

ORIGINAL ARTICLE

Pak1, adjuvant tamoxifen therapy, and breast cancer recurrence risk in a Danish population-based study

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ABSTRACT

Background Adjuvant tamoxifen therapy approximately halves the risk of estrogen receptor-positive (ER+) breast cancer recurrence, but many women do not respond to therapy. Observational studies nested in clinical trial populations suggest that overexpression or nuclear localization of p21-activated kinase 1 (Pak1) in primary tumors predicts tamoxifen failure.

Material and methods We measured the association between Pak1 expression and breast cancer recurrence in a Danish population-based case-control study. Pak1 cytoplasmic expression level and nuclear positivity were determined by immunohistochemical staining of primary breast tumors from recurrence cases and matched controls from two breast cancer populations; women diagnosed with ER-positive tumors who received at least one year of tamoxifen therapy (ER+/TAM+), and women diagnosed with ER-negative tumors who survived for at least one year (ER–/TAM–). Pak1 staining was assessed by a single, blinded pathologist, and associations were estimated with conditional logistic regression models.

Results We included 541 recurrence cases and 1:1 matched controls from the ER+/TAM+ group and 300 recurrence cases and 1:1 matched controls from the ER–/TAM– group. Pak1 cytoplasmic intensity was not associated with breast cancer recurrence in either group (ER+/TAM+ OR_{adj} for strong vs. no cytoplasmic staining = 0.91, 95% CI 0.57, 1.5; ER–/TAM– OR_{adj} for strong vs. no cytoplasmic staining = 0.74, 95% CI 0.39, 1.4). Associations between Pak1 nuclear positivity and breast cancer recurrence were similarly near null in both groups.

Conclusion Pak1 positivity in primary breast tumors was neither predictive nor prognostic in this prospective, population-based study.

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Tamoxifen is a selective estrogen receptor (ER) modulator that binds and stabilizes ER α to prevent estradiol (E2)-mediated transactivation and tumor promotion [1]. Five years of adjuvant tamoxifen therapy approximately halves the risk of breast cancer recurrence among women diagnosed with ER-positive (ER+) disease [2]. Current guidelines specify tamoxifen as adjuvant hormonal therapy for premenopausal ER+ breast cancer patients [3]. Unfortunately, some tamoxifen-treated women experience a breast cancer recurrence. Identification of biomarkers that predict tamoxifen failure is therefore an urgent research and clinical objective.

P21-activated kinase 1 in breast cancer

P21-activated kinase 1 (Pak1) is a serine/threonine kinase involved in signaling cascades with endpoints in cell migration, survival, and proliferation [4]. Pak1 directs normal mammary tissue development [5], and deregulation of its activity results in breast tumor formation in mice [6]. Laboratory evidence also implicates deregulated Pak1 activity in loss of

tamoxifen sensitivity. In the absence of E2, Pak1-mediated phosphorylation of serine-305 in ER α (ER α S305-P) precipitates auto-phosphorylation on serine-118 (ER α S118-P) and leads to E2-independent transactivation and increased transcription of estrogen responsive genes [7,8].

The impact of Pak1 amplification on tamoxifen effectiveness was studied in a Stockholm-based, tamoxifen trial-nested cohort of 224 postmenopausal women with breast tumors that had either metastasized to lymph nodes or that were >30 mm in diameter [9]. Among women without Pak1 gene amplification, tamoxifen reduced the recurrence rate compared with untreated women [hazard ratio (HR) = 0.53, 95% confidence interval (CI) 0.32, 0.88] [9]. Among women with Pak1 gene amplification, recurrence rates were similar between women in the tamoxifen and control arms (recurrence HR = 1.6, 95% CI 0.47, 5.6) [9].

Among premenopausal women with ER α -positive stage II breast cancer who participated in a second Swedish trial (SBI:2a) of two years of adjuvant tamoxifen therapy, tamoxifen treatment was associated with a lower recurrence rate in the stratum of patients whose tumors had low cytoplasmic

Pak1 staining (HR for tamoxifen-treated compared with untreated women = 0.50, 95% CI 0.33, 0.76) [10]. Tamoxifen treatment was not substantially associated with recurrence rate in the stratum of patients whose tumors had either high cytoplasmic Pak1 staining (HR = 0.89, 95% CI 0.42, 1.9), or any nuclear Pak1 staining (HR = 0.96, 95% CI 0.41, 2.3) [10]. In the same trial population, similar associations were observed when nuclear ER α S305-P was evaluated as a predictive biomarker: tamoxifen was associated with a lower recurrence rate among women whose tumor nuclei were free of ER α S305-P (HR = 0.53, 95% CI 0.32, 0.86), but was not protective among women whose tumor nuclei were positive for ER α S305-P (HR = 1.0, 95% CI 0.33, 3.1) [11].

In another study—also nested in the Stockholm trial population—the combination of nuclear Pak1 and nuclear ER α S305-P resulted in no recurrence benefit of tamoxifen therapy (HR = 1.33, 95% CI 0.42, 4.19), whereas tamoxifen was protective against recurrence among women whose tumors lacked both markers (HR = 0.43, 95% CI 0.30, 0.62) [12]. A similar pattern was observed in another study that evaluated both a Dutch metastatic breast cancer case series and participants in the SBI:2a trial [13].

Given the combined evidence, a commentator suggested that Pak1 could be adopted in clinical practice as a predictive biomarker of tamoxifen resistance [14]. As the evidence base upon which this recommendation rested is relatively sparse, we conducted a Danish population-based study of tamoxifen resistance to examine the potential association between breast tumor Pak1 and ER α S305-P cytoplasmic staining intensity, Pak1 nuclear localization, and breast cancer recurrence [15].

Material and methods

Study population

A detailed report of the source population, sampling of cases and controls, and exclusion criteria is given in an earlier publication [15]. Briefly, the source population consisted of all female residents of Denmark's Jutland Peninsula between 1985 and 2001 who were diagnosed with non-metastatic invasive breast cancer between the ages of 35 and 69, and whose diagnoses were reported to the Danish Breast Cancer Cooperative Group (DBCG) Registry [16]. Members of this source population were divided into two groups: women who were diagnosed with ER+ tumors and were treated with tamoxifen for at least one year (ER+/TAM+), and women who were diagnosed with ER-negative tumors and were not treated with tamoxifen, but survived for at least one year after primary therapy (ER-/TAM-). Women meeting neither group definition were excluded. We included the ER-/TAM- group to evaluate whether Pak1 expression was prognostic for breast cancer recurrence in the absence of ER expression and tamoxifen treatment. Absence of a prognostic effect in the ER-/TAM- group would allow attribution of a non-null association in the ER+/TAM+ group to a tamoxifen/ER-dependent mechanism, and would demonstrate the predictive utility of the biomarker.

Cases were women who were diagnosed with a contralateral tumor or local, regional, or distant recurrence within 10

years, as recorded in the DBCG Registry [17]. We then randomly selected one control who matched the case on follow-up time, group membership (ER+/TAM+ or ER-/TAM-), menopausal status at diagnosis (pre- or postmenopausal), date of breast cancer surgery (caliper matched ± 12 months), county of residence at diagnosis, and Union for International Cancer Control (UICC) stage at diagnosis (I, II, or III).

Data collection from Danish registries

The DBCG Registry provided data on patients' demographics (age, menopausal status, county of residence, hospital of diagnosis), tumor (UICC stage, histological grade, ER expression), and treatment characteristics (type of primary surgery, receipt of adjuvant radiotherapy and chemotherapy, and receipt of tamoxifen therapy). Medical history data required for calculation of Charlson Comorbidity Index (CCI) scores [18,19] were collected from the Danish National Patient Registry [20].

Tissue processing, genotyping, and immunohistochemistry

Details on the collection and processing of archived breast tumor tissue, extraction and amplification of DNA, and central re-assay of tumor ER status are provided in an earlier publication [15]. A commercially available TaqMan kit was used to determine CYP2D6*4 genotype (rs3892097; Applied Biosystems kit number C-27102431-D0).

Hematoxylin- and eosin-stained whole tumor slides were reviewed by a pathologist to identify regions of invasive carcinoma. From these regions, up to three 1 mm cores were removed and placed into tissue microarrays (TMA). Thirty-five TMAs accommodated all cases and controls. Sections (2.5 μ m) were cut from each TMA block and placed on glass slides for immunohistochemical assays. Mounted sections were incubated at 60°C for one hour before paraffin removal with Tissue-Clear reagent (Sakura Finetek Europe B.V., The Netherlands) and serial ethanol baths. Antigen retrieval was accomplished by incubating the sections with pH 9 Tris/EDTA buffer (VWR-Bie & Bertsen, Denmark) in a microwave oven. The sections were stained in one batch on a LabVision Autostainer (ThermoFisher Scientific, Fremont, CA, USA) using the EnVision+ detection system (Dako Corporation, Denmark). The sections were enhanced with copper sulfate and visualized with diaminobenzidine chromogen. Pak1 was detected with a monoclonal antibody (Millipore clone #EP795Y, Lot #DAM1498518) at a 1:300 dilution. ER α S305-P detection was attempted with three phosphorylation site-specific antibodies (Millipore catalog numbers 05-922 and 07-962, and Bethyl catalog number A300-598A) at varying dilution factors and under several automated and manual staining protocols.

Pak1 and ER α S305-P expression were evaluated by a single pathologist blinded to the case/control status of the tissue cores. For each core, Pak1 cytoplasmic intensity was classified on an ordinal scale similar to that used by Holm et al. [10]. Pak1 nuclear staining was classified as negative, ambivalent, or positive, with positivity defined as $\geq 10\%$ of tumor nuclei

exhibiting Pak1 staining. Each subject's cytoplasmic and nuclear Pak1 status was defined as the maximum value across replicate tumor cores.

Definitions of analytic variables

The DBCG defines breast cancer recurrence as any diagnosis of a new local, regional, or distant lesion following primary therapy [21]. Ordinal Pak1 cytoplasmic staining intensity was collapsed into categories of none, light, and strong (Figure 1). Given the importance of even low levels of nuclear Pak1 reported in earlier studies, we maximized sensitivity by defining Pak1 nuclear positivity as ambivalent positives plus strong positives. In sensitivity analyses, alternative definitions of nuclear positivity included a specific definition (in which ambivalent cases were counted as negative) and an exclusive definition (in which nuclear status was set to missing for ambivalent cases). Cytoplasmic Pak1 intensity was modeled with design variables representing light staining and strong staining. Nuclear Pak1 positivity was modeled as a dichotomous variable.

Year of diagnosis (the year in which subjects underwent breast cancer surgery) was categorized into the following periods and modeled with design variables: 1985–1993, 1994–1996, and 1997–2001. Menopausal status (pre- or post-) was defined on the diagnosis date. Age at diagnosis was modeled as a continuous variable in regression analyses. UICC

stage (I, II or III) was modeled with design variables. Type of primary surgery was defined dichotomously as receipt of either a mastectomy or a breast conserving procedure. Adjuvant chemotherapy and radiotherapy were both defined dichotomously (received/did not receive). Genotype at the *CYP2D6**4 locus was defined as homozygous wild-type, heterozygous, or homozygous variant according to the auto-call feature of the analytic software (MXPro QPCR version 4.1; Stratagene), and was modeled with design variables.

Statistical analysis

We tabulated the frequency and proportion of cases and controls according to demographic, clinical and tumor characteristics within categories of analytic group (ER+/TAM+ or ER-/TAM-). Observed *CYP2D6**4 genotypes in controls were compared with frequencies expected under Hardy-Weinberg equilibrium by calculating the χ^2 -test statistic, both overall and within analytic groups [22].

Within ER+/TAM+ and ER-/TAM- groups, we fit conditional and multivariate logistic regression models to estimate associations between Pak1 cytoplasmic intensity, nuclear positivity and breast cancer recurrence. Multivariate models accounted for matched factors by modeling them alongside the following candidate confounders: histological grade, age at diagnosis, receipt of adjuvant chemotherapy, receipt of

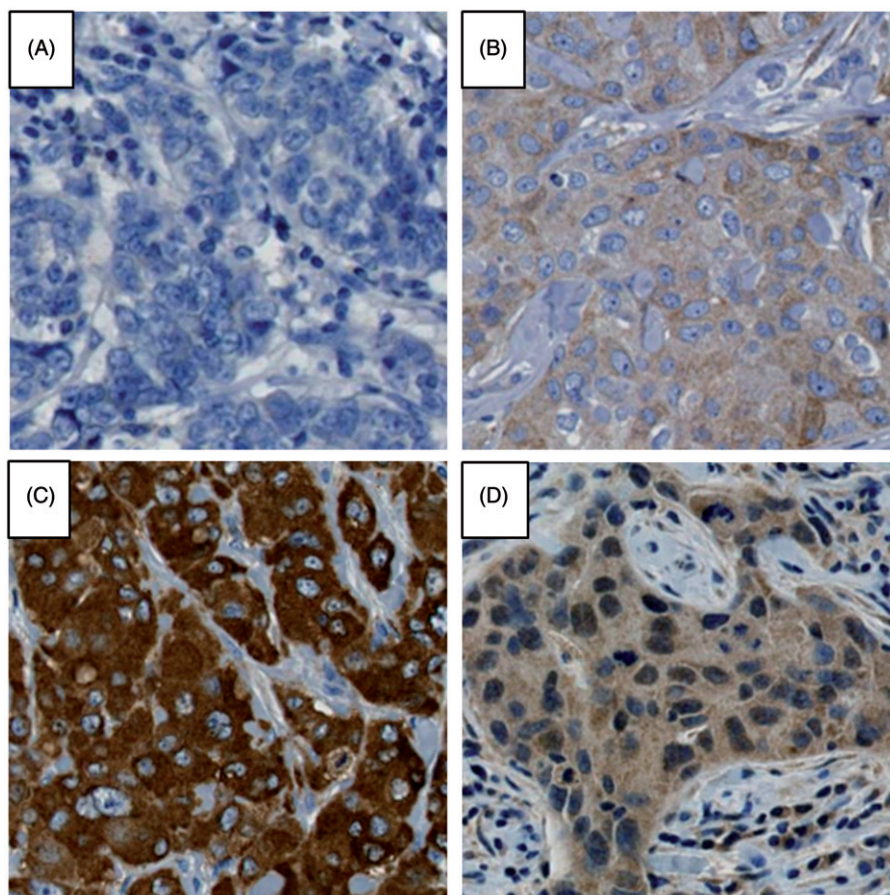


Figure 1. Images of tumor cores representative of the categories used to classify Pak1 cytoplasmic staining intensity and nuclear positivity. A: negative cytoplasmic and negative nuclear staining; B: light cytoplasmic and negative nuclear staining; C: strong cytoplasmic and negative nuclear staining; D: light cytoplasmic and positive nuclear staining.

adjuvant radiotherapy, type of primary surgery and *CYP2D6**4 genotype.

We also fit multivariate models in subsets of women with two normal alleles at the *CYP2D6**4 locus, and women whose tumor ER status was concordant between the original diagnostic assay and the centralized re-assay for this study. Associations are reported as odds ratios (ORs) with accompanying 95% CIs. As controls were sampled from cases' risk sets, ORs approximate recurrence rate ratios [23].

Data on histological grade were missing for approximately 42% of cases and controls. We therefore conducted a secondary multivariate analysis in which missing grade data were multiply imputed based on the cumulative logistic regression of observed grade on age at diagnosis, menopausal status at diagnosis, stage at diagnosis, tumor ER status, year of diagnosis, recurrence status, and receipt of adjuvant chemotherapy [24]. We performed a total of five imputations, fit multivariate models separately to each imputed data set, then pooled association estimates over all imputations [25]. Analyses were performed with SAS version 9.4 (SAS Institute, Cary, NC, USA); statistical tests were two-sided ($\alpha = 5\%$).

This study was approved by the Regional Committee on Biomedical Research Ethics of Denmark's Central Region (record number 1-10-72-16-15), the Danish Data Protection Board (record number 2012-41-4979), and relevant US Institutional Review Boards.

Results

Characteristics of cases and controls

In the ER+/TAM+ group there were 541 cases of recurrence, to whom 541 recurrence-free controls were matched. From the ER-/TAM- group we sampled 300 cases of recurrence, to whom 300 recurrence-free controls were matched. Within both the ER+/TAM+ and ER-/TAM- groups, identical or similar proportions of cases and controls fell into each category of diagnosis year, menopausal status at diagnosis, age group at diagnosis, UICC stage, type of breast cancer surgery, receipt of adjuvant chemotherapy and radiotherapy, and *CYP2D6**4 genotype (Table 1).

Genotypes of all controls at the *CYP2D6**4 locus were in Hardy-Weinberg equilibrium ($p = 0.21$). Findings were similar when we assessed equilibrium among controls within ER/TAM strata.

Pak1 cytoplasmic intensity and nuclear status in breast tumors

Table 2 shows the distribution of Pak1 cytoplasmic staining intensity and nuclear positivity according to other tumor characteristics and clinical/demographic factors. Strong cytoplasmic Pak1 staining was more prevalent among ER+/TAM+ women and was associated with lower histological grade. Histological grade was negatively associated with nuclear Pak1 status, whereas number of variant alleles at *CYP2D6**4 was positively associated with Pak1 nuclear staining. Otherwise, staining distributions were similar across levels of the factors examined.

Table 1. Characteristics of breast cancer recurrence cases and matched breast cancer controls registered in the DBCG according to patient, treatment, and tumor characteristics, Denmark's Jutland Peninsula, 1985–2001.

Characteristics	ER+/TAM+, n (%)		ER-/TAM-, n (%)	
	Cases (n = 541)	Controls (n = 541)	Cases (n = 300)	Controls (n = 300)
Year of diagnosis ^a				
1985–1993	235 (43)	234 (43)	107 (36)	100 (33)
1994–1996	113 (21)	112 (21)	81 (27)	83 (28)
1997–2001	193 (36)	195 (36)	112 (37)	117 (39)
Menopausal status at diagnosis ^a				
premenopausal	34 (6.3)	34 (6.3)	121 (40)	121 (40)
postmenopausal	507 (94)	507 (94)	179 (60)	179 (60)
Age group at diagnosis, years				
35–44	16 (3.0)	13 (2.4)	68 (23)	58 (19)
45–54	116 (21)	111 (21)	120 (40)	113 (38)
55–64	286 (53)	281 (52)	82 (27)	86 (29)
65–70	123 (23)	136 (25)	30 (10)	43 (14)
Stage ^a				
I	9 (1.7)	9 (1.7)	25 (8.3)	25 (8.3)
II	250 (46)	250 (46)	153 (51)	153 (51)
III	282 (52)	282 (52)	122 (41)	122 (41)
Histological grade				
1	108 (25)	144 (35)	27 (11)	23 (10)
2	234 (54)	215 (52)	125 (49)	98 (43)
3	92 (21)	57 (14)	103 (40)	106 (47)
(missing)	107	125	45	73
Type of surgery				
breast conserving	58 (11)	71 (13)	47 (16)	56 (19)
mastectomy	483 (89)	470 (87)	252 (84)	244 (81)
(missing)	0	0	1	0
Adjuvant chemotherapy				
received	70 (13)	65 (12)	248 (83)	188 (63)
did not receive	471 (87)	476 (88)	52 (17)	112 (37)
Adjuvant radiotherapy				
Received	183 (34)	191 (35)	128 (44)	123 (47)
did not receive	358 (66)	350 (65)	166 (56)	137 (53)
(missing)	0	0	6	40
<i>CYP2D6</i> *4 genotype				
homozygous wild-type	299 (61)	308 (62)	167 (60)	173 (62)
heterozygous	154 (31)	159 (32)	91 (33)	94 (34)
homozygous variant	41 (8.3)	30 (6.0)	19 (6.9)	13 (4.6)
(missing)	47	44	23	20
Pak1 cytoplasmic intensity				
none	135 (30)	130 (28)	95 (36)	102 (41)
light	190 (42)	185 (40)	111 (43)	94 (37)
strong	129 (28)	142 (31)	55 (21)	55 (22)
(missing)	87	84	39	49
Pak1 nuclear localization				
negative	260 (55)	278 (57)	164 (60)	163 (60)
positive	215 (45)	209 (43)	108 (40)	108 (40)
(missing)	66	54	28	29

^aVariable on which controls were matched to cases.

ER α S305-P cytoplasmic intensity

No combination of antibody and assay protocol yielded variability in the ER α S305-P staining pattern across tumor cores; all cores were either completely negative or showed positive nuclear and/or cytoplasmic staining (Figure 2; details available from corresponding author). This lack of variability precluded evaluation of associations between ER α S305-P and breast cancer recurrence.

Pak1 cytoplasmic intensity, nuclear localization, and breast cancer recurrence

Table 3 shows associations between Pak1 cytoplasmic intensity, nuclear localization, and breast cancer recurrence. Results

Table 2. Distribution of Pak1 cytoplasmic and nuclear staining patterns according to patient and tumor characteristics, breast cancer controls from Denmark's Jutland Peninsula, 1985–2001.

Characteristic	Pak1 cytoplasmic staining intensity			Pak1 nuclear status	
	None	Light	Strong	Negative	Positive
ER/TAM group					
ER+/TAM+	130 (28)	185 (40)	142 (31)	278 (57)	209 (43)
ER-/TAM-	102 (41)	94 (37)	55 (22)	163 (60)	108 (40)
Year of diagnosis					
1985–1993	73 (27)	112 (41)	90 (33)	157 (54)	134 (46)
1994–1996	44 (27)	70 (42)	51 (31)	96 (53)	84 (47)
1997–2001	115 (43)	97 (36)	56 (21)	188 (66)	99 (34)
Menopausal status					
premenopausal	52 (39)	48 (36)	33 (25)	79 (56)	62 (44)
postmenopausal	180 (31)	231 (40)	164 (29)	362 (59)	255 (41)
Age group at diagnosis					
35–44	24 (39)	25 (40)	13 (21)	45 (68)	21 (32)
45–54	66 (34)	73 (38)	53 (28)	111 (55)	90 (45)
55–64	97 (32)	121 (40)	87 (29)	190 (58)	139 (42)
65–70	45 (30)	60 (40)	44 (30)	95 (59)	67 (41)
Stage					
I	13 (52)	7 (28)	5 (20)	19 (63)	11 (37)
II	92 (28)	145 (44)	95 (29)	203 (57)	156 (43)
III	127 (36)	127 (36)	97 (28)	219 (59)	150 (41)
Histological grade					
1	39 (28)	47 (33)	55 (39)	76 (50)	75 (50)
2	97 (36)	114 (42)	59 (42)	179 (63)	105 (37)
3	53 (38)	59 (42)	27 (19)	95 (64)	53 (36)
(missing)	43	59	56	91	84
Type of surgery					
breast conserving	33 (31)	47 (44)	26 (25)	75 (64)	43 (36)
mastectomy	199 (33)	232 (39)	171 (28)	366 (57)	274 (43)
Adjuvant chemotherapy					
received	71 (34)	88 (43)	48 (23)	128 (57)	95 (43)
did not receive	161 (32)	191 (38)	149 (30)	313 (59)	222 (42)
Adjuvant radiotherapy					
received	87 (33)	106 (41)	67 (26)	171 (60)	115 (40)
did not receive	130 (32)	163 (40)	119 (29)	250 (57)	185 (43)
(missing)	15	10	11	20	17
CYP2D6*4 genotype					
wild-type	136 (32)	177 (42)	113 (27)	277 (61)	179 (39)
heterozygote	74 (33)	80 (36)	67 (30)	133 (57)	101 (43)
variant	13 (5.8)	15 (39)	10 (26)	21 (51)	20 (49)
(missing)	9	7	7	10	17

did not differ appreciably between conditional models, which adjusted only for matching factors, and multivariate models, which adjusted for matching factors and candidate confounders.

Pak1 cytoplasmic intensity was not associated with breast cancer recurrence in either the ER+/TAM+ or ER-/TAM- groups (in the ER+/TAM+ group: OR_{adj} , strong cytoplasmic Pak1 vs. no cytoplasmic Pak1 = 0.91, 95% CI 0.57, 1.5; in the ER-/TAM- group: OR_{adj} , strong cytoplasmic Pak1 vs. no cytoplasmic Pak1 = 0.74, 95% CI 0.39, 1.4). Associations between nuclear Pak1 positivity and breast cancer recurrence were near null and very similar in both analytic groups (in the ER+/TAM+ group: OR_{adj} = 1.3, 95% CI 0.89, 1.8; in the ER-/TAM- group: OR_{adj} = 1.2, 95% CI 0.72, 1.9). Multivariate models using multiply imputed values of histological grade yielded similar results (Table 3).

Associations were also similar when we restricted to women with two normal alleles at CYP2D6*4 or to women whose tumor ER status was concordant between original diagnosis and centralized re-assay for this study

(Table 4). Associations between recurrence and nuclear Pak1 status were essentially unchanged in models using the specific and exclusive alternative definitions (data not shown).

Discussion

We observed no association between primary tumor cytoplasmic Pak1 intensity and breast cancer recurrence among both ER+, tamoxifen-treated and ER-, tamoxifen-untreated women diagnosed with non-metastatic breast cancer. Associations between tumor Pak1 nuclear positivity and breast cancer recurrence were near null, and very similar in the ER+/TAM+ and ER-/TAM- groups. Although it may be tempting to interpret the association as marginally positive in the ER+/TAM+ group, it was also indistinguishable from that in the ER-/TAM- group. These findings support neither a predictive nor prognostic role of nuclear or cytoplasmic Pak1 in primarily postmenopausal early-stage breast cancer patients.

To our knowledge this is the largest study yet (in terms of the number of recurrence events) of Pak1 and tamoxifen effectiveness, and the first to report a null association. Study strengths include virtual immunity to selection bias through identification of recurrence cases and controls from the prospective and nearly complete population-based DBCG Registry, and characterization of exposures on the basis of breast tumor specimens obtained from independent hospital pathology departments. Furthermore, data were of high quality: the positive predictive value of the DBCG Registry's recurrence classification was reported to be 99.4% [26], and this finding was confirmed by medical record review for a subset of cases in this study [15]. As our study covered a time period of evolving ER assay methodologies, we centrally re-assayed ER status for cases and controls using current standard clinical methods [27]. Our use of TMAs allowed Pak1 and ERαS305-P to be assayed in one batch with single reagent lots, eliminating two major sources of staining variability common to immunohistochemical assays. While TMAs are sometimes criticized for their potential to misrepresent histological and molecular characteristics of whole tumors, we were careful to select regions of invasive breast cancer and included up to three such cores for each subject. Furthermore, validation studies indicate that multi-core TMAs closely approximate the molecular information gleaned from whole tumor breast carcinoma slides [28,29]. CYP2D6*4 genotype data were in Hardy-Weinberg equilibrium, which demonstrates the representativeness of our control population and the technical proficiency of our laboratory.

Of the covariates we evaluated, only histological grade substantially changed association estimates upon adjustment. For example, the unadjusted association between strong Pak1 cytoplasmic intensity and breast cancer recurrence in the ER+/TAM+ group increased by 31% when grade was added to the model; no other covariate changed the crude estimate by more than 4% when modeled singly with Pak1 cytoplasmic and nuclear scores (data not shown). Directions of estimate displacement upon adjustment for grade followed expectations based on univariate associations

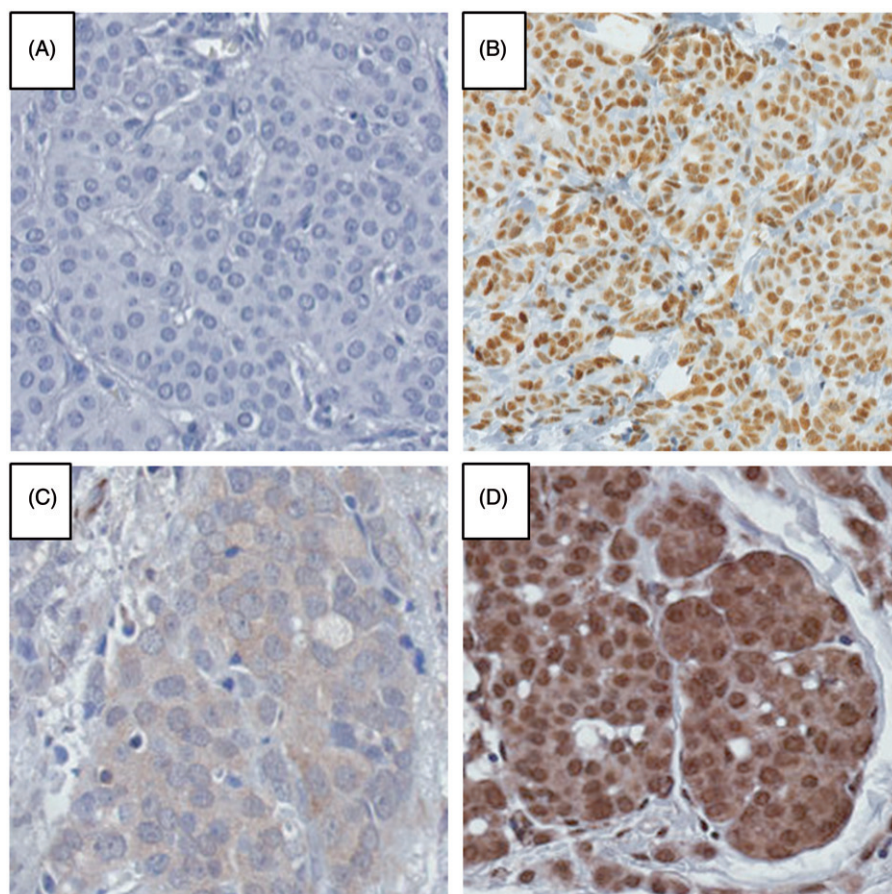


Figure 2. Images depicting staining patterns observed for ERαS305-P using different commercial antibodies and assay protocols. No variability was observed across tumor cores under any given combination of antibody and assay protocol. A: Negative staining using Millipore antibody #05-922 at 1:20 dilution with manual protocol and overnight incubation; B: Positive nuclear staining using Millipore antibody #07-962 at 1:50 dilution with manual protocol and 30 minute incubation; C: Weak cytoplasmic and nuclear staining using Bethyl antibody #A300-598A at 1:300 dilution with manual protocol and overnight incubation; D: Strong cytoplasmic and nuclear staining using Bethyl antibody #A300-598A at 1:100 dilution with manual protocol and overnight incubation.

Table 3. Associations between cytoplasmic Pak1 expression level, nuclear Pak1 localization and breast cancer recurrence.

Group	Pak1 expression and localization	Cases/controls	Matched OR ^a (95% CI)	Adjusted OR ^b (95% CI)	Grade imputed adjusted OR ^c (95% CI)
ER+/TAM+	Cytoplasmic intensity				
	None	135/130	1. ref	1. ref	1. ref
	Light	190/185	0.91 (0.63, 1.3)	1.0 (0.68, 1.5)	0.87 (0.62, 1.2)
	Strong	129/142	0.80 (0.51, 1.3)	0.91 (0.57, 1.5)	0.74 (0.49, 1.1)
	Nuclear status				
	Negative	260/278	1. ref	1. ref	1. ref
ER-/TAM-	Positive	215/209	1.2 (0.88, 1.7)	1.3 (0.89, 1.8)	1.4 (1.0, 1.9)
	Cytoplasmic intensity				
	None	95/102	1. ref	1. ref	1. ref
	Light	111/94	1.2 (0.79, 1.9)	1.1 (0.69, 1.8)	0.87 (0.73, 1.8)
	Strong	55/55	0.94 (0.54, 1.6)	0.74 (0.39, 1.4)	0.92 (0.52, 1.7)
	Nuclear status				
	Negative	164/163	1. ref	1. ref	1. ref
	Positive	108/108	1.1 (0.74, 1.7)	1.2 (0.72, 1.9)	1.2 (0.77, 1.9)

^aAdjusted for matched factors (year of diagnosis, menopausal status at diagnosis, UICC tumor stage, county of residence at diagnosis, and follow-up time) using conditional logistic regression;

^bAdjusted for UICC stage at diagnosis (I, II, or III, design variables), histological grade (1, 2, or 3, design variables), age at diagnosis (continuous), menopausal status at diagnosis (pre- or post-), year of diagnosis (1985–1993, 1994–1996, 1997–2001, design variables), receipt of adjuvant chemotherapy (dichotomous), receipt of adjuvant radiotherapy (dichotomous), type of primary surgery (mastectomy or breast conserving), CYP2D6*4 genotype (2 normal alleles, 1 variant allele, or 2 variant alleles, design variables), and follow-up time;

^cAdjusted for the variables listed in Footnote b in multivariate models fit to 5 data sets in which missing histological grade values were multiply imputed. See Statistical analysis section for details.

between Pak1 scores, grade, and recurrence status. While grade data were missing for a substantial proportion of cases and controls, our secondary analysis using multiply imputed histological grade—an analytic technique recommended for molecular epidemiology studies [30]—did not yield different results.

Study limitations include its restriction to primarily postmenopausal women (96% in the ER+/TAM+ stratum), and the fact that some women in the ER+/TAM+ stratum received less than the recommended five-year course of tamoxifen treatment. About two-thirds of ER+/TAM+ women were initially assigned to one or two years of tamoxifen therapy,

Table 4. Association between cytoplasmic Pak1 staining intensity, nuclear localization, and breast cancer recurrence within strata of women with (a) no variant allele at the *CYP2D6**4 locus (rs3892097), and (b) concordant estrogen receptor status upon centralized re-assay.

	Group	Pak1 expression and localization	Cases/controls	Adjusted OR ^a (95% CI)
2 normal function alleles at <i>CYP2D6</i> *4	ER+/TAM+	Cytoplasmic intensity		
		None	77/71	1. ref
		Light	112/128	0.85 (0.52, 1.4)
		Strong	77/79	0.95 (0.51, 1.8)
		Nuclear status		
		Negative	156/179	1. ref
	ER-/TAM-	Positive	123/117	1.4 (0.89, 2.3)
		Cytoplasmic intensity		
		None	60/65	1. ref
		Light	60/49	1.1 (0.58, 2.2)
		Strong	31/34	0.67 (0.29, 1.5)
		Nuclear status		
Concordant ER result	ER+/TAM+	Negative	94/98	1. ref
		Positive	63/62	1.1 (0.57, 2.1)
	ER-/TAM-	Cytoplasmic intensity		
		None	118/124	1. ref
		Light	182/179	1.0 (0.71, 1.5)
		Strong	124/139	0.92 (0.57, 1.5)
	ER+/TAM+	Nuclear status		
		Negative	236/268	1. ref
		Positive	203/198	1.3 (0.90, 1.9)
	ER-/TAM-	Cytoplasmic intensity		
		None	74/74	1. ref
		Light	85/80	0.93 (0.53, 1.6)
		Strong	31/30	0.69 (0.31, 1.6)
		Nuclear status		
		Negative	123/123	1. ref
		Positive	76/77	1.2 (0.65, 2.1)

^aAdjusted for UICC stage at diagnosis (I, II, or III, design variables), histological grade (1, 2, or 3, design variables), age at diagnosis (continuous), menopausal status at diagnosis (pre- or post-), year of diagnosis (1985–1993, 1994–1996, 1997–2001, design variables), receipt of adjuvant chemotherapy (dichotomous), receipt of adjuvant radiotherapy (dichotomous), type of primary surgery (mastectomy or breast conserving), and follow-up time. Analyses in the concordant ER result stratum were additionally adjusted for *CYP2D6**4 genotype (2 normal alleles, 1 variant allele, or 2 variant alleles, design variables).

while one-third of ER+/TAM+ women were assigned to five years of therapy. Our earlier validation study compared original assignment with actual duration of tamoxifen therapy abstracted from medical records, and estimated that 70% of women initially assigned to one or two years of tamoxifen actually received five years of therapy because of evolving treatment guidelines [15]. Therefore, tamoxifen use in our study population was likely closer to current guidelines than tamoxifen use in the previous studies that reported associations between Pak1 expression and recurrence among tamoxifen users [9–13]. We collapsed Pak1 staining categories in our analyses, which resulted in a reference category of unequivocal negatives combined with probable negatives (weak or trace staining). While this had the potential to misclassify true Pak1 status and to attenuate association estimates, our findings were similar when we modeled staining levels individually (data not shown). Finally, we used the DBCG definition of recurrence as our outcome, which comprises local, regional, and distant disease. If Pak1 were associated only with distant recurrence among tamoxifen users, the inclusion of local and regional lesions in the composite outcome would be expected to attenuate that association. However, all of the earlier studies that found an association between Pak1 expression and recurrence on tamoxifen used similar composite definitions of recurrence [9–13], which argues against outcome misclassification as an explanation for our null result. Furthermore, Pak1 is a suggested

predictive marker for tamoxifen effectiveness, and as tamoxifen therapy protects against all types of recurrence, our outcome definition is the most appropriate choice for evaluating that claim.

Unlike earlier reports [11–13], we detected no variability in tumor ER α S305-P staining. Previous studies observed a majority of breast tumors to be negative for nuclear ER α S305-P staining [11–13], and two of these studies used the same monoclonal antibody that we initially used for our assays [11,13].

Most previous clinical evidence concerning Pak1 and tamoxifen failure comes from studies nested in one of two Swedish tamoxifen trials. The SBII:2a trial, which began enrollment in 1986, randomized premenopausal stage II breast cancer patients to either two years of tamoxifen (20 or 40 mg/day) or no treatment [31]. A Stockholm-based trial, which began enrollment in 1976, randomized postmenopausal early-stage breast cancer patients to either two years of tamoxifen (40 mg/day) or no treatment [32]. Other published evidence comes from a relatively small and highly selected case series (n = 142) of women diagnosed with metastatic breast cancer in The Netherlands [33]. Our study therefore provides important diversity in the source populations generating evidence for associations between breast tumor Pak1 status and tamoxifen failure. Given our null result, the earlier recommendation to incorporate Pak1 expression as a predictive biomarker for tamoxifen merits reconsideration.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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