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14-3-3 ζ as a Predictor of Early Time to Recurrence and Distant Metastasis in Hormone Receptor-Positive and -Negative Breast Cancers

Anna Bergamaschi¹, Jonna Frasor², Kristina Borgen³, Adina Stanculescu², Patricia Johnson⁴, Kendrith Rowland⁴, Elizabeth L. Wiley³, and Benita S. Katzenellenbogen¹

¹Department of Molecular and Integrative Physiology, University of Illinois and College of Medicine at Urbana-Champaign, Urbana, IL, 61801

²Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL 60612

³Department of Pathology, University of Illinois at Chicago, Chicago, IL 60612

⁴Mills Breast Cancer Institute, Carle Foundation Hospital, Urbana, IL 61801

Abstract

The 14-3-3 ζ gene, on 8q22, is often amplified in breast cancer and encodes a survival factor that interacts with and stabilizes many key signaling proteins. We examined the relationship between the expression of 14-3-3 ζ , estrogen receptor (ER α), and other parameters (tumor size, grade, nodal status, progesterone receptor, HER2, EGFR, and p53) in matched primary and recurrence tumor tissue and how these factors impact time to recurrence, properties of the recurred tumors, and site of metastasis. In this cohort of over 100 patients, median time to recurrence was 3 years (range 1-17 yrs). Our analyses of primary tumor microarray cores revealed that 14-3-3 ζ status was significantly correlated with tumor grade, size, and ER α . Women with 14-3-3 ζ -positive and ER α -negative tumors had the earliest time to recurrence (median 1yr, $p < 0.001$, hazard ratio 2.89), while median time to recurrence was 7 yrs for 14-3-3 ζ -negative and ER-positive tumors. Of recurred tumors, 70-75% were positive for 14-3-3 ζ , up from the 45% positivity of primary tumors. High expression of 14-3-3 ζ also correlated with site of recurrence and showed a propensity for distant metastases to lung and chest wall. Multifactor correlation regression analysis revealed 14-3-3 ζ to be a non-redundant, independent variable that adds clinical strength in predicting risk for early recurrence in ER-positive and -negative breast cancers, providing information beyond that of all other clinical pathological features examined. Thus, high expression of 14-3-3 ζ in the primary tumor was significantly associated with earlier time to recurrence and with distant metastasis. Furthermore, even when the primary breast cancers were negative-low for 14-3-3 ζ , the majority acquired increased expression in the recurrence. The findings underscore the detrimental role played by 14-3-3 ζ in tumor aggressiveness and suggest that reducing its expression or interfering with its actions might substantially improve the clinical outcome for breast cancer patients.

Keywords

estrogen receptor; recurrence; 14-3-3 ζ ; Tamoxifen

Corresponding Author: Prof. Benita S. Katzenellenbogen, Department of Molecular and Integrative Physiology, University of Illinois and College of Medicine at Urbana-Champaign, Urbana, IL, 61801 katzenel@illinois.edu, Phone: 217-333-9769; Fax: 217-244-9906.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

INTRODUCTION

Breast cancer is the most common malignancy in women and the second leading cause of cancer deaths in women. Despite the increase in earlier detection, the survival rate has not improved proportionately [1]. Therefore, a major focus has been directed to the identification and introduction into clinical analyses of suitable markers that can determine the risk of recurrence and help oncologists to choose the most appropriate treatments for individual patients.

We have shown previously by gene expression analysis that the scaffold adaptor protein 14-3-3 ζ is associated with a poor clinical outcome for women with ER-positive breast tumors on tamoxifen treatment, and that it is a novel predictive biomarker for tamoxifen therapeutic resistance [2,3]. 14-3-3 ζ is a member of a family of seven highly conserved proteins that control key aspects of cellular function, including proliferation, apoptosis, and cell survival [4]. 14-3-3 ζ , on 8q22, is often amplified in breast cancers and in other cancers as well [5]. Interestingly, we observed that 14-3-3 ζ was up-regulated by tamoxifen and that high levels of 14-3-3 ζ in primary breast tumors were associated with a poor patient clinical outcome on tamoxifen [3]. In addition, among estrogen receptor α (ER α)-positive breast tumors, in large data sets from over 400 women [3,6-8], high expression of 14-3-3 ζ was found predominantly in luminal B breast tumors and also in some ER-positive basal breast cancers, suggesting 14-3-3 ζ association with more aggressive ER-containing breast cancers [2]. In addition to 14-3-3 ζ association with endocrine resistance, high expression of 14-3-3 ζ in ER-negative breast tumors was found to be associated with chemoresistance to anthracyclines in a neoadjuvant trial in women with primary breast cancer [5,9,10], implying that this protein has a detrimental influence also in tumors that lack estrogen receptors. However, the direct associations between 14-3-3 ζ presence in the primary breast tumor and time to recurrence, and hormone receptor status, and site and extent of metastasis, are not known.

14-3-3 ζ serves a pivotal role in enhancing growth factor and protein kinase signaling, and promoting cell survival by regulating the activity and stability of key signaling proteins including HER2, EGFR, IRS1, PKC, β -catenin, and RAF1 [2,11-14]. Our studies revealed that knockdown of 14-3-3 ζ in breast cancer cells in culture was able to restore sensitivity to endocrine therapies and to reduce the proliferation, antiapoptotic activity, and invasiveness observed when the level of 14-3-3 ζ was high [2,15]. Furthermore, we found 14-3-3 ζ to be associated with a gene signature rich in genes regulating mitosis and cytokinesis [2].

In this study, we have utilized a large cohort of breast cancer patients, all of whom experienced tumor recurrence and for whom matched primary breast tumor and tumor recurrence tissue were available, to explore more fully the value of 14-3-3 ζ as a prognostic marker of early time to breast cancer recurrence. We have investigated the relationships between 14-3-3 ζ in the primary and recurred tumors, and tumor phenotypic properties and clinical behavior, including time to recurrence, receptor status (ER, PR, EGFR, HER2), p53 status, and tumor size and grade. We also assessed whether the recurrence was local or distant and the site of metastasis in relation to 14-3-3 ζ expression. Our findings reveal that in both ER-positive and ER-negative breast cancers, 14-3-3 ζ -positivity was associated with a markedly shortened time to recurrence. Further, multifactor and correlation regression analysis demonstrated 14-3-3 ζ status to be a non-redundant, independent variable that adds clinical strength in predicting risk of recurrence in breast cancer beyond that of any of the other clinical pathological features assessed. The findings underscore the detrimental role played by 14-3-3 ζ in tumor aggressiveness and metastasis, and suggest that reducing its

expression or interfering with its actions might substantially improve the clinical outcome for breast cancer patients.

MATERIAL AND METHODS

Patient Cohort and Tumor Characteristics

In this study, we examined tumor tissue from 139 women with breast cancer and the cohort tumor characteristics are described in Table 1. Residual tissue sections from previously constructed tissue microarrays were available for study. The arrays were previously constructed from formalin fixed paraffin embedded positive primary tumors and recurrences of patients with breast cancer recurrence over the 17 year period to characterize biochemical impact of tamoxifen on estrogen receptor positive tumors. Patient samples had been collected and processed in compliance with protocols approved by the University of Illinois Cancer Center Institutional Review Board and Northwestern University Institutional Review Board. FFPE blocks from primary breast cancers and recurrences were retrieved for examination and paired. Two one millimeter core samples were obtained from each primary tumor and recurrence for construction of paraffin tissue microarrays. Tissue sample annotations included tumor type, grade and stage, recurrence interval, recurrence site, Tamoxifen treatment, and various molecular markers indicated below [16,17].

Immunohistochemistry

To determine the expression of 14-3-3 ζ , ER α or other proteins, 5 μ m thick paraffin-embedded tissue sections were de-paraffinized in xylene for 2 $\times$ 5 min, then hydrated by soaking in graded ethanol (100%, 95%, and 70%). Antigen retrieval was performed by submerging the slides in a low pH Antigen Unmasking Solution (H-3300, Vector Laboratories, Burlingame, CA) and incubating for 5 min in the Decloaking Chamber electric pressure cooker at 20 psi and 120°C (Biocare Medical, Walnut Creek, CA). Endogenous peroxidase was quenched in 0.3% H₂O₂ in methanol for 30 min and the tissue blocked in TBS containing 5% goat serum (Vectastain Elite ABC Kit, Vector Laboratories). Primary rabbit polyclonal antibody or a 5:1 mixture of the corresponding blocking peptide with primary antibody was incubated overnight at room temperature at a dilution of 1:5000 (14-3-3 ζ sc-1019 and sc-1019P, Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, tissue sections were incubated with avidin- biotin peroxidase (Vector Elite ABC Kit, Vector Laboratories), color was developed by addition of DAB substrate and slides were counterstained with hematoxylin. Antibodies were optimized in the staining of tissue sections, and negative controls were performed by omission of the primary antibody. Digital images were acquired with a Nikon Eclipse E400 microscope equipped with a Spot 4.1 color cooled digital camera and SPOT Advanced software (Diagnostic Instruments, Sterling Heights, MI). Staining was scored by two independent pathologists (EW and KB) and all cases were scored without knowledge of the clinical-pathological or outcome data. Scoring of estrogen and progesterone receptor was based on the percentage of tumor cells staining positive with greater than 10% of nuclei as positive, 1 to 10% weak positive and less than 1% as negative. Her2/neu staining was scored according to ASCO/CAP guidelines and EGFR staining was also scored using the guidelines for Her2/neu with both 2+ and 3+ staining considered positive. Staining for p53 was positive if more than 20% of tumor nuclei stained strongly (clearly visible at 5x magnification). 14-3-3 ζ staining was scored as high if a majority of tumor cells stained with intensity enough to be clearly visible at 5x magnification. Hormonal therapy (Tamoxifen) was prescribed to most of the patients with ER α -positive tumors.

Cell Culture and Invasion Assays

BT474 and MDA-MB-231 cells, from the American Type Culture Collection (Manassas, VA), were grown in DMEM (Invitrogen) and 10% fetal bovine serum. Tamoxifen-resistant MCF-7 cells [21] were grown and treated as described [2, 10]. At 24h after 14-3-3 ζ knockdown, cells were seeded and matrigel invasion assays were carried out using modified Boyden chambers with polycarbonate Nucleopore membrane (Corning, Corning, NY) as described [2]. In brief, 2×10^4 cells in 500 μ L were seeded on precoated filters (8- μ m pore size) after membrane rehydration. Following incubation for 48h at 37°C, cells were fixed in 10% formalin buffer and stained using crystal violet. Non-invaded cells on the upper surface of the filter were removed with a cotton swab. Invasiveness was determined by counting invaded cells [2,15].

Statistical Analyses

The expression of 14-3-3 ζ was compared with tumor grade, tumor size, nodal status and expression of hormone receptors (ER α , PgR), p53, HER2, and EGFR in Spearman's two-tailed correlation test. Multifactor analysis was computed using WinStat software (WinStat for Excel R.Fitch software). Cox proportional hazard ratio (HR) model was used for the multivariate survival analysis. Kaplan-Meier analysis was done using log-rank to linearly compare trends of cluster groups to time to recurrence in Fig 3. All tests were computed using WinStat software.

RESULTS

Clinical significance of 14-3-3 ζ protein expression

Patients in this study experienced recurrence of their breast cancer, and matched primary and recurrence tumor samples were available for analysis in almost all cases. Patient breast tumor characteristics are listed in Table 1. Of the 139 women with breast cancer, 53% of the primary tumors were estrogen receptor (ER)-positive, 29% were progesterone receptor (PR)-positive, 38% were HER2-positive, and 20% were triple negative (TN). Almost all tumors were ductal (97%) and most were of high tumor grade (69% grade 3). Forty-five percent of primary breast tumors had high 14-3-3 ζ protein expression based on IHC. Bivariate analysis showed that 14-3-3 ζ positivity correlated significantly with ER α ($p = 0.050$), tumor size ($p = 0.045$), and histologic grade ($p = 0.039$) (Table 2).

Next, using multifactor analysis, we determined that 14-3-3 ζ is a non-redundant variable that provides clinical strength in predicting recurrence that is independent of all other variables assessed (Fig. 1). Fig.1 examines the relatedness of these factors to each other, and shows that 14-3-3 ζ is not linearly related to any of the other factors evaluated (ER, PR, p53, EGFR, HER2, tumor size, grade, i.e. the Y and X variables are not linearly related to each other).

Over the maximum follow-up time of 17 years, there were 110 patients who manifested recurrences, including 64 to distant sites. Of these 110 tumors that recurred, we obtained 14-3-3 ζ protein IHC staining information for a total of 100. Fig. 2A shows representative IHC staining of two 14-3-3 ζ -negative/low tumors and two 14-3-3 ζ -positive tumors. Further, in the multivariate analysis based on the Cox proportional hazard model (Fig. 2B), we found that 14-3-3 ζ , histological grade, and ER α status were significant predictors of risk of cancer-specific recurrence.

Prognostic significance of 14-3-3 ζ in combination with other markers and time to recurrence

Using hierarchical clustering based on clinical markers in breast tumors (14-3-3 ζ , ER α , PR, p53 status, and tumor grade), we identified 4 groups of patients (Fig. 3A) with tumor characteristics that were significantly associated with time to recurrence. Among them, by Kaplan-Meier analysis, we identified a good prognostic group (green) represented by low/negative 14-3-3 ζ expression, ER α positivity and low grade tumors (median time of recurrence = 6.5 yrs and HR= 0.37, $p < 0.001$) (Fig. 3B, C). The poorest prognostic group (red) was represented by patients with tumors positive for 14-3-3 ζ , negative for ER α , and with high grade breast cancer (median time to recurrence = 1yr and HR= 2.89, p value= < 0.001) (Fig. 3B, C). Time to recurrence in ER-positive breast cancers clearly separated into 2 distinctly different groups, with greatly different times to recurrence based on 14-3-3 ζ positive or negative status. 14-3-3 ζ expression also stratified ER-negative tumors into 2 groupings with different time course patterns of recurrence (Fig. 3B, C).

14-3-3 ζ positivity is associated with recurrence and increased invasiveness, and correlates with the development of distant metastasis

As a further analysis, we stratified tumors into subgroups based on 14-3-3 ζ IHC staining status in primary tumors and in tumors that recurred (Fig. 4A). Interestingly, we observed that 70-75% of recurred tumors showed high expression of 14-3-3 ζ . This represented a large increase in 14-3-3 ζ positivity compared to the 45% positivity of the primary tumors in the same women. But most of note, we observed that many primary tumors that were low/negative for 14-3-3 ζ became positive in the recurrence, so that only 29% of 14-3-3 ζ -low/negative primary tumors remained 14-3-3 ζ - low/negative in the recurrence (Fig.4A). Thus, high 14-3-3 ζ expression was characteristic of about three-fourths of recurred tumors regardless of whether 14-3-3 ζ was low/negative or high positive in the primary tumor, implying that increased expression of this protein was a common trait acquired by the majority of the cancers that recurred.

To investigate the role of 14-3-3 ζ in cancer cell invasiveness, we used an *in vitro* approach in which we knocked down 14-3-3 ζ using siRNA in three different cell lines (BT474, MCF-7-Tam^R and MDA-MB-231), and we measured the capacity of these cells to invade using a transwell system (Fig. 4B). BT474 and MCF-7-Tam^R (tamoxifen resistant MCF7 cells) are ER α -positive cells that are also positive for HER2, whereas MDA-MB-231 are triple negative breast cancer cells. In these three cell types, depletion of 14-3-3 ζ resulted in a greatly reduced invasion capacity compared to their control cells ($p < 0.0001$).

We next examined the relationship between tumor level of 14-3-3 ζ and site of metastasis as shown in Fig. 4C. Primary tumors that were positive for 14-3-3 ζ showed a propensity for distant sites of recurrence, tumor metastases to lung and chest wall.

DISCUSSION

Our observations reveal that 14-3-3 ζ is strongly associated with increased risk of early recurrence in ER-positive and ER-negative breast cancer, and with risk of distant metastasis. We found 14-3-3 ζ to be a negative prognostic factor, increasing the hazard ratio almost 3-fold in women with ER α -positive and ER α - negative tumors. Moreover, we found association of 14-3-3 ζ with histological grade and tumor size, consonant with our prior findings that 14-3-3 ζ was disproportionately associated with ER-positive luminal B vs. luminal A breast cancers, and supportive of this protein being associated with a more aggressive subtype of ER-containing breast cancer [2].

In this unique cohort of patients who experienced recurrence of their breast cancer and in which matched primary and recurrence breast cancer tissue was available, 45% of primary tumors expressed high 14-3-3 ζ , whereas 70-75% of recurred tumors had high expression of this protein, indicating that a significant proportion of the tumors gain expression of 14-3-3 ζ . Furthermore, of note was our observation that many primary tumors that were low/negative for 14-3-3 ζ became positive in the recurrence, so that only 29% of 14-3-3 ζ -low/negative primary tumors remained 14-3-3 ζ -low/negative in the recurrence. Thus, high 14-3-3 ζ expression was characteristic of about three-fourths of recurred tumors regardless of whether 14-3-3 ζ was low/negative or high positive in the primary tumor, implying that increased expression of this protein was a common trait acquired by the majority of the cancers that recurred. This suggests that 14-3-3 ζ plays an important role in promoting tumor aggressiveness. Invasion studies conducted in three breast cancer cell lines, either ER α -positive or ER α -negative, clearly supported this hypothesis by showing that depletion of 14-3-3 ζ greatly reduced cell invasiveness. In addition, positive 14-3-3 ζ status of breast tumors was correlated with site of recurrence and a propensity for distant sites such as lung and chest wall versus loco-regional sites. The switch in 14-3-3 ζ status to increased expression in many recurred tumors versus their primary breast cancers was notably much more prevalent than the switch in ER, PR or HER2 status between primary and recurrent breast cancer, reported to occur in only 1 in 6 patients [18].

Breast cancer prognostic assessments and consideration of therapeutic options are routinely based on the detection of three biomarkers: ER α , PR and HER2, in addition to histopathological parameters [19,20]. It is clear that there is need for advances in the discovery of additional prognostic and predictive markers [21]. We observed that patients with 14-3-3 ζ +ER-negative tumors had the worst prognosis, whereas patients with tumors that were 14-3-3 ζ low-negative/ER α -negative and 14-3-3 ζ high/ER α -positive showed a similar time to recurrence profile. While complete clinical treatment details were not available for all patients in our cohort, among the ER α -positive tamoxifen-treated patients with tumors positive for 14-3-3 ζ (n= 36), we observed an earlier time to recurrence than in 14-3-3 ζ -negative ER α -positive (n=14) tumors (p = 0.041). These findings imply that further follow-up studies would be warranted to carefully assess the relationship between specific treatments and their benefit in women with breast cancers with high expression of 14-3-3 ζ . Thus, simultaneous analysis of 14-3-3 ζ and ER α may provide improved discrimination in selecting patients for adjuvant treatment options. Assessment of 14-3-3 ζ might also complement and likely improve upon the assessment of risk of recurrence in ER-positive, lymph node negative patients on tamoxifen treatment that is often based solely on the OncoType DX[®] assay [22]. This assay determines risk of recurrence over a 10-year period based on the expression of 21 genes profiled on RNA isolated from formalin fixed paraffin embedded (FFPE) tumor tissue [23,24]. It is of note that 14-3-3 ζ is detected not only in tumor tissue, but also is secreted from some tumors and has been identified in sera of patients with head and neck small cell carcinomas [25] and in ascites from ovarian cancers [26]. Hence, it might prove to be a valuable biomarker in serum of disease state or prognosis in patients with certain cancers.

A pivotal role for 14-3-3 ζ in tumor progression was suggested by work of others showing that 14-3-3 ζ promotes cancer cell survival [10] and that it down-regulates p53 in mammary epithelial cells, disrupting the architecture of acini and conferring luminal filling [27]. Further, the strong association of 14-3-3 ζ -positive status with resistance to endocrine and chemotherapeutic agents may be explained by the many important cell signal regulating properties of 14-3-3 ζ [2,3,28]. Indeed, this protein is known to bind and stabilize many protein kinases and key signaling proteins, including EGFR, HER2, IRS1, PKC, β -catenin, and RAF1 [11-14,29]; 14-3-3 ζ thus serves as a critical scaffold and integrator of multiple signaling networks in cells that can intensify cellular functions that underlie resistance to

endocrine and chemotherapeutic agents. Our observations extend prior findings by discovering a clear correspondence between 14-3-3 ζ positivity and early breast cancer recurrence in women with both ER-positive and ER-negative primary breast cancers.

We previously showed that 14-3-3 ζ was up-regulated by tamoxifen in ER-positive breast cancer cells and that 14-3-3 ζ was elevated in patient breast tumors that acquired resistance to tamoxifen [2,3]. These changes were found to be mediated by the down-regulation of miR-451 by the tamoxifen-ER complex, resulting in increased 14-3-3 ζ gene expression and promotion of breast cancer cell survival and endocrine resistance [15]. Thus, 14-3-3 ζ and miR-451 showed an inverse relationship in breast cancers [15], implying that increasing miR-451 expression in breast tumors or reducing or inhibiting 14-3-3 ζ functional activities might prove to be of potential therapeutic utility. There has been some success in the development of inhibitors for the 14-3-3 family, but achieving selectivity for specific family members has proven to be challenging [30]. Therefore, intercepting downstream effector targets of 14-3-3 ζ , such as FOXM1 [2], might prove to be of benefit.

In conclusion, from this study analyzing primary tumors and their matched tumor sample at recurrence, 14-3-3 ζ has been documented to be an independent predictor of time to recurrence in ER α -positive and ER α -negative breast cancers, and to be associated with risk of metastasis. Although the predictive value of 14-3-3 ζ in the response of patients to specific treatment regimens needs further exploration in randomized clinical trials, the present findings support the importance of 14-3-3 ζ -positive status as a valuable biomarker for identifying breast cancer patients at high risk for early disease recurrence.

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Abbreviations

ERα	estrogen receptor alpha
E2	estradiol
PgR	progesterone receptor
Tam	Tamoxifen

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Fig. 1. Multifactor analysis was conducted to assess independent dimensions underlying the existing variables and their possible independence in predicting clinical outcome.

Fig. 2.

A) Representative primary breast tumor cores stained for 14-3-3 ζ IHC, showing two low/negative tumors and two high expressing (positive) tumors. B) Multivariate Cox regression analysis of several variables and their association with time to recurrence. HR, hazard ratio; CI, confidence interval; significance indicates p value.

Fig. 3.
A) Hierarchical clustering of primary tumors based on their 14-3-3 ζ status and ER α status, and other available clinical data for tumors (PR status, p53 status, and tumor grade). Red denotes high expression and blue denotes low. For Disease Specific Survival (DSS) color-coding: bright red square denotes recurrences detected in <1 year; dark red square, recurrences in 1-5yrs; dark blue square, recurrences in 6-10 yrs; and blue square >10 yrs. Four groups (green, black, red, and blue) are shown. B) The 4 identified groups were then used as grouping criteria in Kaplan-Meier analysis. This disease-free survival analysis shows a highly significant correlation of cluster groups and time to recurrence (p value <0.0001). C) Hazard ratios (HR), 95% confidence intervals (CI), and p values are shown for each cluster group.

Fig. 4.

A) Pie chart (center) shows percentage of primary breast tumors 14-3-3 ζ positive (i.e. high expression) or low-negative based on IHC staining. Sub-pie charts (at left and right) give representation of 14-3-3 ζ positive or low-negative staining in recurred tumors stratified based on the positive or low-negative staining in primary tumors. B) Invasion assay was performed in 3 breast cancer cell lines after siRNA knockdown of 14-3-3 ζ . Invasive cells were counted after 48h for BT474 cells and MCF-7 TamR (tamoxifen resistant) cells, and after 36h for MDA-MB-231 cells. C) Percent recurrence at the indicated sites based on 14-3-3 ζ expression in primary tumors.

Table 1**Cohort Tumor Characteristics***

Estrogen Receptor	Number	%
Pos	69	(53)
Neg	61	(47)
Progesterone Receptor		
Pos	33	(29)
Neg	80	(71)
HER2		
Pos	38	(38)
Neg	63	(62)
Tumor Histology		
Ductal	130	(97)
Lobular	4	(3)
Tumor Grade		
1	10	(7)
2	33	(24)
3	96	(69)

* Primary tumor tissue was examined from 139 women. Analyses for the indicated parameter were not available for all tumors so that the numbers given in the table are somewhat fewer than n=139 for the various parameters.

Table 2

Bivariate analysis of 14-3-3 ζ status in primary breast tumors (n=122) with other disease variables. Highlighted in bold are variables with significance ≤ 0.05 .

Variable	Correlation coefficient	Significance (two-tailed)	No. of Patients
ER	0.318	0.050	118
PR	0.107	0.141	103
p53	-0.009	0.465	95
HER2	-0.082	0.205	101
Grade	-0.121	0.039	118
Tumor Size	0.163	0.045	112
Nodal Status	0.161	0.273	103
EGFR	-0.015	0.463	118