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Jake Thistle Date

The Role of 14-3-3ζ in Tamoxifen Resistance and Breast Cancer Recurrence

By

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Epidemiology

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The Role of 14-3-3ζ in Tamoxifen Resistance and Breast Cancer Recurrence

By

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B.S., Microbiology
California State University, Long Beach
2012

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An abstract of
A thesis submitted to the Faculty of the
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Abstract

The Role of 14-3-3ζ in Tamoxifen Resistance and Breast Cancer Recurrence
By Jake Thistle

Background. Overexpression of 14-3-3ζ has been linked to breast cancer recurrence in several studies, including studies assessing its effect on tamoxifen resistance.

Methods. A case-control study, nested in a population 11,251 females of the Jutland Peninsula aged 35-69 diagnosed with stage I, II, or III breast cancer between 1985 and 2001 registered with the Danish Breast Cancer Cooperative Group, was performed to estimate the effect of 14-3-3ζ expression on tamoxifen resistance. 541 recurrent breast cancer cases with estrogen receptor-positive disease treated with tamoxifen for at least 1 year (ER+/TAM+) and 300 recurrent breast cancer cases in women with estrogen receptor-negative disease never treated with tamoxifen (ER-/TAM-) were identified. 1:1 matching was performed on group membership (ER+/TAM+ or ER-/TAM-), date of surgery, menopausal status, stage, and county. 14-3-3ζ expression was assessed in the nucleus and cytoplasm using tissue microarray immunohistochemistry. The odds ratio (OR) associating 14-3-3ζ expression with breast cancer recurrence was estimated adjusted for confounding using logistic regression. Bias due to expression assay methods was accounted for in an analysis controlling for misclassification and confounding. Inverse-variance weighting Bayesian analysis was performed to further differentiate 14-3-3ζ expression as predictive of tamoxifen resistance or prognostic of breast cancer recurrence.

Results. Patients with above the 50^{th} percentile combined cytoplasmic and nuclear staining showed an association in the ER+/TAM+ group (OR = 1.44, 95% confidence interval = 1.05, 1.99) and a near null association in the ER-/TAM- group (OR = 1.22, 95% confidence interval = 0.82, 1.82). After quantitative bias analysis, associations increased slightly, indicating non-null results are not explained by exposure misclassification, assuming a valid bias model. Evidence was lacking from inverse-variance Bayesian analysis to make conclusions about combined staining as a marker of tamoxifen resistance. Associations for above the 75th percentile of combined staining were moderate in ER+/TAM+ patients (OR = 1.93, 95% confidence interval = 1.15, 3.24) and ER-/TAM- patients (OR = 1.93, 95% confidence interval = 1.03, 3.62), indicating potential prognostic utility.

Conclusion. 14-3-3ζ is a potentially useful prognostic marker of breast cancer recurrence. Further research is needed to determine if 14-3-3ζ has utility beyond established prognostic markers.

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Introduction

Breast cancer is the most common type of invasive cancer in women and the second leading cause of cancer-related death. In the US, breast cancer accounts for 29% of newly diagnosed cancer cases and results in nearly 40,000 deaths annually [1]. Incidence rates have increased on average 0.3% per year between 1992 and 2011 [2]. Treatment options for breast cancer include surgery, radiation therapy, systemic therapies or a combination of these. Although many patients benefit from initial treatments, approximately 30% of early stage breast cancer patients develop recurrence [3]. In 2013, there were estimated to be more than 3.1 million US women living with a history of invasive breast cancer [4].

About 75% of breast cancers express estrogen receptor α (ERα) and its expression is associated with highly distinct gene patterns [5]. ERα is a transcription factor for genes extensively involved in cell cycle regulation that act by binding DNA directly or through modes involving other transcription factors. Carcinogenesis in some breast cancers may result from a deregulation of ERα stimulation, which gives rise to a proliferative phenotype [6].

Patients whose tumors express ERα are candidates for treatments that target growth stimulation by estrogen. Two primary classes of drugs are used to treat ERα-positive breast cancer patients, the aromatase inhibitors and the selective estrogen receptor modulators (SERMs). Aromatase inhibitors interfere with the production of estrogen by suppressing the enzyme aromatase, responsible for converting androgens to circulating estrogen [7]. For treatment of breast cancers, SERMs are antagonists to estrogen; occupying the ligand-binding domain of ERα [8]. Due to the success of these

treatments, cancer patients with tumors that express ERα have a reduced risk of mortality independent of age, ethnicity, and tumor characteristics (stage, grade, and histology) [9, 10].

ERα-positive breast cancer patients are commonly treated with the SERM tamoxifen. Five years of treatment with tamoxifen reduces the risk of recurrence by half in ERα-positive women [11]. Current National Comprehensive Cancer Network (NCCN) guidelines recommend tamoxifen in premenopausal women, either tamoxifen, aromatase inhibitors, or courses that combine these therapies in postmenopausal women, and tamoxifen in postmenopausal women with contraindications to aromatase inhibitors [12]. Therefore, tamoxifen remains a cornerstone of breast cancer treatment for ERα-positive women.

Identifying patients at high-risk for recurrence is a priority of breast cancer research [13]. Patients at high risk when treated with tamoxifen may be better suited for treatment with aromatase inhibitors, as these drugs are sometimes better tolerated and show efficacies comparable or greater than tamoxifen [14]. The American Society of Clinical Oncology identified research to develop biomarkers for selection of endocrine strategies in ERα-positive women as a significant goal of clinical research [15]. Despite numerous studies, status of ERα expression remains the sole predictor of an individual patient's response to treatment with tamoxifen [11].

Overexpression of the 14-3-3ζ protein has been linked to recurrence of breast cancer in several studies [16-22]. The 14-3-3 proteins comprise a highly conserved group of proteins widely expressed in human tissue [23] and have been associated with progression in several cancer types [24-26]. 14-3-3 proteins serve as scaffolds that

integrate signaling proteins with targets involved in important biological processes, including cell cycle regulation [27].

14-3-3ζ has been implicated in breast cancer recurrence in three studies using microarray analysis of gene expression profiles [16-18]. Two of these studies identified the gene for 14-3-3ζ among a set of genes involved in cell cycle regulation [17, 18]. Two protein profiling studies have identified 14-3-3ζ as differentially expressed, one comparing normal breast tissue and cancerous breast tissue from the same patient [19], and another comparing chemotherapy-sensitive and chemotherapy-resistant patients [20]. Studies using immunohistochemistry (IHC) have demonstrated an increased risk of recurrence for those overexpressing 14-3-3ζ [24, 25]. In a 2009 study by Neal et al. of 121 women with invasive breast cancer, 42% of patients overexpressed 14-3-3ζ and level of expression was related to disease free survival [24]. A 2013 study by Bergamaschi et al. of 139 women found 14-3-3ζ to be an independent predictor of time to recurrence [25]. This study also found different time course patterns for recurrence based on both 14-3-3ζ expression and ERα status.

Despite these findings, these studies are limited by a small sample size and insufficient control of confounding, with confounders, at most, restricted to tumor characteristics. Only two of these studies have directly examined the effect of 14-3-3ζ on resistance to tamoxifen. To address the limitations of earlier research and to obtain a precise estimate of its effect on tamoxifen sensitivity in a large, well-defined cohort, we aim to measure 14-3-3ζ expression and estimate its association with breast cancer recurrence in a population-based registry of breast cancer patients in Denmark with sufficient control for demographics, treatment information, and tumor characteristics.

This study will differentiate the effects of expression in subcellular locations (cytoplasm vs. nuclear) and address possible sources of bias due to 14-3-3ζ assay methods.

Materials and Methods

Study Population

The Danish Breast Cancer Cooperative Group (DBCG) maintains a population-based registry that has collected data on nearly all breast cancer patients in Denmark since 1977. The DBCG registry was used to collect information on 11,251 females of the Jutland Peninsula aged 35–69 diagnosed with stage I, II, or III breast cancer as defined by the Union for International Cancer Control (UICC) between 1985 and 2001 [28]. All registered patients follow the same 10-year follow-up protocol [29]. Follow-up time began 1 year from the diagnosis date and continued until the date of the first recurrence, death from any cause, loss to follow-up, 10 years after follow-up, or September 1st, 2006. Datasets were linked using Danish Civil Personal Registration (CPR) numbers, a unique identifier assigned to each resident of Denmark.

The source population was divided into two groups defined by combined ERα and tamoxifen treatment (ER/TAM) status: 1) patients whose tumor expressed ERα and were treated with tamoxifen for at least one year (ER+/TAM+) and 2) patients whose tumor did not express ERα, were not treated with tamoxifen, and who survived at least one year (ER-/TAM-). All registry patients not in these two groups were excluded from analysis (Figure 1). Patients were also excluded if their level of 14-3-3ζ staining could not be accurately determined using tissue microarray (TMA) IHC. Tumor cores were determined to be unsatisfactory if insufficient readable material remained after specimen processing, staining, and digital imaging, if staining and imaging artifacts precluded

scoring of tumor cells, if minimal invasive cancer cells were seen in the core (<20 cells), if tumor cells were an *in situ* carcinoma, or if the core contained predominantly benign epithelial cells.

Cases were patients with local or distant breast cancer recurrence or contralateral breast cancer occurrence during their follow-up time. For each case patient, one control was selected without replacement from the source population who were alive and had no recurrence or contralateral breast cancer after the same amount of follow-up time. Controls were matched to cases based on group membership (ER+/TAM+ or ER-/TAM-), menopausal status at diagnosis (premenopausal or postmenopausal), date of breast cancer surgery (caliper matched ± 12 months), county of residence at the time of diagnosis, and cancer stage at diagnosis (UICC stage I, II, or II).

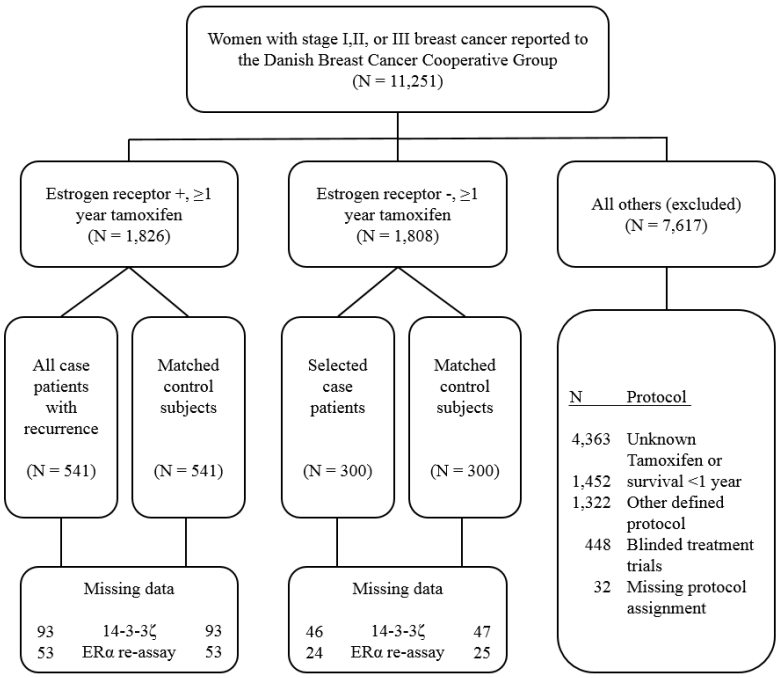

Figure 1. Design used to select the study sample and determine grouping based on the inclusion criteria. The source population consisted of 11,251 female residents of the Jutland Peninsula in Denmark aged 35–69 years who were diagnosed with stage I, II, or III breast cancer between 1985 and 2001. Most of the women (n = 4363) excluded because of unknown protocol had stage I breast cancer treated without a guideline protocol from the Danish Breast Cancer Cooperative Group. 14-3-3ζ results were missing if tissue was not available or if tumor core was unsatisfactory after processing, staining and imaging. ERα re-assay results were missing if tissue was not available or if assay results were indeterminate. ERα = estrogen receptor α.

Data Collection from Danish Registries

The DBCG registry was used to collect information on demographic characteristics (age, menopausal status, and hospital of diagnosis), tumor information (UICC stage at diagnosis, histologic grade and ERα status), local therapy (surgical management and radiation therapy), and systemic therapy (receipt of chemotherapy and tamoxifen).

Data Collection from Archived Tissue Sample

Laboratory personnel were blinded to all clinical information, including case or control status, ERα status, and receipt of tamoxifen therapy.

Immunohistochemistry

Tissue sections were processed from formalin-fixed paraffin-embedded blocks. TMA IHC was used for high-throughput *in situ* 14-3-3ζ expression and subcellular localization determination. All techniques used sterile laboratory protocols to avoid cross-contamination. 14-3-3ζ expression was measured using the anti-human 14-3-3ζ monoclonal mouse antibody (MAB2669) from R&D systems. Cylindrical samples were taken from each patient tumor and re-embedded into a paraffin block. Each patient tumor donated three representative tumor cores and one marginal tissue core, if possible. TMAs of 1 mm core diameter were constructed using the TMA Master from 3DHistech. Liver and placental cores were added to each microarray to serve as a positive and negative control, respectively. TMAs were scanned at 40x magnification with the Hamamatsu Nanozoomer 2.0HT in .ndpi format. Slides were converted to conform to the 3DHistech software and uploaded to Panoramic Viewer TMA Module software.

TMA Core Scoring

A pathologist of the Aarhus University Institute of Pathology trained a masters level epidemiology student (JT) to identify structures commonly seen in breast tissue, differentiate invasive epithelial cells from *in situ* epithelial cells and benign epithelial cells, and score immunohistochemical staining in breast tumor tissue. Level of 14-3-3ζ staining was determined using the zoom feature in Panoramic Viewer to visualize and score invasive epithelial cells and to differentiate subcellular location of staining (Figure 2).

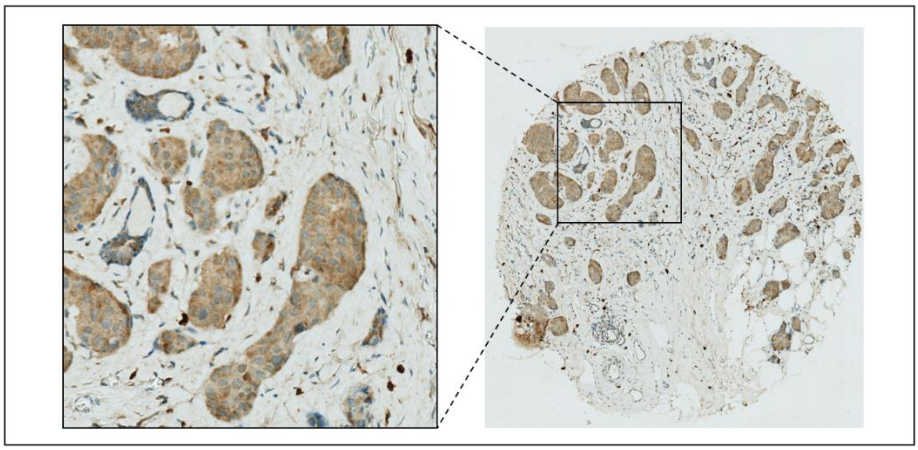

Figure 2. Determination of 14-3-3ζ expression level by scanning at 40x magnification in Panoramic Viewer to evaluate invasive epithelial cells and differentiate subcellular location of staining.

A preliminary scoring system was developed by reading 600 cores by both scorers. Cores were flagged if a scorer could not determine an accurate score and discuss**e**d later with the other scorer until a consensus was reached.

Scoring was divided into a cytoplasmic and a nuclear component as staining intensity in the two cell structures differed (Figure 3). Cytoplasmic scoring used a semi-quantitative system that combines staining intensity with its relative proportion known as an "H-score". To calculate H-scores, cytoplasmic staining intensity was divided into four categories: 0 (no staining), 1 (light staining), 2 (moderate staining), 3 (heavy staining).

The staining intensity was then multiplied by the proportion of cancer cells that exhibit that staining intensity in a given core. For example, the calculation for a core with 5% no staining, 50% light staining, 35% moderate staining, and 10% heavy staining would be (0x5) + (1x50) + (2x35) + (3x10) yielding an H-score of 150. H-scores could range from 0, for 100% no staining, to 300, for 100% heavy staining. Between 1 and 4 cores were processed per patient and the final cytoplasmic score was an average H-score of the satisfactory cores for a given patient.

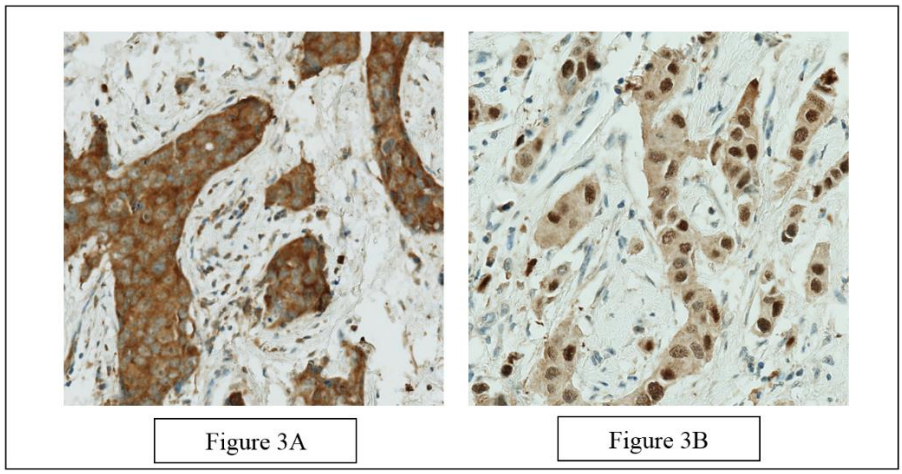

Figure 3. Difference in cytoplasmic and nuclear 14-3-3ζ staining intensity. Figure 3A shows typical staining concentrated to the cytoplasm. This core was scored as 45% moderate staining and 55% heavy staining for the cytoplasm and light staining in the nucleus. Figure 3B shows typical staining concentrated to the nucleus. This core was scored as 70% light staining and 30% moderate staining in the cytoplasm and heavy staining in the nucleus.

Nuclear staining was scored on an ordinal scale for the entire core in a simplified metric reflective of staining intensity and proportion: 0 (no staining), 1 (light staining), 2 (moderate staining), 3 (heavy staining). The final nuclear score was the average ordinal score of the satisfactory cores for a given patient. All cores were evaluated by the epidemiology student and cytoplasmic and nuclear scores were determined for 3,904 of 5,280 stained cores. The remaining cores were determined to be unsatisfactory and excluded from the analysis. To assess the reliability of the cytoplasmic and nuclear 14-3-

3ζ staining level determination, scores were determined independently by the pathologist. Final cytoplasmic 14-3-3ζ scores were determined for 476 patients and final nuclear 14-3-3ζ scores were determined for 475 patients, by the pathologist, and compared to the results obtained by the epidemiology student. Agreement was generally good for both cytoplasmic and nuclear scores (Table 1).

Table 1. Inter-rater agreement of above the 50th percentile of cytoplasmic and nuclear 14-3-3ζ expression*

		Rater 1			
		Cytoplasmic 14-3-3ζ expression		Nuclear 14-3-3ζ expression	
	Quartile	50th percentile or lower	Above the 50th percentile	50th percentile or lower	Above the 50th percentile
Rater 2	50th percentile or lower	184	32	221	60
	Above the 50th percentile	55	205	37	157

*Scores were determined independently between the epidemiology student (Rater 1) and the pathologist (Rater 2) and compared. Cytoplasmic 14-3-3ζ expression level was determined for 476 patients and nuclear 14-3-3ζ expression level was determined for 475 patients.

Validation Substudy

Because TMA IHC may produce different results compared to whole section (WS) IHC, five whole tumor sections from randomly selected patients were scored for both cytoplasmic and nuclear 14-3-3ζ staining to estimate sensitivity and specificity parameters for TMA IHC against the WS IHC gold standard. The area of the stained tumor section with invasive epithelial cells were overlaid with a grid of 1 mm "virtual" TMAs meant to simulate cores that would have been selected had TMA IHC been performed (Figure 4). On each whole section, virtual TMAs were numbered and bisected into two groups. A single core was randomly sampled from each of the two groups and scored as previously described for TMA IHC, then averaged. Sampling and scoring was

replicated six times per whole section. An H-score and nuclear score was also determined for the whole tissue section itself as the gold-standard measure.

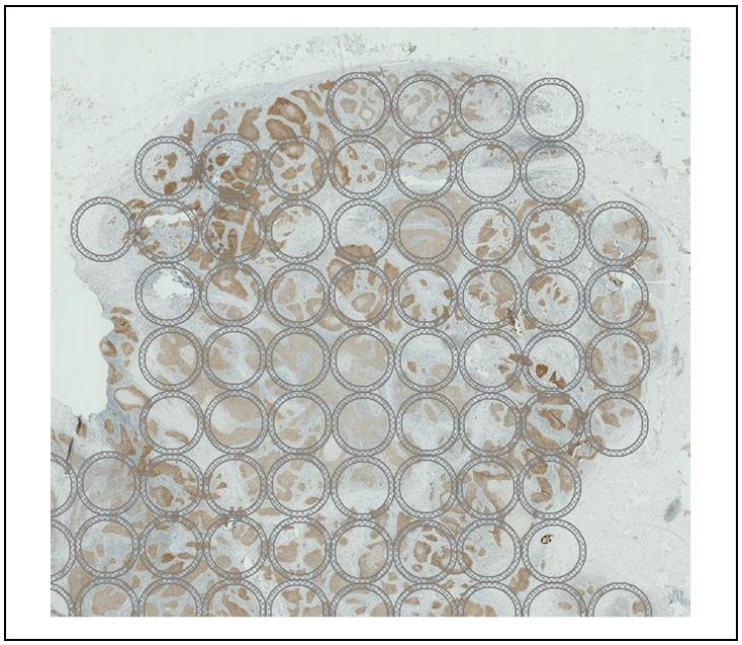

Figure 4. Grid of 1 mm virtual TMAs for validation substudy. Five whole tumor sections from randomly selected patients were stained and overlaid with a grid meant to simulate cores that would have been selected had TMA IHC been performed. Virtual TMAs were randomly selected and scored to estimate sensitivity and specificity parameters.

Statistical Analysis

Analytic Variables

14-3-3ζ Cytoplasmic Staining

Cytoplasmic staining was categorized in two ways: 1) as a dichotomous variable of low staining vs. high staining where high staining is a final cytoplasmic score above the 50th percentile and low staining is a final cytoplasmic score at or below the 50th percentile, and 2) as a four category variable of quartiles to assess a dose-response where the first category is a final score above the 75th percentile, the second quartile is a final score above the 50th percentile up to and including the 75th percentile, the third quartile is a final score above the 25th percentile up to and including the 50th percentile, and the fourth

quartile is a final score at or below the 25th percentile. The quartile score was analyzed with and without the assumption of a proportional change for differences between quartiles.

14-3-3ζ Nuclear Staining

Nuclear staining was categorized in two ways: 1) as a dichotomous variable of low staining vs. high staining where high staining is a final nuclear score above the 50th percentile and low staining is a final nuclear score at or below the 50th percentile, and 2) as a four category variable of quartiles to assess a dose-response where the first category is a final score above the 75th percentile, the second quartile is a final score above the 50th percentile up to and including the 75th percentile, the third quartile is a final score above the 25th percentile up to and including the 50th percentile, and the fourth quartile is a final score at or below the 25th percentile. The quartile score was analyzed with and without the assumption of a proportional change for differences between quartiles.

Combined 14-3-3ζ Staining

Patients above the 50th percentile for both nuclear and cytoplasmic 14-3-3ζ staining were classified as having combined 14-3-3ζ staining compared to patients at or below the 50th percentile for cytoplasmic, nuclear, or both. To assess a dose-response, patients were classified into four categories of combined 14-3-3ζ staining where the first category is patients above the 75th percentile for cytoplasmic and nuclear staining, the second category is values above the 50th percentile for both cytoplasmic and nuclear staining but not above the 75th percentile for both, the third category is values above the 25th percentile for cytoplasmic and nuclear staining but not above the 50th percentile for both, and the fourth category is any patient with staining at or below the 25th percentile for

cytoplasmic or nuclear staining. The quartile score was analyzed with and without the assumption of a proportional change for differences between quartiles.

Recurrence

Information on recurrences was collected from the DBCG. The DBCG defines a recurrence as any breast cancer or distant metastases diagnosed after the initial course of therapy. In our study population, cases were defined as a recurrence that occurred within 1 to 10 years after the initial diagnosis.

Covariates

Covariates were UICC stage, grade, menopausal status at diagnosis, receipt of chemotherapy, receipt of radiotherapy, surgery type (mastectomy vs. breast-conserving surgery), diagnosis year, age at diagnosis, and county.

Conventional Analysis

All analyses were performed stratified by ER/TAM grouping to evaluate whether the association between 14-3-3ζ and breast cancer recurrence is predictive of tamoxifen resistance, prognostic of breast cancer recurrence, or neither [30]. The frequency and proportion of cases and controls were calculated within categories of 14-3-3ζ cytoplasmic, nuclear, and combined expression and within all categories of covariates. Conditional logistic regression was used to estimate the measure of association between breast cancer recurrence, the outcome, and 14-3-3ζ staining level, conditioned on the matched factors. Measures of association were also estimated using logistic regression with adjustment for UICC stage, grade, menopausal status, receipt of chemotherapy, receipt of radiotherapy, surgery type (mastectomy vs. breast-conserving surgery),

diagnosis year, age at diagnosis, and county. All analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC).

Categorization Method

Upon scoring of cores, it was observed that the sections used for TMAs showed marked variation in staining intensity (Table 2). To address concerns of non-differential misclassification of 14-3-3ζ staining level due to variable staining intensity, patients were categorized based on their percentile in the entire cohort and their percentile in the section their tumor cores were stained (Table 3). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for each method of categorization across ER/TAM grouping and compared to evaluate potential bias due to variable staining intensity.

Table 2. Variation in cytoplasmic staining intensity by section used for tissue microarray immunohistochemistry*

Section Number	Median H-score	IQR	Section Number	Median H-score	IQR	Section Number	Median H-score	IQR
1	95	(65, 112)	13	130	(110, 157)	25	107	(100, 128)
2	113	(104, 135)	14	149	(133, 162)	26	102	(78, 110)
3	118	(107, 138)	15	118	(98, 128)	27	118	(112, 128)
4	121	(113, 140)	16	175	(145, 203)	28	113	(103, 122)
5	125	(115, 150)	17	127	(98, 160)	29	77	(45, 100)
6	121	(113, 140)	18	107	(93, 125)	30	123	(105, 138)
7	116	(111, 139)	19	115	(102, 133)	31	118	(103, 122)
8	125	(113, 140)	20	110	(88, 148)	32	128	(112, 150)
9	120	(110, 124)	21	128	(113, 147)	33	95	(52, 108)
10	115	(100, 127)	22	108	(99, 147)	34	120	(110, 125)
11	124	(100, 155)	23	51	(32, 68)	35	100	(75, 108)
12	113	(107, 140)	24	115	(105, 137)			

*35 sections were used in tissue microarray immunohistochemistry to perform the staining of the tumor cores. Each section had roughly 150 cores with each patient donating three representative tumor cores and one marginal tissue core, if possible. The final cytoplasmic score was the average H-score of the satisfactory cores for a given patient. IQR = inter-quartile range.

Table 3. Comparison of cytoplasmic 14-3-3ζ percentile categorization between the percentile in the entire cohort and the percentile in the section their tumor cores were stained*

Cytoplasmic 14-3-3ζ percentile in the entire cohort (Quartile)	Cytoplasmic 14-3-3ζ percentile in the section the tumor cores were stained: 25th percentile or lower	Above the 25th percentile up to the 50th percentile	Above the 50th percentile up to the 75th percentile	Above the 75th percentile
25th percentile or lower	272	83	21	4
Above the 25th percentile up to the 50th percentile	92	161	51	16
Above the 50th percentile up to the 75th percentile	10	69	177	71
Above the 75th percentile	9	17	86	261

*Percentiles were determined using the final cytoplasmic score for each patient. The final cytoplasmic score was the average H-score of the satisfactory cores for a given patients.

Validation Data and quantitative bias analysis

Validation of 14-3-3ζ Staining Determination

Although TMA IHC allows high-throughput analysis of a large number of samples, it is limited due to intratumoral heterogeneity of gene expression in breast tissue [31] . Several studies have demonstrated a discordance between the results of TMA IHC compared to WS IHC in breast tumor tissue although they still consider TMA IHC an adequate method for expression determination [31-33]. To address potential bias due to using TMA IHC for expression determination, a quantitative bias analysis was performed [34]. Using the results of virtual TMA scoring compared to whole sections they were derived from in the validation substudy, sensitivity and specificity values were estimated for cytoplasmic 14-3-3ζ staining level (sensitivity = 16/18 (0.89), specificity = 12/12 (1.0)), nuclear 14-3-3ζ staining level (sensitivity = 9/12 (0.75), specificity = 17/18 (0.94)), and combined 14-3-3ζ staining level (sensitivity = 4/6 (0.67), specificity = 23/24 (0.96)). Trapezoidal distributions were assigned to sensitivities and specificities based on estimated values for cytoplasmic, nuclear, and combined 14-3-3ζ staining level for use in

a probabilistic bias analysis. The trapezoidal distributions were applied to summary-level data stratified by ER/TAM categorization using the macro published by Fox, Lash, and Greenland to obtain an odds ratio adjusted for potential confounding and misclassification due to using TMA IHC, as well as a 95% simulation interval (SI) incorporating both random and systematic error [35, 36].

Estrogen Receptor Re-assay

As methods for ERα status determination have improved, the DBCG recommendations for assays evolved during the course of the study [37]. To account for improvements in ERα determination and potential variability across diagnosing hospitals, ERα status was centrally re-assayed from the original tumor of all patients. Whole sections were sampled from the diagnostic paraffin-embedded tissues and a primary antibody against ERα (clone 6F11; Novocastra, Newcastle Upon Tyne, UK) was used to measure expression. Heat-induced epitope retrieval for ERα was achieved by incubation in a Tris–EDTA buffer, pH 9 (Target Retrieval Solution, pH 9; Dako, Glostrup, Denmark) using a microwave oven. Sections were enhanced using copper sulphate and visualized with horseradish peroxidase and diaminobenzadine. ERα positivity was set at a cutoff point of at least 10% of positive tumor nuclei in accordance with previous DBCG recommendations for the diagnostic period of patients included in the current study [37].

Inverse-Variance Weighting Approximate Bayesian Analysis

To further differentiate 14-3-3ζ staining as predictive of tamoxifen resistance or prognostic of breast cancer recurrence, inverse-variance weighting approximate Bayesian analysis was performed [38]. Logistic regression modeling for the interaction of ERα and staining of 14-3-3ζ staining was used to obtain the regression coefficient ($\hat{\beta}$) and the

standard error ($se_{\hat{\beta}}$) for the interaction term, controlling for covariates. Prior distributions were plotted as normal with a mean (β_i = ln(OR_i) and variance ($\sigma_i{}^2$) corresponding to a marker of tamoxifen resistance (β_1 = ln(2.0)), the predictive marker, and a marker of breast cancer recurrence (β_2 = ln(1.0)), the prognostic marker (Figure 5). The variance was determined by examining prior plots that provided sufficient separation ($\sigma_1{}^2 = \sigma_2{}^2 = 0.15^2$) of the prior hypotheses. The posterior mean ($\beta_i{}'$) and the posterior variance ($\sigma_i{}^{2\prime}$) were estimated based on inverse-variance weighting equations used for Bayesian approximation [38].

Posterior mean for ln(OR); $$\beta_i{}' \approx \frac{(\beta_i / \sigma_i^2) + (\hat{\beta} / se_{\hat{\beta}}^2)}{(1.0/\sigma_i^2) + (1.0/se_{\hat{\beta}}^2)}$$

Posterior variance for ln(OR); $$\sigma_i^{2}{}' \approx \frac{1}{(1.0/\sigma_i^2) + (1.0/se_{\hat{\beta}}^2)}$$

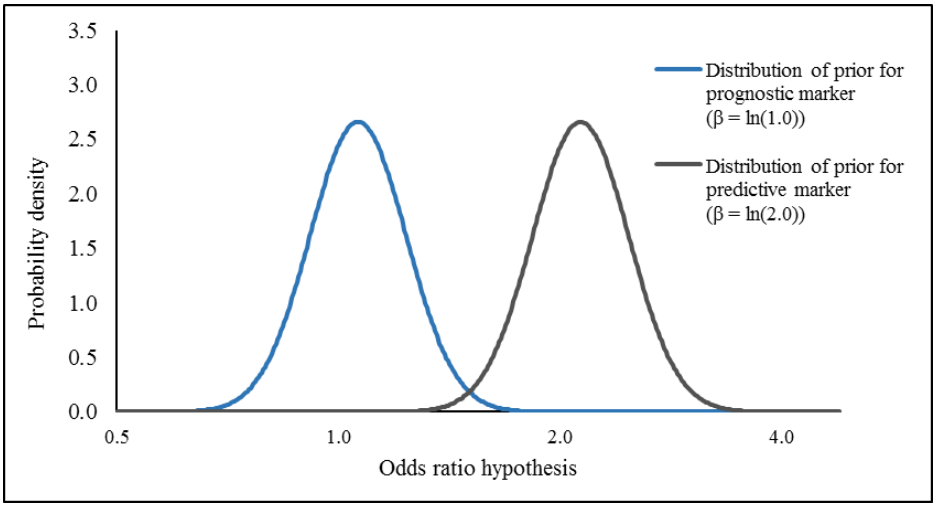

Figure 5. Plots for normal prior distributions used to compare two hypotheses corresponding to a predictive marker (β_1 = ln(2.0)) and a prognostic marker (β_2 = ln(1.0)). The mean values corresponded to the natural log of the interaction of ERα and combined 14-3-3ζ. The variance was determined by examining plots that provided sufficient separation between the two hypotheses ($\sigma_1{}^2 = \sigma_2{}^2 = 0.15^2$).

In order to show that approximate Bayesian analysis can discriminate 14-3-3ζ staining as predictive of tamoxifen resistance or prognostic of breast cancer recurrence, posterior

distributions were plotted as normal with the calculated posterior mean and variance corresponding to the predictive and prognostic marker. Expected posterior distributions were plotted (Figure 6) corresponding to precisely measured ($se_{\hat{\beta}i}$ = 0.05) regression coefficients for a predictive marker ($\hat{\beta}_1$ = ln(2.0)) and a prognostic marker ($\hat{\beta}_2$ = ln(1.0)).

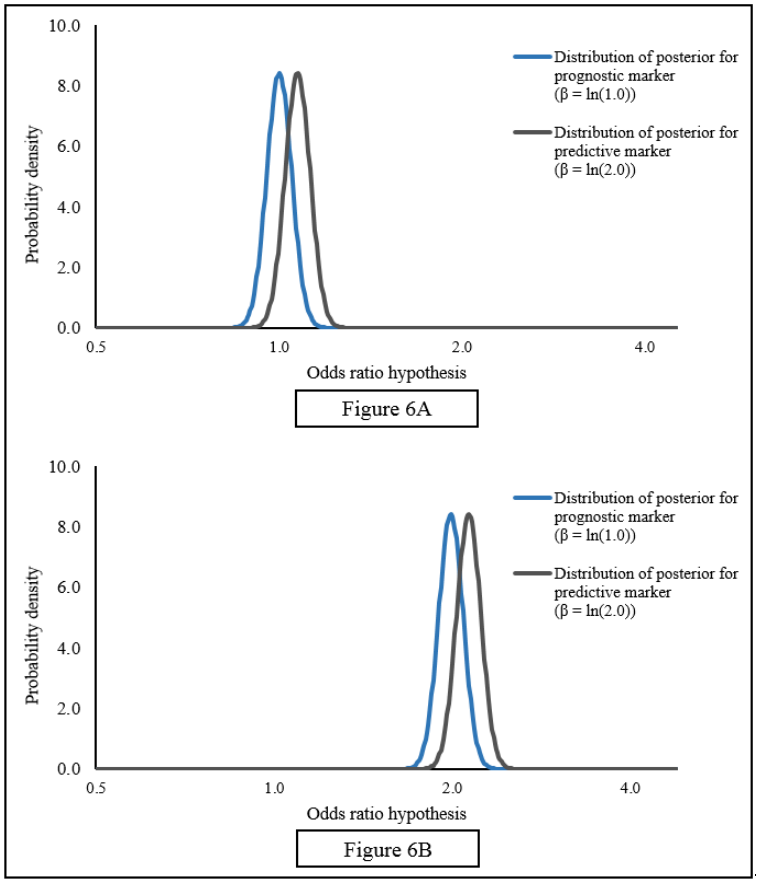

Figure 6. Expected plots for normal posterior distributions by inverse-variance weighting used to compare two prior hypotheses corresponding to a predictive marker (β_1 = ln(2.0)) and a prognostic marker (β_2 = ln(1.0)). The posterior distributions were plotted corresponding to precisely measured ($se_{\hat{\beta}i}$ = 0.05) regression coefficients. Figure 6A is the expected normal posterior distribution for a precisely measured prognostic marker ($\hat{\beta}_2$= ln(1.0)). Figure 6B is the expected normal posterior distribution for a precisely measured predictive marker ($\hat{\beta}_1$ = ln(2.0)).

Results

Descriptive Statistics

The majority of patients in the study population were Stage II (48%) or Stage III (48%) at time of diagnosis per UICC standards (Table 4) as a consequence of DBCG criteria for

tamoxifen therapy during the period of diagnosis of the population [29]. Among ER+/TAM+ patients, the majority were aged 55–65 years (52%) whereas the most common age in ER-/TAM- patients was 45–54 years (39%). 85% of the study population underwent mastectomy and 39% had radiation therapy. 12% of the ER+/TAM+ patients had chemotherapy whereas 63% of the ER-/TAM- patients received chemotherapy, consistent with indications for breast cancer treatment in these groups. Our study population consisted of diagnoses from 1985 to 2001; 40% occurred between 1985 and 1993, 23% occurred between 1994 and 1996, and 37% occurred between 1997 and 2001. Among ER+/TAM+ patients, 47% were assigned a tamoxifen protocol of 1 year, 18% had a protocol of 2 years, and 35% had a protocol of 5 years or more. Medical records often indicate that patients on 1 or 2 year tamoxifen therapies had longer durations of therapy as evidence favoring a 5 year protocol grew [39].

Categorization Method

In the ER+/TAM+ group, above the 50^{th} percentile of cytoplasmic 14-3-3ζ staining in the entire cohort yielded an unadjusted odds ratio of 1.12 (95% CI = 0.94, 1.59; Table 5). When the percentile was determined in the section tumor cores were stained, the unadjusted odds ratio moved up and away from the null to 1.22 (95% CI = 0.94, 1.59). In the ER-/TAM- group, the two methods yielded comparable null measures (OR = 1.06, 95% CI = 0.74, 1.52; OR = 1.06, 95% CI = 0.74, 1.51). As expected, variable staining intensity on different TMA sections likely resulted in non-differential misclassification of cytoplasmic 14-3-3ζ staining level with the expectation of bias toward the null being realized. To account for variable staining intensity, all subsequent results use percentile in the section the tumor cores were stained to determine 14-3-3ζ staining level.

Table 4. Frequency and proportion of breast cancer recurrence case patients within ER/TAM group*

	ER+/TAM+, No. (%)		ER-/TAM-, No. (%)	
Patient characteristic	Case patients	Control subjects	Case patients	Control subjects
Cytoplasmic 14-3-3ζ expression				
50th percentile or lower	246 (55)	266 (60)	99 (39)	102 (40)
Above the 50th percentile	202 (45)	179 (40)	155 (61)	151 (60)
Missing†	93	96	46	47
Nuclear 14-3-3ζ expression				
50th percentile or lower	273 (61)	294 (66)	117 (46)	130 (52)
Above the 50th percentile	175 (39)	151 (34)	140 (54)	120 (48)
Missing†	93	96	46	47
Combined 14-3-3ζ expression				
50th percentile or lower	327 (73)	353 (79)	156 (61)	161 (64)
Above the 50th percentile	121 (27)	92 (21)	101 (39)	89 (36)
Missing†	93	96	46	47
Diagnosis year				
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)
Age category at diagnosis, y				
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 - 69	123 (23)	136 (25)	30 (10)	43 (14)
Menopausal Status at diagnosis§				
Premenopausal	34 (6.3)	34 (6.3)	121 (40)	121 (40)
Postmenopausal	507 (94)	507 (94)	179 (60)	179 (60)
UICC tumor stage at diagnosis§				
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				
I	108 (25)	144 (35)	27 (11)	23 (10)
II	234 (54)	215 (52)	125 (49)	98 (43)
III	92 (21)	57 (14)	103 (40)	106 (47)
Missing	107	125	45	73
Surgery type				
Breast-conserving surgery	58 (11)	71 (13)	47 (16)	56 (19)
Mastectomy	483 (89)	470 (87)	252 (84)	244 (81)
Missing	0	0	1	0
Radiation therapy				
Yes	183 (34)	191 (35)	128 (47)	123 (47)
No	358 (66)	350 (65)	166 (56)	137 (53)
Missing	0	0	6	40
Tamoxifen protocol, y				
1	257 (48)	261 (48)		
2	98 (18)	92 (17)		
5	186 (34)	188 (34)		
Systemic adjuvant chemotherapy				
Yes	70 (13)	65 (12)	248 (83)	188 (63)
No	471 (87)	476 (88)	52 (17)	112 (37)
Current ER expression				
Positive	451 (92)	474 (96)	72 (26)	70 (25)
Negative	37 (7.6)	19 (3.9)	204 (74)	205 (75)
Not available	53	48	24	25

*The source population consisted of 11,251 female residents of the Jutland Peninsula in Denmark aged 35–69 years who were diagnosed with stage I, II, or III breast cancer between 1985 and 2001. Subjects were estrogen receptor α (ERα) positive and received at least 1 year of tamoxifen therapy (ER+/TAM+) or ERα negative and never received tamoxifen therapy and survived at least 1 year after diagnosis (ER-/TAM-). UICC = Union for International Cancer Control.

†No tissue available or tissue material unsatisfactory after processing, staining, and imaging.

§Variable included in risk set sampling to match control subjects to case patients.

Table 5. Associations between 14-3-3ζ cytoplasmic expression and breast cancer recurrence within ER/TAM group using two methods to determine percentiles*

14-3-3ζ cytoplasmic expression categorization method	ER+/TAM+ Case patients	ER+/TAM+ Control subjects	ER+/TAM+ OR (95% CI)	ER-/TAM- Case patients	ER-/TAM- Control subjects	ER-/TAM- OR (95% CI)
Percentile among cohort†						
50th percentile or lower	248	259		96	97	
Above the 50th percentile	200	186	1.12 (0.86, 1.46)	161	153	1.06 (0.74, 1.52)
Percentile among section‡						
50th percentile or lower	246	266		99	102	
Above the 50th percentile	202	179	1.22 (0.94, 1.59)	155	151	1.06 (0.74, 1.51)

*The source population consisted of 11,251 female residents of the Jutland Peninsula in Denmark aged 35–69 years who were diagnosed with stage I, II, or III breast cancer between 1985 and 2001. Subjects were estrogen receptor α (ERα) positive and received at least 1 year of tamoxifen therapy (ER+/TAM+) or ERα negative and never received tamoxifen therapy and survived at least 1 year after diagnosis (ER-/TAM-). UICC = Union for International Cancer Control.
†Patients were categorized based on the 14-3-3ζ cytoplasmic expression percentile in the entire cohort.
‡Patients were categorized based on the 14-3-3ζ cytoplasmic expression percentile in the slide their tumor cores were stained on.

Using percentiles in the section tumor cores were stained, above the 50th percentile cytoplasmic 14-3-3ζ staining was observed in 43% of ER+/TAM+ patients and in 60% of ER-/TAM- patients, and above the 50th percentile nuclear 14-3-3ζ staining was observed in 37% of ER+/TAM+ patients and in 51% of ER-/TAM- patients. Fewer patients were above the 50th percentile in nuclear staining due to the frequency of patients at the 50th percentile cut-off, which is more common in the simplified nuclear categorical scoring system. Above the 50th percentile combined 14-3-3ζ staining was observed in 24% of ER+/TAM+ patients and in 37% of ER-/TAM- patients.

Conventional Results

In ER+/TAM+ patients, the associations of above the 50th percentile cytoplasmic 14-3-3ζ staining were near null (matched OR = 1.27, 95% CI = 0.95, 1.70; adjusted OR = 1.22, 95% CI = 0.94, 1.60; Table 6). Near null associations were also seen in ER-/TAM- patients (matched OR = 0.95, 95% CI = 0.65, 1.37; adjusted OR = 1.09, 95% CI = 0.74,

1.62). Although the measures of the matched analysis were compatible with the expected results of tamoxifen prediction for cytoplasmic staining (association in ER+/TAM+ patients, no association in ER-/TAM- patients), the 95% confidence intervals across ER/TAM grouping widely overlap and the effect is near null in ER+/TAM+ patients. The associations of above the 50th percentile nuclear 14-3-3ζ staining were comparable in ER+/TAM+ patients (matched OR = 1.21, 95% CI = 0.89, 1.63; adjusted OR = 1.21, 95% CI = 0.92, 1.60) but were larger in ER-/TAM- patients (matched OR = 1.38, 95% CI = 0.95, 2.01; adjusted OR = 1.33, 95% CI = 0.90, 1.96) compared to cytoplasmic staining. Patients with combined 14-3-3ζ staining showed associations in the ER+/TAM+ group (matched OR = 1.51, 95% CI = 1.08, 2.11; adjusted OR = 1.44, 95% CI = 1.05, 1.99) and nearer to null associations in the ER-/TAM- group (matched OR = 1.22, 95% CI = 0.82, 1.80; adjusted OR = 1.22, 95% CI = 0.82, 1.82).

Table 6. Associations between 14-3-3ζ expression and breast cancer recurrence within ER/TAM group*

	ER+/TAM+				ER-/TAM-			
14-3-3ζ expression	Case patients	Control subjects	Matched OR (95% CI)ǂ	Adjusted OR (95% CI)ǂ	Case patients	Control subjects	Matched OR (95% CI)ǂ	Adjusted OR (95% CI)ǂ
Cytoplasmic 14-3-3ζ expression								
50th percentile or lower	246	266			99	102		
Above the 50th percentile	202	179	1.27 (0.95, 1.70)	1.22 (0.94, 1.60)	155	151	0.95 (0.65, 1.37)	1.09 (0.74, 1.62)
Nuclear 14-3-3ζ expression								
50th percentile or lower	273	294			117	130		
Above the 50th percentile	175	151	1.21 (0.89, 1.63)	1.24 (0.95, 1.64)	140	120	1.38 (0.95, 2.01)	1.31 (0.89, 1.92)
Combined 14-3-3ζ expression								
50th percentile or lower	327	353			156	161		
Above the 50th percentile	121	92	1.51 (1.08, 2.11)	1.44 (1.05, 1.98)	101	89	1.22 (0.82, 1.80)	1.22 (0.82, 1.82)

*The source population consisted of 11,251 female residents of the Jutland Peninsula in Denmark aged 35–69 years who were diagnosed with stage I, II, or III breast cancer between 1985 and 2001. Subjects were estrogen receptor α (ERα) positive and received at least 1 year of tamoxifen therapy (ER+/TAM+) or ERα negative and never received tamoxifen therapy and survived at least 1 year after diagnosis (ER-/TAM-). OR = odds ratio; CI = confidence interval; UICC = Union for International Cancer Control.
ǂEstimated using conditional logistic regression with conditioning on the matched factors (time to recurrence or control selection, county, menopausal status, and stage).
ǂEstimated using logistic regression with adjustment for UICC stage, menopausal status, receipt of chemotherapy, receipt of radiotherapy, surgery type, diagnosis year, age at diagnosis, and county.

In dose-response analysis, cytoplasmic, nuclear, and combined 14-3-3ζ staining generally showed near null associations for above the 25th percentile up to the 50th percentile and above the 50th percentile up to the 75th percentile, but an increased odds of recurrence risk for staining above the 75th percentile, when analyzed without an assumption of proportional change (Table 7). A moderate association was observed in above than 75th percentile staining of combined 14-3-3ζ in both ER+/TAM+ patients (matched OR = 1.77, 95% CI = 1.01, 3.11; adjusted OR = 1.93, 95% CI = 1.15, 3.24) and ER-/TAM- patients (matched OR = 1.64, 95% CI = 0.92, 2.94; adjusted OR = 1.93, 95% CI = 1.03, 3.62). When analyzed assuming proportionality, associations for a one quartile increase in staining level were generally near null for cytoplasmic, nuclear and combined 14-3-3ζ staining. All estimated measures of association had substantially overlapping confidence intervals between ER+/TAM+ patients and ER-/TAM- patients in dose-response analysis. All results were similar with ER/TAM grouping based on the results of the ERα re-assay.

Bias-Adjusted Results

Bias-adjusted estimates of above the 50th percentile staining, accounting for misclassification, by TMA IHC instead of WS IHC, and confounding, were slightly further from the null compared to estimates only adjusted for confounding (Table 8). The uncertainty in bias-adjusted results was greater than those conveyed in the conventional 95% CI, with percent increases in the width of the simulation interval ranging from 6.7% to 30%. The associations changed marginally for cytoplasmic 14-3-3ζ staining in both ER+/TAM+ patients (bias-adjusted OR = 1.26, 95% SI = 0.94, 1.71) and ER-/TAM- patients (bias-adjusted OR = 1.09, 95% SI = 0.69, 1.71). Associations were larger for

Table 7. Analysis of the dose-response between 14-3-3ζ expression and breast cancer recurrence within ER/TAM group*

	ER+/TAM+				ER-/TAM-			
14-3-3ζ expression	Case patients	Control subjects	Matched OR (95% CI)ǂ	Adjusted OR (95% CI)ǂ	Case patients	Control subjects	Matched OR (95% CI)ǂ	Adjusted OR (95% CI)ǂ
Cytoplasmic 14-3-3ζ expression								
25th percentile or lower	136	142			54	51		
Above the 25th percentile up to the 50th percentile	110	124	0.80 (0.54, 1.20)	0.95 (0.67, 1.35)	48	48	1.03 (0.55, 1.94)	0.98 (0.54, 1.79)
Above the 50th percentile up to the 75th percentile	104	107	0.99 (0.67, 1.47)	1.04 (0.72, 1.49)	57	67	0.93 (0.53, 1.62)	0.88 (0.49, 1.56)
Above the 75th percentile	98	72	1.43 (0.93, 2.21)	1.43 (0.97, 2.11)	98	84	0.98 (0.59, 1.65)	1.26 (0.74, 1.56)
Increase by one quartile¥			1.11 (0.97, 1.27)	1.11 (0.98, 1.25)			0.99 (0.84, 1.16)	1.08 (0.91, 1.27)
Nuclear 14-3-3ζ expression								
25th percentile or lower	169	172			77	83		
Above the 25th percentile up to the 50th percentile	104	122	0.64 (0.43, 0.95)	0.87 (0.62, 1.23)	40	47	0.99 (0.54, 1.82)	0.80 (0.45, 1.42)
Above the 50th percentile up to the 75th percentile	74	84	0.79 (0.52, 1.21)	0.88 (0.60, 1.29)	60	64	1.10 (0.66, 1.83)	0.94 (0.56, 1.56)
Above the 75th percentile	101	67	1.29 (0.85, 1.94)	1.57 (1.07, 2.30)	80	56	1.72 (1.03, 2.87)	1.57 (0.93, 2.63)
Increase by one quartile¥			1.06 (0.93, 1.20)	1.12 (0.98, 1.25)			1.18 (1.01, 1.39)	1.14 (0.97, 1.34)
Combined 14-3-3ζ expression								
25th percentile or lower	221	224			97	102		
Above the 25th percentile up to the 50th percentile	106	129	0.71 (0.50, 1.00)	0.84 (0.61, 1.17)	59	59	1.15 (0.69, 1.89)	1.05 (0.64, 1.61)
Above the 50th percentile up to the 75th percentile	73	66	1.17 (0.78, 1.77)	1.14 (0.77, 1.68)	55	57	1.06 (0.63, 1.77)	0.98 (0.59, 1.61)
Above the 75th percentile	48	26	1.77 (1.01, 3.11)	1.93 (1.15, 3.24)	46	32	1.64 (0.92, 2.94)	1.93 (1.03, 3.62)
Increase by one quartile¥			1.13 (0.97, 1.30)	1.15 (1.00, 1.32)			1.13 (0.95, 1.34)	1.15 (0.96, 1.37)

*The source population consisted of 11,251 female residents of the Jutland Peninsula in Denmark aged 35–69 years who were diagnosed with stage I, II, or III breast cancer between 1985 and 2001. Subjects were estrogen receptor α (ERα) positive and received at least 1 year of tamoxifen therapy (ER+/TAM+) or ERα negative and never received tamoxifen therapy and survived at least 1 year after diagnosis (ER-/TAM-). OR = odds ratio; CI = confidence interval; UICC = Union for International Cancer Control.
ǂEstimated using conditional logistic regression with conditioning on the matched factors (time to recurrence or control selection, county, menopausal status, and stage).
ǂEstimated using logistic regression with adjustment for UICC stage, menopausal status, receipt of chemotherapy, receipt of radiotherapy, surgery type, diagnosis year, age at diagnosis, and county.
¥Estimate for a one quartile increase assuming a proportional change for differences between quartiles.

Table 8. Bias-adjusted associations between 14-3-3ζ expression and breast cancer recurrence within ER/TAM group*

	Trapeziodal Sensitivity Parameters				Trapeziodal Specificity Parameters				ER+/TAM+	ER-/TAM-
14-3-3ζ expression	Min	Lower Mode	Upper Mode	Max	Min	Lower Mode	Upper Mode	Max	Bias-adjusted OR (95% SI)ŧ	Bias-adjusted OR (95% SI)ŧ
Cytoplasmic 14-3-3ζ expression										
50th percentile or lower										
Above the 50th percentile	0.78	0.83	0.95	1.00	0.93	0.95	1.00	1.00	1.26 (0.94, 1.71)	1.09 (0.69, 1.71)
Nuclear 14-3-3ζ expression										
50th percentile or lower										
Above the 50th percentile	0.65	0.69	0.81	0.85	0.88	0.90	0.98	1.00	1.36 (0.97, 1.92)	1.57 (0.95, 2.67)
Combined 14-3-3ζ expression										
50th percentile or lower										
Above the 50th percentile	0.56	0.61	0.73	0.78	0.92	0.94	0.98	1.00	1.63 (1.11, 2.43)	1.33 (0.81, 2.19)

*The source population consisted of 11,251 female residents of the Jutland Peninsula in Denmark aged 35–69 years who were diagnosed with stage I, II, or III breast cancer between 1985 and 2001. Subjects were estrogen receptor α (ERα) positive and received at least 1 year of tamoxifen therapy (ER+/TAM+) or ERα negative and never received tamoxifen therapy and survived at least 1 year after diagnosis (ER-/TAM-). OR = odds ratio; SI = simulation interval; UICC = Union for International Cancer Control.

ŧEstimated using logistic regression with the sensitivity macro to account for misclassification based on the specified values for the classification parameters with adjustment for UICC stage, grade, menopausal status, receipt of chemotherapy, receipt of radiotherapy, surgery type, diagnosis year, age at diagnosis, and county.

nuclear 14-3-3ζ staining in both ER+/TAM+ patients (bias-adjusted OR = 1.36, 95% SI = 0.97, 1.92) and ER-/TAM-patients (bias-adjusted OR = 1.57, 95% SI = 0.95, 2.67). The association of combined 14-3-3ζ staining increased slightly for combined staining in both ER+/TAM+ patients (bias-adjusted OR = 1.63, 95% SI = 1.11, 2.43) and ER-/TAM- patients (bias-adjusted OR = 1.33, 95% SI = 0.81, 2.19). Therefore, misclassification of 14-3-3ζ staining, by use of TMA IHC instead of WS IHC, does not completely explain any non-null association with breast cancer recurrence, assuming a valid bias model.

Approximate Bayesian Analysis Results

Because the results from logistic regression for combined above the 50th percentile 14-3-3ζ did not align with the results of either a predictive or prognostic marker, we used estimates for the interaction of ERα and combined 14-3-3ζ staining for the coefficient ($\hat{\beta}$ = 0.16) and the standard error ($se_{\hat{\beta}}$ = 0.25) in inverse-variance weighting to plot posterior distributions comparing hypothesis based on a predictive marker and a posterior marker. Compared to their respective prior distributions, the posterior distributions move toward each other to a value between the expected posterior distributions for a predictive marker and a prognostic marker (Figure 7), and do not converge at a single value for the odds ratio as depicted in the distributions in Figure 6. Because we do not see convergence, evidence is lacking to make conclusions about combined 14-3-3ζ staining and its ability to be used as a predictive marker of tamoxifen resistance.

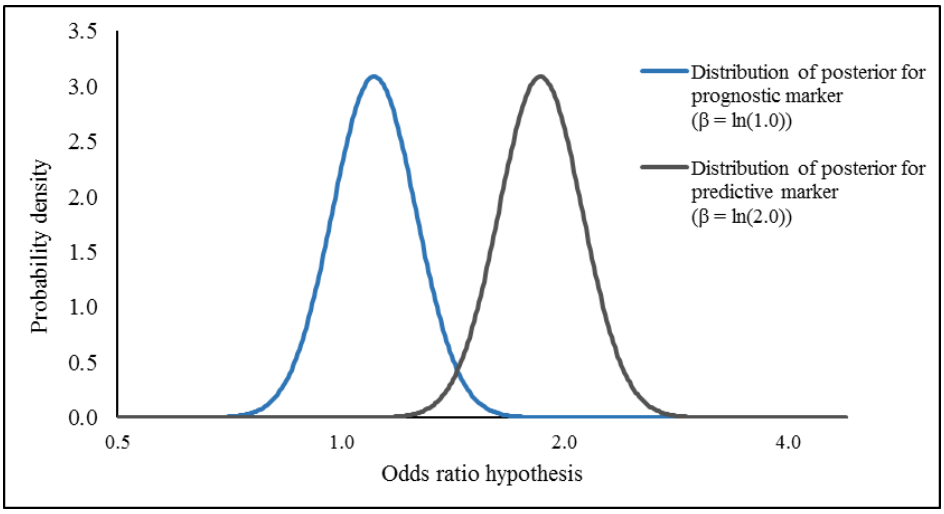

Figure 7. Plots for normal posterior distributions used to compare two hypothesis corresponding to a predictive marker (β_1 = ln(2.0)) and a prognostic marker (β_2 = ln(1.0)). The mean values corresponded to the natural log of the interaction of ERα and combined 14-3-3ζ ($\hat{\beta}$= 0.16, $se_{\hat{\beta}}$ = 0.25). The variance was determined by examining plots that provided sufficient separation between the two hypotheses (${\sigma_1}^2 = {\sigma_2}^2 = 0.15^2$).

Discussion

In this population-based study, the associations between recurrence and cytoplasmic 14-3-3ζ staining or nuclear 14-3-3ζ staining were near null. When we examined patients who had both cytoplasmic and nuclear 14-3-3ζ staining, the adjusted association between combined 14-3-3ζ staining and recurrence increased in ER+/TAM+ patients (adjusted OR = 1.44, 95% CI = 1.05, 1.99) but the confidence intervals widely overlapped those of the ER-/TAM- patients (adjusted OR = 1.22, 95% CI = 0.82, 1.82). These results caution against any interpretation of 14-3-3ζ staining as a predictive marker of tamoxifen resistance as any association would be limited to the ER+/TAM+ group for a predictive marker. The approximate Bayesian analysis provides quantitative support for this inference as the calculated posterior distributions did not match the expected posterior distributions of either a predictive or prognostic marker. In dose-response analysis, the confidence intervals between the ER+/TAM+ group and the ER-/TAM- group

widely overlapped in all categorizations of 14-3-3ζ staining, further suggesting a lack of association between 14-3-3ζ and an ERα dependent pathway.

A moderate association was observed for above the 75th percentile staining of combined 14-3-3ζ staining in both ER+/TAM+ patients (adjusted OR = 1.93, 95% CI = 1.15, 3.24) and ER-/TAM- patients (adjusted OR = 1.93, 95% CI = 1.03, 3.62), indicating possible utility as a prognostic marker of breast cancer recurrence. In the population, 5.8% of ER+/TAM+ patients and 13.0% of ER-/TAM- patients have this level of 14-3-3ζ staining.

The bias analysis yielded estimates of above the 50th percentile cytoplasmic, nuclear, and combined 14-3-3ζ staining that were further from the null compared to the conventional analysis. These results indicate that misclassification of 14-3-3ζ staining, due to using TMA IHC compared to WS IHC, do not completely explain any non-null findings, assuming a valid bias model.

This study is the largest to examine the relationship of 14-3-3ζ staining and breast cancer recurrence. Subcellular staining in the nucleus and cytoplasm was accounted for to examine differential effects of 14-3-3ζ localization. Analyses were stratified by ER/TAM status to differentiate 14-3-3ζ as a marker predictive of breast cancer recurrence and one prognostic of breast cancer recurrence. Bias due to using TMA IHC compared to WS IHC was accounted for in an analysis controlling for both misclassification and confounding, using internal data from a validation substudy. Concordance between ERα status determined at diagnosis and during central re-assay was good and results were similar when using the ERα re-assay for ER/TAM grouping. The use of a population-based registry, containing nearly all cases under age 70 at diagnosis, linked to tumor archives provides results likely devoid of selection bias.

Inter-rater agreement was good for both cytoplasmic and nuclear 14-3-3ζ staining level determination. Recurrences have been previously confirmed in a validation study using medical record review thus eliminating bias due to misclassification of the outcome [40]. All covariates showed perfect agreement expect menopausal status in a single patient. Review of medical record did identify discrepancies of duration of tamoxifen therapy, with the DBCG often indicating shorter durations of therapy compared to the medical record as patients likely switched to longer protocols as evidence grew favoring a 5 year protocol during the study period. However, this strengthens our results, as patients with tamoxifen therapy longer than indicated by DBCG are less likely to have a recurrence due to lack of therapy.

A limitation of this study is ascertaining a cutoff for positivity as staining will be observed in all samples when examining housekeeping genes, such as 14-3-3ζ. Also, the level of staining that showed the strongest odds of recurrence had the smallest sample sizes, resulting in poorer precision compared to other estimates of association. Another limitation of this study was the variable staining intensity observed in sections used for TMA IHC. To account for this, percentiles were determined within a patient's section as opposed to the entire cohort, but in doing so, percentiles would be based on a smaller number of samples. This study also lacks information on adherence to tamoxifen therapy. In a previous study, 20 ER+/TAM+ patients from this cohort were reviewed by medical record and six were found to have not completed their intended duration of tamoxifen therapy, 2 because of a recurrent breast cancer [39].

The results of this study serve to precisely estimate the association between 14-3-3ζ and breast cancer recurrence and differentiate potential predictive and prognostic utility. Despite the findings of previous studies, evidence is lacking to conclude that 14-3-3ζ is a useful marker of tamoxifen resistance. High levels of combined 14-3-3ζ staining is a potentially useful prognostic

marker of breast cancer recurrence in both ER+/TAM+ and ER-/TAM- patients. Further research is be needed to determine if 14-3-3ζ has independent utility in a clinical setting beyond established prognostic markers of breast cancer recurrence.

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