mortality by 30%, half of the treated patients at risk still develop a relapse despite adjuvant tamoxifen treatment [30]. It has been cumbersome to identify the endocrine agent most effective for an individual patient at high risk for recurrent disease. At present, the only validated predictive biomarker for tamoxifen response used in the

clinic is ERα expression, but this test has suboptimal positive predictive value. Data on the predictive value of PR are conflicting [22, 31, 32].

We confirmed the findings of Holm et al. that PAK1 levels are correlated with tamoxifen sensitivity [17]. Our study indicated no direct link between PAK1 and ERαS305-P, since outcome of the PAK1-positive group was not significantly affected by implementing ERαS305-P for the identification of tumors with poor outcome (see **Figure 2E**). In addition, our expression analysis showed no clear link between ERαS305P and PAK1-related pathways (see **Table 2**). Finally, *in vitro* PAK1 over-expression did not induce ERαS305P (see **Figure 3**). Although our data indicated that PAK1 is not directly involved in phosphorylation of ERαS305, PAK1 remained still an important additional marker in the identification of tamoxifen non-responders.

Our gene expression analysis revealed that pathways including PKA activity are overrepresented in tumors with an ERα phosphorylated at serine 305. This mechanism of PKA activation was first described by Miller et al., who showed a correlation between tamoxifen resistance and mRNA downregulation of a negative regulator of PKA (PKA-RIα) [33], and was also supported by our previous work in which we correlated mRNA levels of PKA-RIα to outcome after adjuvant tamoxifen treatment in breast cancer patients. More importantly, we showed that PKA activity induced a modification of ERα which in turn is causal for tamoxifen resistance *in vitro* [11]. Although phosphorylation of ERαS305 by PKA is clearly associated with tamoxifen resistance *in vitro*, pPKA expression alone appeared not to be sufficient to predict tamoxifen response (**Figure 2B**). The majority of breast tumors

expressed pPKA, while approximately 20% of the tumors showed ERαS305-P. This suggests that additional factors like phosphatases, may play a role in causing detectable ERαS305-P. The weak association between the markers may also be due to the application of antibodies detecting phospho-proteins in archived samples. This may, in some cases, be complicated by fixation procedures that might affect the stability of phospho-proteins. On the other hand, the PAK1-PKA/ ERαS305-P Score was validated in an independent dataset from another hospital with stainings performed in a different laboratory. This indicated that, though the designs and patient selections were quite different between the training and validation series, the association of the PAK1-PKA/ ERαS305-P Score with RFS after tamoxifen treatment was roughly similar in both patient series, although it was somewhat weaker and no longer significant in the validation study. The differences between the training set and validation set regarding design and patient selection may limit the interpretation of our results. Lack of a placebo group in the training set did not allow a data-driven definition of a marker combination so that we therefore relied mainly on a biological-driven definition based on functional experiments. However, the main effect of the PAK1-PKA/ ERαS305-P Score in the validation set can be calculated from **Table 3B** as (.71*1.22)/(1.0*.44)=1.97, and is very similar to the 1.91 in **Table 3A** (training set).

Further research is needed to determine the diagnostic accuracy of the PAK1-PKA/ ERαS305-P Score, such as sensitivity and specificity. Since patients in the validation series received only two years of tamoxifen treatment, further validation of the PAK1-PKA/ ERαS305-P Score has to be done for the

currently prescribed five years of adjuvant endocrine treatment. In addition, our data are based on a subgroup of patient that had predominantly lymph-node positive disease, resulting in a relatively poor survival even in the subgroup predicted as 'tamoxifen sensitive' by our Predictive Score. Consequently, our study design allows the selection of patients who may have sufficient benefit of tamoxifen monotherapy in the adjuvant setting. Finally, the PAK1-PKA/ ERαS305-P Score identified between 54 and 76% of the resistant cases in the respective breast cancer series. The remainder fraction has yet to be identified, but is still present in the tamoxifen-responsive subgroup of patients (**Figures 2F** and **5D**). The PAK1-PKA/ ERαS305-P Score identifies therefore, a subfraction of the patients who benefit less from tamoxifen treatment.

Each of the markers, PAK1 and pPKA/ERαS305P, identified a separate group of patients in both series of breast cancer patients that showed a reduced response to tamoxifen. The combination of these markers, however, enabled us to identify a major proportion of the less tamoxifen responsive cases. The combined marker identified 38% and 27% of the total number of breast cancer patients in the test and validation series, respectively, as tamoxifen resistant. In each set of patients, approximately half of the patients are expected to become resistant to tamoxifen. This implies that the combined marker identified respectably 76% and 54% of all tamoxifen resistant cases in these two breast cancer patient series.

In this study, we confirmed the correlation between PAK1 and tamoxifen resistance and provided evidence for the relationship between pPKA and

ERαS305-P that is relevant for tamoxifen response in patients. The proportion of tumors that have both PAK1 as well as pPKA-associated ERαS305-P was limited (9% in training, 0.4% in validation, **Figure 4**) suggesting that the three markers reflect two different mechanisms. We have shown previously that the effects of tamoxifen on RFS in subgroups defined by PAK1 alone were different [17]. The current study provides evidence that pPKA/ERαS305-P is a marker for tamoxifen sensitivity that is not related to PAK1. Using both markers resulted in the identification of an increased proportion of patients (27% based on the PAK1-PKA/ ERαS305-P Score versus 14% based on PAK1 alone) who are less sensitive to tamoxifen (**Figure 4**).

Only a few candidate biomarkers predicting drug response progress from laboratory to the clinic. Accurate patient stratification into responders and non-responders on the basis of one single biomarker is rare. The strength of the predictive PAK1-PKA/ ERαS305-P Score presented here is not only that it combined three markers and consequently captures PKA/PAK1-pathway activities at different levels in the signaling cascade, but that the implication of all three markers in tamoxifen sensitivity is supported by extensive functional experiments [11-15, 17]. This PAK1-PKA/ ERαS305-P Score may provide an important step towards personalized anti-estrogen therapy as patients who have less benefit from tamoxifen have alternative treatment options such as fulvestrant or aromatase inhibitors and thus may improve the outcome of breast cancer.

LEGENDS

Figure 1

Expression of PAK1, pPKA and ERαS305-P in human breast tumors.

Proportion of tumors expressing nuclear PAK1, pPKA and/or ERαS305-P. Below, the co-expression of PAK1 and pPKA with ERαS305P is depicted.

Figure 2

Association of ERαS305-P, pPKA and PAK1 with outcome after tamoxifen treatment for metastatic disease (training series)

Kaplan-Meier analysis according to ERαS305-P, pPKA and nuclear PAK1 expression in 103 patients. All HR and p-values are based on univariate Cox regression analysis. **A)** Time to tumor progression (TTP) according to ERαS305-P, **B)** TTP according to pPKA, **C)** TTP according to ERαS305-P and pPKA. Red line represents patients with a tumor co-expressing ERαS305-P and pPKA. Blue line represents patients with a tumor expressing pPKA but no ERαS305-P. HR and p-value estimate the difference between the groups depicted in blue and red, **D)** TTP according to nuclear PAK1, **E)** TTP according to ERαS305-P and nuclear PAK1. Red line represents patients with a tumor co-expressing ERαS305-P and PAK1. Blue line represents patients with a tumor expressing PAK1 but no ERαS305-P. HR and p-value estimate the difference between the groups depicted in blue and red. **F)** TTP according to the ERαS305-P/pPKA and PAK1. Red line represents patients with a tumor expressing pPKA-associated ERαS305-P, and/or nuclear PAK1. Green line

represents patients with a tumor that is expressing neither nuclear PAK1 nor pPKA-associated ERαS305-P.

Figure 3

PAK1 does not directly phosphorylate ERαS305 in MCF-7 cells

Western blot analysis of MCF-7 breast cancer cells expressing kinase-active PAK1-T423E (lanes 3,4,7,8) either or not in combination with the catalytic subunit of PKA, PKA-cat, (lanes 5-8). In the even lanes, cells were treated for 30 minutes prior to lysis with 10μM forskolin for PKA activation, whereas cells were untreated in uneven lanes. Protein was analyzed for expression of ERα, ERαS305-P, PAK1, PKA and α-tubulin (loading control). While both PKA activation and PKA-cat overexpression induced phosphorylation of ERαS305-P, this did not occur when overexpressing PAK1.

Figure 4

The PAK1-PKA/ ERαS305-P Score affects outcome of tamoxifen treatment

A) Categories defined by the PAK1-PKA/ ERαS305-P Score. **B)** and **C)** Illustration of the predictive PAK1-PKA/ ERαS305-P Score and the proportion of patients classified as less sensitive to tamoxifen.

Figure 5

Association of the PAK1-PKA/ ERαS305-P Score with outcome after adjuvant tamoxifen treatment (validation series)

Kaplan-Meier analysis according to the PAK1-PKA/ ERαS305-P Score in 231 patients. Recurrence-free survival (RFS) of patients who had been randomly assigned to tamoxifen or no adjuvant systemic treatment. Tumors with no PAK1 and no pPKA-associated ERαS305-P expression (**A**), and tumors with either PAK1 and/or pPKA-associated ERαS305-P expression (**B**) were analyzed separately. (**C**) RFS according to the PAK1-PKA/ ERαS305-P Score among patients who did not receive any adjuvant treatment (controls). (**D**) RFS according to the PAK1-PKA/ ERαS305-P Score among patients who did receive adjuvant tamoxifen.

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Table 1. Patient characteristics

		Training (n=103)		Validation (n=231)	
Variable	**Category**	**N**	**%**	**N**	**%**
Year of diagnosis	Range	1977-1997		1986-1991	
Time to tumor progression*	Median in months (range)	14 (1-169)			
Follow-Up	Median in years (range)			12 (0-17)	
Age at surgery	Median in years (range)	60 (36-83)		45 (26-57)	
Grade**	I/II	71	69%	151	66%
	III	32	31%	77	34%
	Unknown			3	
Lymph Node Status	Negative	68	67%	55	24%
	Positive	33	33%	176	76%
	Unknown	2			
WHO subtype	Invasive Ductal Carcinoma	87	88%	196	89%
	Invasive Lobular Carcinoma	12	12%	23	11%
	Unknown or other subtype	4		12	
Size	≤ 20 mm	61	59%	97	42%
	>20 mm	42	41%	134	58%
Progesterone Receptor (IHC)	≤ 10 %	36	35%	22	10%
	>10 %	67	65%	196	96%
	Unknown			13	
ERαS305-P (IHC)	Negative	83	81%	178	84%
	Positive	20	19%	35	16%
	Unknown			18	
pPKA (IHC)	Negative	30	29%	25	13%
	Positive	73	71%	165	87%
	Unknown			41	
PAK1 (IHC)	Negative	75	73%	191	85%
	Positive	28	27%	34	15%
	Unknown			6	

* Measured from start until stop tamoxifen treatment.
** According Nottingham Grading system (Elston et al. Histopathology 1993).
IHC= immunohistochemistry, ERαS305-P= phosphorylation of ER at serine 305, pPKA= phosphorylated PKA, PAK1= p21-activated kinase

Table 2. Pathways which have genes differentially expressed in ERαS305-P positive tumors

Rank	Biocarta pathway	PKA subunit in pathway	LS permuted p-value *
1	Rho-selective guanine exchange factor AKAP13 mediates stress fiber formation	PRKAR2A, PRKAR2B, PRKACA	0.006
2	AKAP95 role in mitosis and chromosome dynamics	PRKAR2A, PRKAR2B, PRKACA	0.007
3	Transcription regulation by methyltransferase of CARM1	PRKAR1B	0.008
4	Inhibition of Matrix Metalloproteinases		0.012
5	Y branching of actin filaments		0.012
6	Role of MAL in Rho-mediated activation of SRF		0.014
7	IFN alpha signaling pathway		0.022
8	mCalpain and friends in Cell motility	PRKAR1B	0.023
9	BCR signaling pathway		0.023
10	Steps in the glycosylation of mammalian N-linked oligosaccarides		0.025
11	Actions of Nitric Oxide in the Heart	PRKAR1B	0.031
12	EGF signaling		0.035
13	Generation of amyloid b-peptide by PS1		0.036
14	HIV Induced T cell apoptosis		0.038
15	Hypoxia and p53 in the cardiovascular system		0.042
16	Phosphorylation of MEK1 by cdk5/p35 downregulates the MAP kinase pathway		0.042
17	CDK regulation of DNA replication		0.044
18	Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription in carcinoma cells		0.046
19	Bone remodelling		0.046

ERαS305-P positive tumours (n=11) were compared with ERαS305-P negative tumours (n=47) using gene expression profiling.
42,034 genes were used for the analysis. 302 pathways as defined by Biocarta were tested using the Gene Set Comparison Tool in BRB array tools. The pathways that had a permuted p-value <0.05 are included in the table. In the 302 pathways tested, 27 are known to be related to PKA (Cancer Genome Anatomy Project). The expression of 5 out of the 27 pathways turned out to be significantly associated with ERαS305-P status.
Enrichment, within the significant pathways, for PKA-related pathways was tested using Fisher's Exact test: odds ratio= 4.24, p=0.019

* Based on 100,000 permutations. The Fishers's LS (least squares) summary statistic uses the average log P values for the genes in that class.

Table 3. Multivariable Cox regression analysis of the risk of progression after tamoxifen according to the PAK1-PKA/ ERαS305-P Score

A. Trainingset, NKI, tamoxifen for metastatic disease

Variable	Category	HR	95% CI	P-value
Grade*	I/II III	1 1.38	 1.09-1.76	 0.008
Lymph node status	Negative Positive	1 1.30	 0.83-2.05	 0.26
Age	Continuous (per year)	1.00	0.98-1.02	0.68
PAK1-PKA/ ERαS305-P Score	Negative Positive	1 1.91	 1.23-2.95	 0.004

Due to missing values in the factors used for adjustment, the analysis was based on 101 cases with 89 events. Variables included as previously described for this series (16,17). Including variables that performed significant in this trainingset in univariate analyses (progesterone receptor, HER2 and disease-free interval) did not substantially change the HR for the Algorithm.
Ki67 was not available for this series. * Nottingham grading system

B. Validationset, Lund, adjuvant tamoxifen versus no systemic treatment in randomized trial

Variable	Category		HR	95% CI	P-value
Grade*	I/II III		1 1.74	 1.07-2.84	 0.026
Lymph node status	Negative Positive		1 0.94	 0.58-1.55	 0.82
Age	Continuous (per year)		0.95	0.91-1.00	0.052
Ki67	≤25 % > 25%		1 1.22	 0.70-2.13	 0.47
PAK1-PKA/ ERαS305-P Score	Negative Positive		1 0.71	 0.37-1.35	 0.29
Tamoxifen	Negative PAK1-PKA/ ERαS305-P Score	-Control	1		
		-Tamoxifen	0.44	0.26-0.74	0.002
	Positive PAK1-PKA/ ERαS305-P Score	-Control	1		
		-Tamoxifen	1.22	0.55-2.74	0.62
Interaction** (PAK1-PKA/ ERαS305-P Score and Tamoxifen)					0.037

Due to missing values in the factors used for adjustment, the analysis was based on 201 cases with 88 events. Variables included as previously described for this series (16,17)
* Nottingham grading system
** The interaction variable indicates whether there is a difference in treatment response in relation to the PAK1-PKA/ ERαS305-P Score.

Figure 1. Expression of PAK1, pPKA and ERαS305-P in human breast tumors

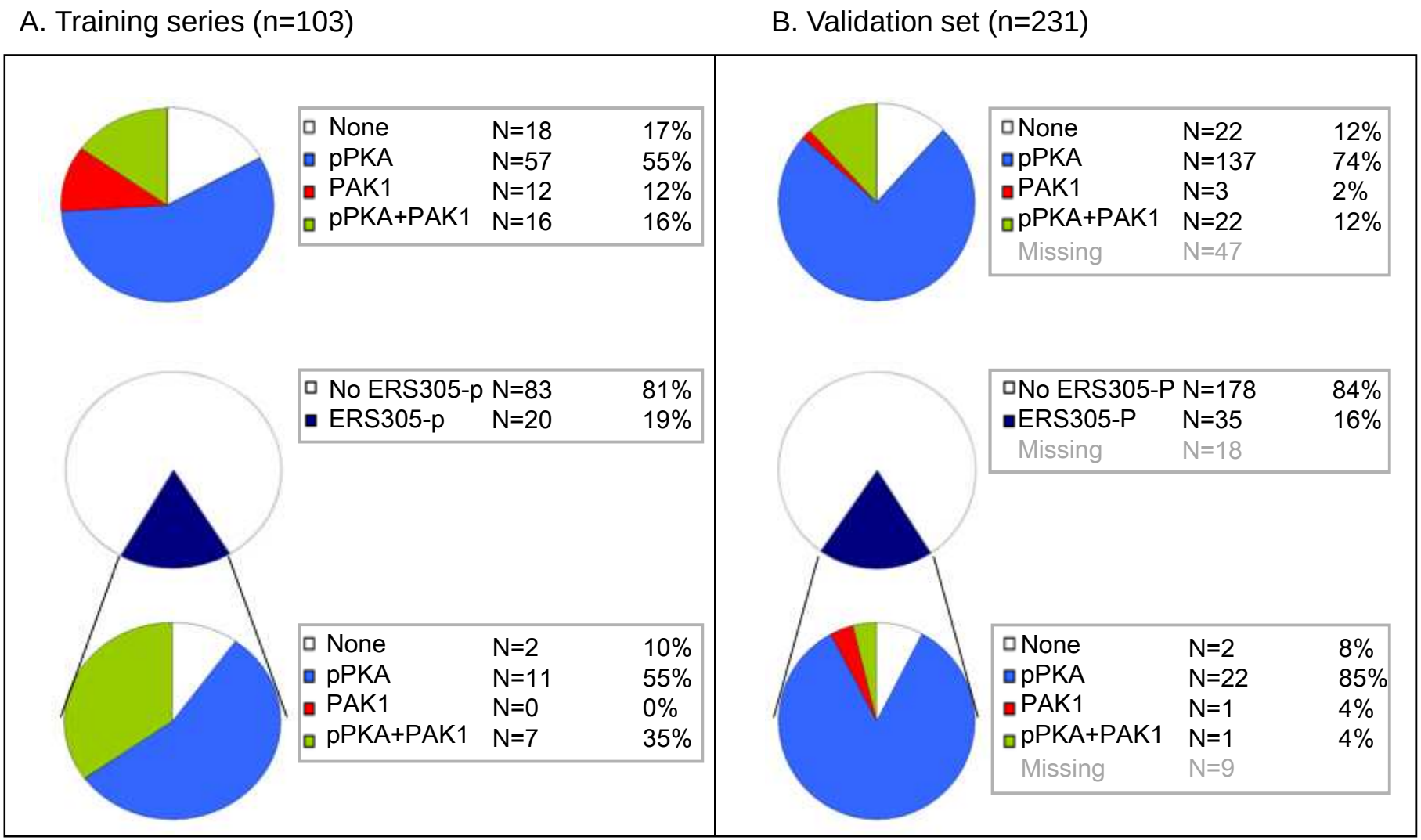
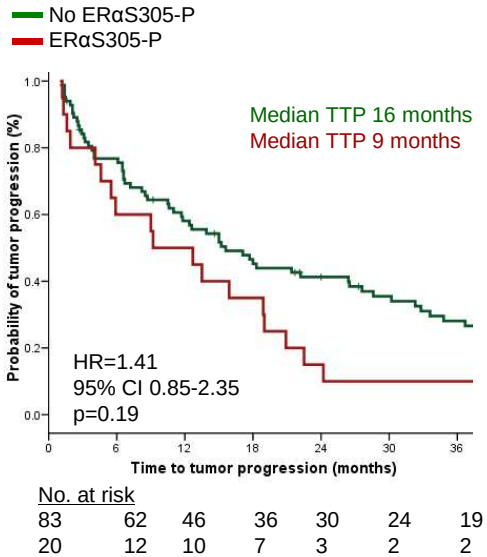


Figure 2

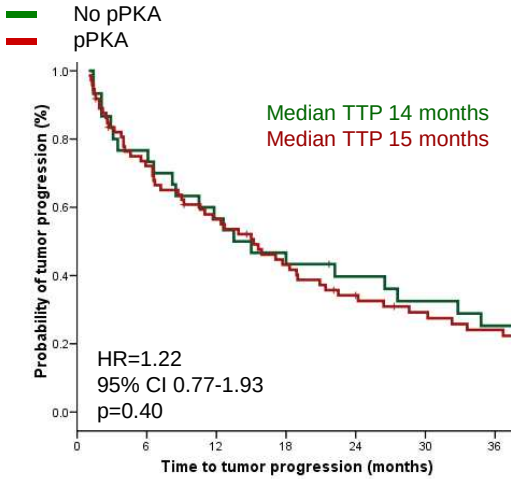
A. ERαS305-P



No. at risk

83	62	46	36	30	24	19
20	12	10	7	3	2	2

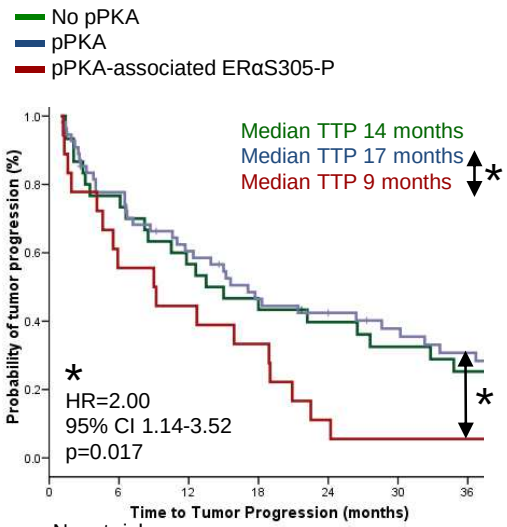
B. pPKA



No. at risk

30	23	17	14	11	9	7
73	51	39	29	22	17	14

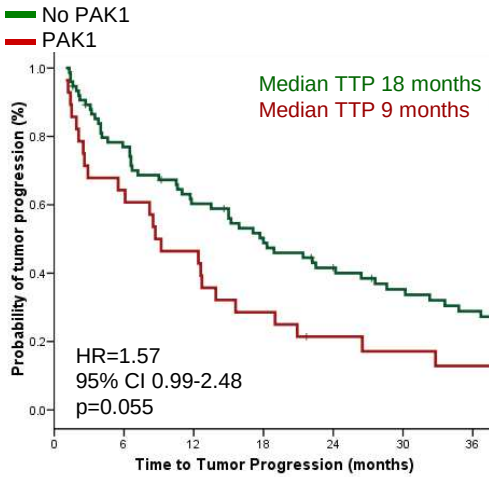
C. ERαS305-P and pPKA



No. at risk

30	23	17	14	11	9	7
55	41	31	23	20	16	13
18	10	8	6	2	1	1

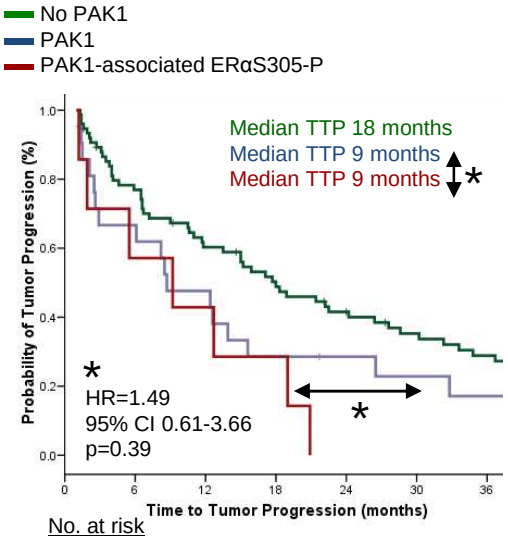
D. PAK1



No. at risk

75	56	43	35	28	22	18
28	18	13	8	5	4	3

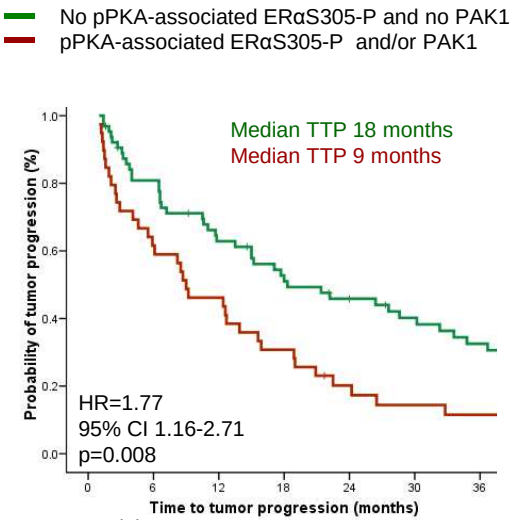
E. ERαS305-P and PAK1



No. at risk

75	56	43	35	28	22	18
21	14	10	6	5	4	3
7	4	3	2	0	0	0

F. ERαS305-P, pPKA and PAK1



No. at risk

64	50	38	31	26	21	17
39	24	18	12	7	5	4

Figure 3

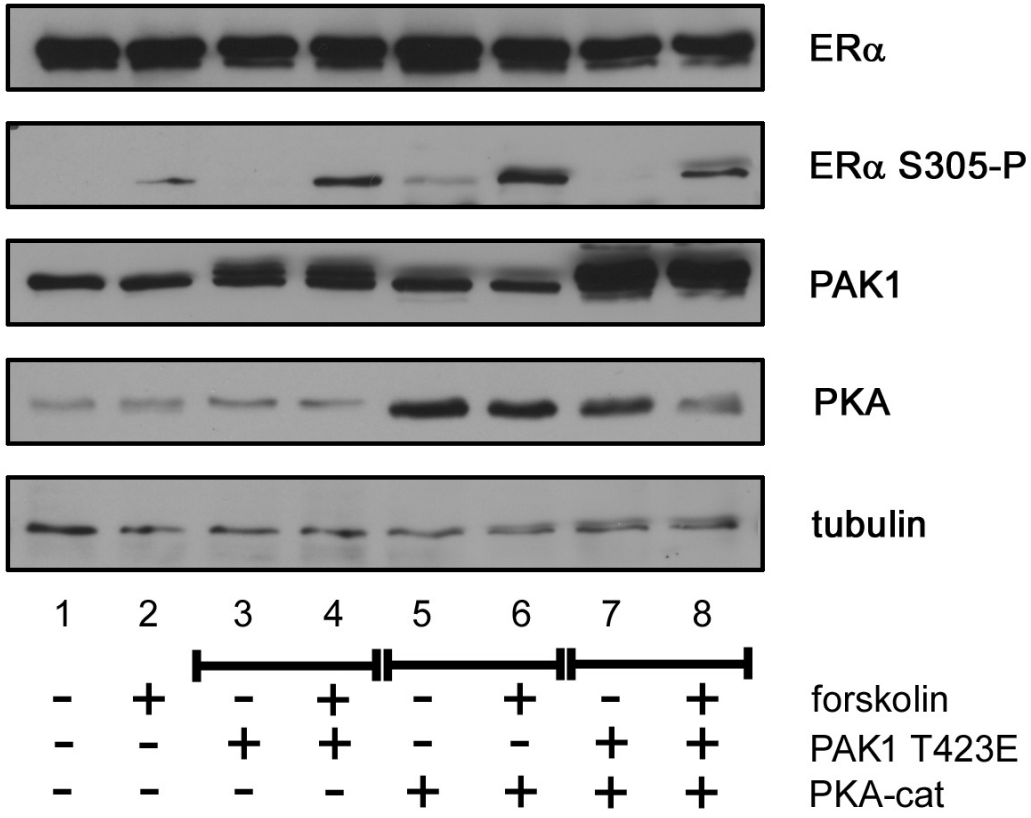
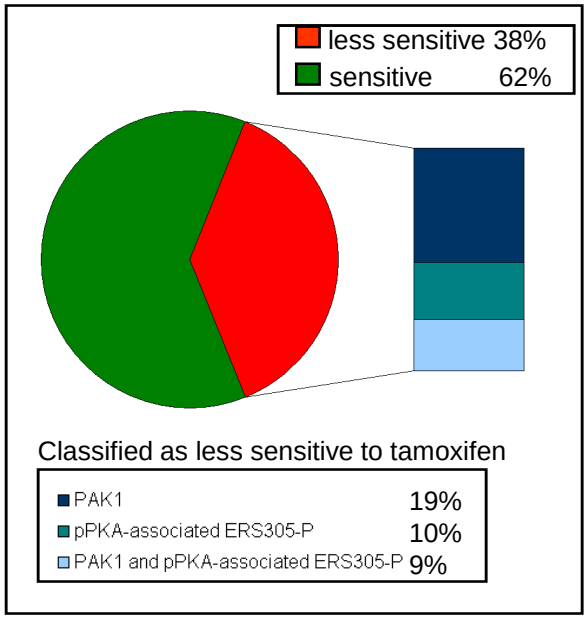

ERα
ERα S305-P
PAK1
PKA
tubulin
1 2 3 4 5 6 7 8
- + - + - + - + forskolin
- - + + - - + + PAK1 T423E
- - - - + + + + PKA-cat

Figure 4

A. PAK1-PKA/ ERαS305-P Score

- PAK1 OR pPKA-associated ERS305-P positive = PAK1-PKA/ ERαS305-P Score positive = tumor classified as less sensitive
- IF (No PAK1) AND (No pPKA-associated ERS305-P) = PAK1-PKA/ ERαS305-P Score negative = tumor classified as sensitive

B. Training series (n=103)



C. Validation set (n=231)

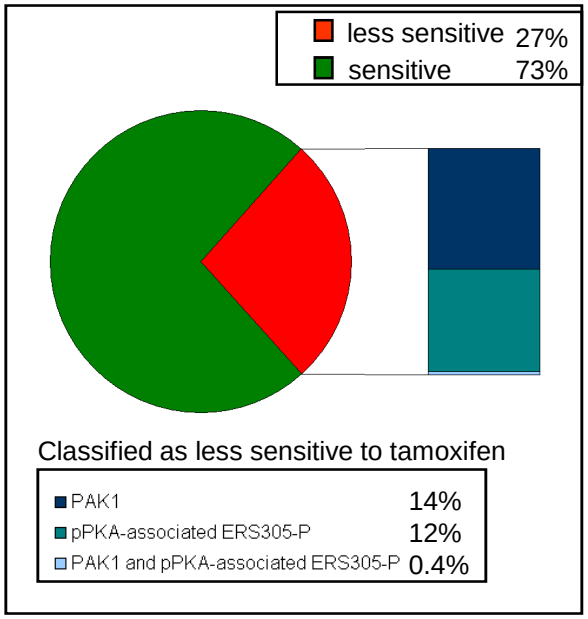
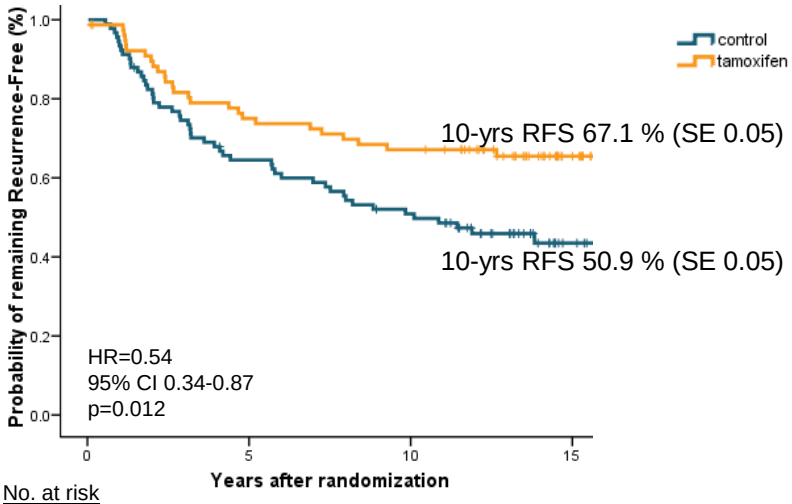


Figure5

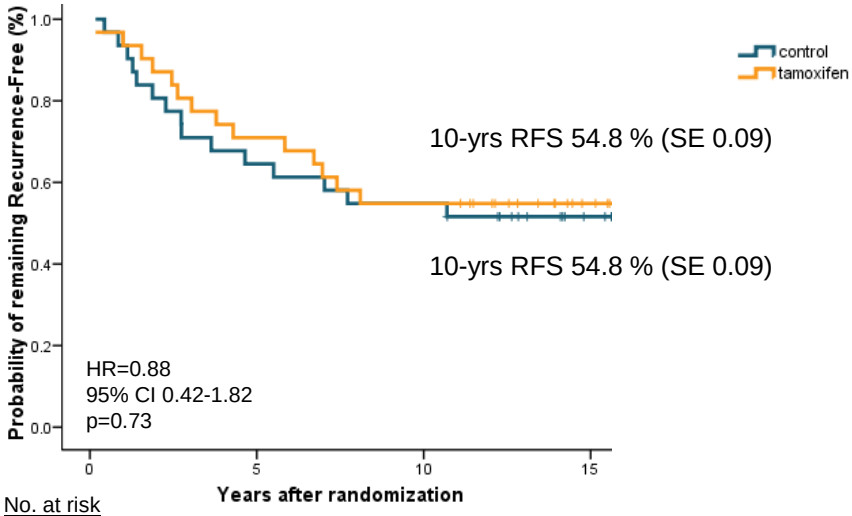
A. No pPKA-associated ERαS305-P and no PAK1 (PAK1-PKA/ ERαS305-P Score negative)



No. at risk

TAM	78	57	51	18
Control	91	57	44	10

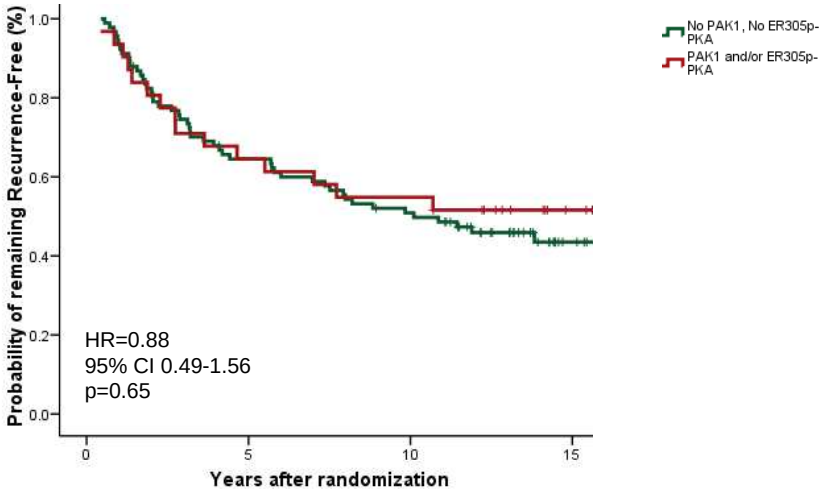
B. pPKA-associated ERαS305-P and/or PAK1 (PAK1-PKA/ ERαS305-P Score positive)



No. at risk

TAM	31	22	17	4
Control	31	20	17	6

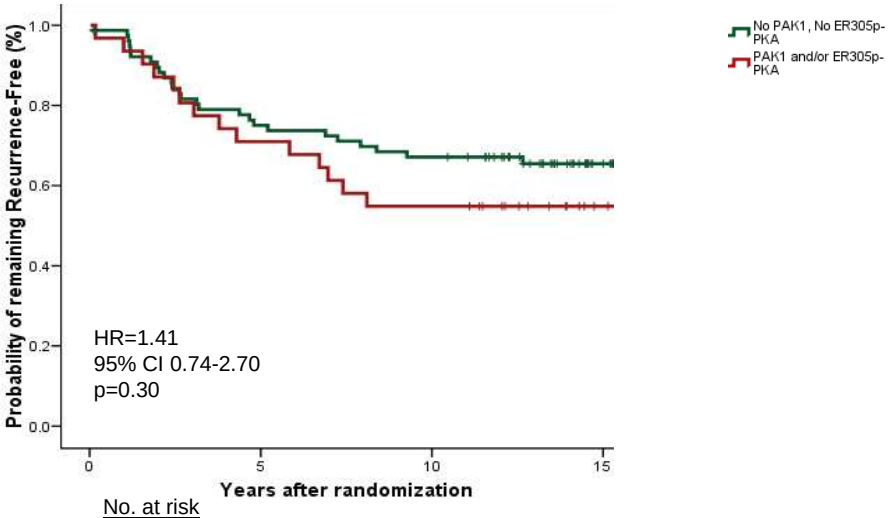
C. Untreated patients



No. at risk

PAK1-PKA/ ERαS305-P Score positive			
31	20	17	6
PAK1-PKA/ ERαS305-P Score negative			
91	57	44	10

D. Tamoxifen treated patients



No. at risk

PAK1-PKA/ ERαS305-P Score positive			
31	22	17	4
PAK1-PKA/ ERαS305-P Score negative			
78	57	51	18