Published in final edited form as:

Cancer Biomark. 2009 ; 5(4): 215–224. doi:10.3233/CBM-2009-0106.

Higher Expression Levels of 14-3-3 σ in Ductal Carcinoma In Situ of the Breast Predict Poorer Outcome

Nam K. Yoon¹, David B. Seligson^{1,2}, David Chia^{1,2}, Yahya Elshimali¹, Giri Sultur¹, Ai Li³, Steve Horvath^{2,3,4}, Erin Maresh¹, Vei Mah¹, Shikha Bose⁶, Benjamin Bonavida^{2,5}, and Lee Goodglick^{1,2}

¹Department of Pathology and Laboratory Medicine, Immunology and Molecular Genetics, David Geffen School of Medicine at UCLA, Los Angeles, California

²Jonsson Comprehensive Cancer Center, Immunology and Molecular Genetics, David Geffen School of Medicine at UCLA, Los Angeles, California

³Department of Biostatistics, Immunology and Molecular Genetics, David Geffen School of Medicine at UCLA, Los Angeles, California

⁴Department of Human Genetics, Immunology and Molecular Genetics, David Geffen School of Medicine at UCLA, Los Angeles, California

⁵Department of Microbiology, Immunology and Molecular Genetics, David Geffen School of Medicine at UCLA, Los Angeles, California

⁶Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, California

Abstract

The protein 14-3-3 σ is involved in the regulation of cellular processes such as apoptosis, cell cycle progression and proliferation. Disruption of protein expression has been implicated in a number of malignancies. Here we examine the expression pattern of 14-3-3 σ in breast cancer and specifically consider whether expression in ductal carcinoma *in situ* (DCIS) lesions is predictive of disease outcome. We examined 14-3-3 σ protein expression and localization using immunohistochemical staining on a high-density tissue microarray consisting of 157 invasive breast cancer patients. Statistical analyses were used to assess the correlation of 14-3-3 σ expression with clinico-pathological parameters and patient outcome. We observed a statistically significant increase in 14-3-3 σ protein expression in ductal hyperplasia, DCIS, and invasive ductal carcinoma (IDC) as compared normal glandular epithelium. In IDC, lower expression of 14-3-3 σ tended to predicted poorer survival time while in DCIS lesions, there was a stronger correlation between relatively higher levels of 14-3-3 σ predicting shorter survival time. Further, of patients who had concurrent DCIS and IDC lesions, those that exhibited a decrease of 14-3-3 σ expression from DCIS to IDC had significantly shorter survival time. Our findings indicate that 14-3-3 σ expression may be a useful prognostic indicator for survival in patients with breast cancer with an elevated 14-3-3 σ in earlier disease predicting a less favorable disease outcome. To our knowledge this is the first published study associating 14-3-3 σ protein expression with breast cancer survival.

To whom correspondence should be addressed: Lee Goodglick, Ph.D., Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, 10833 Le Conte Ave; Center for the Health Sciences, Box 951747, Los Angeles, California, 90095-1747. Phone: (310) 825-9134; lgoodglick@mednet.ucla.edu..

Keywords

Tissue microarray; breast cancer; tumor marker; 14-3-3 σ ; prognostic marker; DCIS

INTRODUCTION

Breast cancer is the second leading cause of cancer deaths in women. It is estimated that this year, more than 200,000 American women will be diagnosed with invasive breast cancer, and another 60,000 with *in situ* cancer lesions [15]. Despite advances in therapy options, accurately assessing and responding to the clinical and molecular heterogeneity of breast cancers remains a key limitation. Thus, extensive research efforts have been devoted to characterizing the molecular abnormalities underlying breast cancer as well as identifying the molecular biomarkers that can characterize disease and serve as prognostic indicators.

14-3-3 σ belongs to the 14-3-3 family of chaperone molecules involved in the regulation of various cellular processes and signal transduction pathways involved in apoptosis, stress response, cell cycle progression, and proliferation. This family of proteins is highly conserved evolutionarily. In humans, there are seven known isoforms (β , γ , ϵ , σ , ζ , τ and η) which can exist as homo or heterodimers. In general 14-3-3 members can bind to a number of protein partners, typically, though not exclusively, at specific phosphoserine or phosphothreonine sites, with a resultant change in activity, conformation, and/or localization of the binding protein [18; 25; 46].

The 14-3-3 σ isoform is unique in function and distribution. This isoform is mostly expressed in epithelium and is thought to help modulate responses to stress and DNA damage and resultant proliferation and/or cell death specifically by binding to proteins such as p53, MDM2, Cdc2-cyclin-B, and Akt [4; 11; 17–19; 33; 40; 45; 46]. 14-3-3 σ has also been implicated in modulation of cell cycle progression in a p53-independent fashion due to insulin-like growth factor-1 stimulation [47].

As might be expected from its function in critical cellular pathways, 14-3-3 σ has also been implicated in a number of human malignancies including breast, lung, prostate, ovarian, endometrial, pancreatic, hepatocellular, and lung cancers [1; 6; 12; 14; 21–24; 28; 37; 41; 43; 44]. Depending on the study and the tumor type, there are conflicting reports on whether 14-3-3 σ expression levels are generally increased or decreased. In breast cancer, for example, silencing of 14-3-3 σ expression has been described in various cell lines and some human tumors [6; 41; 44], however in other studies, silencing is an uncommon and sporadic event [24]. To date, no studies have been published examining the prognostic value of 14-3-3 σ expression and disease outcome in breast cancer.

Here we examine on a population basis using tissue microarray (TMA) technology, the level of 14-3-3 σ in breast cancer with a specific focus on whether expression levels of this protein is indicative of disease course or outcome. Interestingly, we observed that the expression of 14-3-3 σ in ductal carcinoma *in situ* (DCIS) lesions, was highly predictive of survival of these individuals. To our knowledge this is the first study which has examined and correlated 14-3-3 σ protein expression with disease outcome (death due to breast cancer).

MATERIALS AND METHODS

Breast Tissue Microarray

A breast TMA was constructed using formalin-fixed, paraffin embedded breast tissue samples as described previously under IRB approval and guidelines from the University of

California, Los Angeles [16; 35]. The TMA consisted of 243 cases of 210 patients who underwent surgery at the UCLA Medical Center from 1995 to 2000, of which 157 patients had invasive breast cancer of various histologic types. Hematoxylin and eosin-stained slides were reviewed by a pathologist to identify an average of three areas per donor tissue block. Patients who received neoadjuvant therapy were excluded from this study. In total, 59 primary cases of invasive ductal carcinoma, not otherwise specified (IDC) had informative invasive tissue and were supported by disease-specific survival data. Thirty nine primary cases of IDC had informative DCIS lesions adjacent to invasive cancers. Of these, 32 cases had informative IDC and DCIS tissues. All DCIS lesions were from women with invasive breast cancer. For survival analyses, only cases with known outcomes were utilized. An additional TMA constructed at the Cedars-Sinai Medical Center was utilized for marker validation. This array has been previously described [3].

Immunohistochemistry

A standard immunohistochemistry protocol was used to determine 14-3-3 σ expression as previously described [35]. Briefly 4 micron sections were cut from each array block. Following deparaffinization, antigen retrieval, quenching of endogenous peroxidases, and blocking with 5% horse serum, slides were incubated for 30 minutes with 1 μ g/ml mouse anti-human monoclonal anti-14-3-3 σ antibody (RDI-1433Sabm-XP, Research Diagnostics, Inc., Concord, MA). Specific staining was detected using horse anti-mouse horseradish peroxidase-conjugated secondary antibody (Vector ABC, 1:200 dilution standard) followed by diaminobenzidine. For the negative control, a concentration-matched mouse monoclonal isotype control was used for primary incubation.

Tissue array scoring

Staining frequency and intensity of 14-3-3 σ expression on the TMA was assessed as described previously with the percentage of glandular cell staining ranging from 0–100% and the intensity of staining being 0 (below the level of detection) to 3 (strong expression) [35]. An integrated measure of expression for frequency and intensity of staining was calculated using the following formula: $[3 (\%x) + 2(\%y) + 1 (\%z)] / 100$, where x, y, and z represent the percentage of cells staining at intensity 3, 2, and 1, respectively. This value was referred to as the integrated intensity. In this study we did not consider myoepithelial cell expression. For outcome analysis, a pooled value for each case was determined as described previously [29; 34; 36]. For some analyses, a separate variable was defined ($\Delta 14-3-3\sigma$) which was calculated by subtracting the mean integrated intensity of the pooled DCIS lesions from the mean integrated intensity of pooled matched invasive lesions of the same cases ($IDC_{\text{expression}} - DCIS_{\text{expression}}$).

Statistical analysis

Statistical analyses were conducted as previously reported [20; 29; 34; 36]. Briefly, non-parametric two-group and multi-group comparisons were carried out using Mann-Whitney and Kruskal-Wallis tests. Correlations were calculated using Spearman Correlation. Patients were dichotomized at quartiles of 14-3-3 σ expression and Disease-specific survival curves were visualized using the Kaplan-Meier method and the difference between survival distributions tested using the log-rank test. The Cox proportional hazards model was used to test the statistical significance of predictors in a univariate and multivariate setting. All statistical analyses were performed with StatView Version 5.0 (SAS Institute, Cary, NC) or with the software package, R¹.

¹<http://www.r-project.org>

RESULTS

14-3-3 σ Expression is Increased in Breast Cancer Tissue

To evaluate the expression levels of 14-3-3 σ among breast histopathologies, we utilized a high-density breast tissue microarray (TMA) constructed at UCLA. This TMA consisted of 2,040 spots from 243 cases of which 410 spots (147 cases) were invasive ductal carcinoma (IDC), 201 spots (101 cases) were DCIS, 50 spots (32 cases) were ductal hyperplasia without atypia (DH), and 235 spots (118 cases) contained matched nonmalignant epithelia distal to tumor site, and 41 spots (21 cases) were normal glandular epithelium from breast reduction surgery (Table 1). Immunohistochemical analysis was performed and the percentage of glandular cells staining positive or negative as well as the intensity of staining was quantified.

The 14-3-3 σ expression was localized predominantly in the cytoplasm of glandular epithelium and surrounding myoepithelial cells. For this study, we did not quantify expression in the myoepithelial cells. All breast histopathologies examined exhibited some degree of heterogeneity for 14-3-3 σ expression. Representative images of staining are shown in Figure 1. An integrated measure of the frequency and intensity of staining was determined for each array spot (described in Materials and Methods), and the mean integrated intensity was compared across histopathologies. Notably, expression in normal cells was significantly lower than either IDC ($P < 0.0001$), DCIS, ($P < 0.0001$), or DH ($P = 0.0066$) (Figure 2). We observed no significant difference between normal cells from breast reduction surgeries and morphologically normal cells adjacent to cancerous lesions. Further, we compared case-matched spots from patients with both primary and metastatic invasive tissues, and although we observed a slight decrease in average immunoreactivity in metastatic tissues as compared to primary cancer cells, this difference was not statistically significant. Despite these trends, there was substantial variability of expression within each histopathological group (Figure 2).

14-3-3 σ Expression is a Strong Predictor of Patient Outcome

Because of these marked differences in 14-3-3 σ expression with progression to malignancy, we analyzed whether protein levels predicted clinical outcome. While univariate Cox model analysis of 14-3-3 σ expression in IDC as a continuous variable was not a significant predictor of patient survival ($P = 0.29$), if 14-3-3 σ expression in IDC was dichotomized, it showed marginal ability to predict patient survival (Figure 3A; $P = 0.056$). In this case, higher levels of 14-3-3 σ in IDC specimens were associated with better survival and lower levels predicted an increased probability of death due to breast cancer (Figure 3A). This trend were validated on an independent breast cancer TMA constructed at the Cedars-Sinai Medical Center in which 80 patients with breast cancer had informative information. Similar to the population from UCLA, relatively higher levels of 14-3-3 σ tended to predict the probability of longer survival compared to lower levels (data not shown). The 14-3-3 σ expression in invasive lesions was not significantly associated with any of the known clinical-pathological parameters (data not shown).

We next considered whether 14-3-3 σ expression in DCIS lesions was predictive of disease outcome. 14-3-3 σ protein levels were determined in glandular cells in DCIS lesions which were found adjacent to invasive cancers. Significantly, both as a continuous variable (hazard ratio = 4.70; 95% CI = 1.52 – 14.6; $P = 0.0073$), and as a dichotomized variable (Figure 3B; $P = 0.0002$), 14-3-3 σ expression in DCIS lesions was a strong univariate predictor of patient survival. As shown in Figure 3b, when patients were dichotomized based on their 14-3-3 σ protein levels into quartiles, we found that the top quartile of patients with the highest expression of 14-3-3 σ , had a significantly worse prognosis than the remaining patients with

lower expression levels. Interestingly, higher 14-3-3 σ expression in DCIS was correlated with higher stage ($P = 0.0217$), the presence of lymphovascular invasion ($P = 0.0296$) and larger tumor size ($P = 0.0488$). We used multivariate Cox-model regression analysis to assess the association of 14-3-3 σ marker expression in DCIS lesions with patient survival after correcting for known clinico-pathological variables. We included 14-3-3 σ expression in a multivariate Cox model along with known clinico-pathological variables and found 14-3-3 σ to be the only variable significantly associated with outcome ($P = 0.042$, Table 2).

Finally, we examined a subset of patients who had invasive cancer as well as DCIS lesions ($n=32$). We specifically examined whether in these patients there was differential expression of these lesions and whether such information was predictive of disease outcome. To do this, we calculated a new variable $\Delta 14-3-3\sigma$ which represented $14-3-3\sigma_{\text{expression}}^{\text{IDC}}$ minus $14-3-3\sigma_{\text{expression}}^{\text{DCIS}}$ as described in Materials and Methods. Interestingly, individuals who had 14-3-3 σ expression in IDC lesions at levels lower than DCIS, i.e., $\Delta 14-3-3\sigma < 0$, had a much worse prognosis than individuals who maintained higher or equal levels of 14-3-3 σ in IDC lesions, i.e., $\Delta 14-3-3\sigma \geq 0$ ($P = 0.0017$; see Figure 4).

DISCUSSION

In this study we assessed the protein expression of 14-3-3 σ in breast tissues on a population basis using TMA technology. Although the expression level of 14-3-3 σ varied somewhat from case-to-case, there was a general trend towards increased expression frequency and intensity in DH, DCIS and IDC lesions as compared to matched normal ductal epithelium. We further examined whether expression levels had predictive power for disease outcome. When considering cells from IDC samples, relatively lower levels of 14-3-3 σ tended to indicate an increased probability of death due to breast cancer while higher expression predicted longer survival (Figure 3A). Interestingly, an even stronger predictor of survival was the expression level of 14-3-3 σ in DCIS lesions. Somewhat surprisingly, relatively higher expression of 14-3-3 σ in DCIS lesions predicted a much worse prognosis than lower 14-3-3 σ expression (Figure 3B). Finally, we compared the 14-3-3 σ expression in IDC versus DCIS lesions in cases which contained both and found that either a constant or a decreased expression of 14-3-3 σ in IDC compared to DCIS predicted a shortened survival time (Figure 4). To our knowledge this is the first study which has examined and correlated 14-3-3 σ protein expression with disease outcome (death due to breast cancer).

14-3-3 σ and Tumor Progression

Of the seven known human 14-3-3 genes, the σ isoform has been most directly implicated in cancer progression. 14-3-3 σ expression is restricted to epithelial cells, and appears to be directly regulated by p53 and BRCA1 when induced by events such as DNA damage [2; 9; 11]. Upon such induction, 14-3-3 σ can inhibit G2-M cell cycle progression by sequestering CDC2-Cyclin B complexes in the cytoplasm away from its nuclear substrates [10]. 14-3-3 σ may also induce G1-specific cell cycle arrest through interaction with specific cyclin-dependent kinases such as CDK2 and CDK4 [17]. The absence of appropriate damage-induced cell cycle pausing can lead to accumulated DNA damage and induction of chromosomal abnormalities and thus can contribute to tumor development and progression. Thus, decreased expression of 14-3-3 σ in many tumor types is consistent with a tumor suppressor role.

Although mechanistic studies of 14-3-3 σ demonstrate tumor suppressor properties whose loss of expression may be correlated with tumor progression, correlative studies have proven more inconclusive. Protein expression of 14-3-3 σ has been shown to be down-regulated in prostate [22; 43], colorectal [31], hepatocellular [14], oral [7], and small-cell lung carcinomas [28], but up-regulated in pancreatic [12] and non-small cell lung carcinomas [26;

28]. Further, conflicting results have been found even in cancers of the same tissue in gastric, endometrial, and breast carcinomas [5; 13; 27; 39].

With regard to breast cancer, Sukumar and colleagues found that 14-3-3 σ mRNA was undetectable in 45 of 48 primary breast carcinomas by Northern blot analysis due to gene silencing by CpG methylation [6; 9] and that hypermethylation of 14-3-3 σ seemed to be a relatively early event in breast cancer [41]. However, in other tumor systems, methylation does not necessarily result in decreased expression of 14-3-3 σ [8]. Proteomics analysis using 2-D gel electrophoresis also showed that 14-3-3 σ protein was lower in primary breast tumor cells compared to cultured non-malignant breast epithelium [44]. Using immunohistochemical analysis, Simooka et al. found that 14-3-3 σ protein expression in glandular cells and myoepithelium (in DCIS and DH) was lower in IDC compared to DCIS or DH [37]. In contrast, Moreira et al. found that a decrease expression of 14-3-3 σ protein in breast tumors to be a less frequent event than previously reported [5].

It is interesting that in the cohort of patients that we examined, a relatively lower expression of 14-3-3 σ in DCIS lesions predicted a more favorable survival time, while the opposite was true in IDC cells and when considering a decreased expression in IDC compared to DCIS. Although 14-3-3 σ protein functions under the control of p53 to promote cell cycle arrest at the G2/M checkpoint following DNA damage [4; 17], 14-3-3 σ appears to also be regulated by other factors under different circumstances such as p63, estrogen-induced zinc finger protein [42], and insulin-like growth factor receptor (IGF-IR) [47]. Thus, depending on the specific context and protein pathway interactions, 14-3-3 σ may appear to have relative tumor suppressor or oncogenic properties. Examples of the later point prevention of apoptosis through sequestration of Bax following DNA damage [33] and p53-independent promotion of cell cycle progression stimulated by IGF-IR [47].

Hyperactivation of IGF-IR is thought to play a role in early breast cancer development [38]. Pezzino et al. found IGF-IR to be over-expressed in primary tumors, but also observed that its levels appeared to undergo reduction during the course of disease; ironically, in advanced stages, IGF-IR actually marked more differentiated tumors with better outcome [30; 32]. Thus, we hypothesize that 14-3-3 σ over-expression under the control of IGF-IR may dominate earlier lesions such as DCIS and denote lesions which are prime for progression. More advanced invasive lesions may develop IGF-IR independence and, combined with decreasing 14-3-3 σ may promote increased chromosomal instability leading to more aggressive malignancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Stephanie Hanna and Greg Kanter for excellent technical assistance and Jacob Schatz for helpful discussion.

This work was supported in part by the Early Detection Research Network NCI CA-86366 (LG, DC, and DS) the Jonsson Comprehensive Cancer Center (JCCC) Shared Resource Core Grant at UCLA NIH NCI 2 P30 CA16042-29 (DS).

Abbreviations

TMA tissue microarray

DCIS	ductal carcinoma <i>in situ</i>
DH	ductal hyperplasia
IDC	invasive ductal carcinoma
IGF-IR	insulin-like growth factor receptor

REFERENCES

- [1]. Akahira J, Sugihashi Y, Suzuki T, Ito K, Niikura H, Moriya T, Nitta M, Okamura H, Inoue S, Sasano H, Okamura K, Yaegashi N. Decreased expression of 14-3-3 sigma is associated with advanced disease in human epithelial ovarian cancer: its correlation with aberrant DNA methylation. *Clin Cancer Res.* 2004; 10:2687–2693. [PubMed: 15102672]
- [2]. Aprelikova O, Pace AJ, Fang B, Koller BH, Liu ET. BRCA1 is a selective co-activator of 14-3-3 sigma gene transcription in mouse embryonic stem cells. *J Biol Chem.* 2001; 276:25647–25650. [PubMed: 11384963]
- [3]. Bose S, Chandran S, Mirocha JM, Bose N. The Akt pathway in human breast cancer: a tissue-array-based analysis. *Mod Pathol.* 2006; 19:238–245. [PubMed: 16341149]
- [4]. Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature.* 1999; 401:616–620. [PubMed: 10524633]
- [5]. Cunningham SC, Kamangar F, Kim MP, Hammoud S, Haque R, Iacobuzio-Donahue CA, Maitra A, Ashfaq R, Hustinx S, Heitmiller RE, Choti MA, Lillemoe KD, Cameron JL, Yeo CJ, Schlick RD, Montgomery E. Claudin-4, mitogen-activated protein kinase kinase 4, and stratifin are markers of gastric adenocarcinoma precursor lesions. *Cancer Epidemiol Biomarkers Prev.* 2006; 15:281–287. [PubMed: 16492916]
- [6]. Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR, Sukumar S. High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. *Proc Natl Acad Sci U S A.* 2000; 97:6049–6054. [PubMed: 10811911]
- [7]. Gasco M, Bell AK, Heath V, Sullivan A, Smith P, Hiller L, Yulug I, Numico G, Merlano M, Farrell PJ, Tavassoli M, Gusterson B, Crook T. Epigenetic inactivation of 14-3-3 sigma in oral carcinoma: association with p16(INK4a) silencing and human papillomavirus negativity. *Cancer Res.* 2002; 62:2072–2076. [PubMed: 11929827]
- [8]. Henrique R, Jeronimo C, Hoque MO, Carvalho AL, Oliveira J, Teixeira MR, Lopes C, Sidransky D. Frequent 14-3-3 sigma promoter methylation in benign and malignant prostate lesions. *DNA Cell Biol.* 2005; 24:264–269. [PubMed: 15812243]
- [9]. Hermeking H. The 14-3-3 cancer connection. *Nat Rev Cancer.* 2003; 3:931–943. [PubMed: 14737123]
- [10]. Hermeking H, Benzinger A. 14-3-3 proteins in cell cycle regulation. *Semin Cancer Biol.* 2006; 16:183–192. [PubMed: 16697662]
- [11]. Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein B. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell.* 1997; 1:3–11. [PubMed: 9659898]
- [12]. Iacobuzio-Donahue CA, Ashfaq R, Maitra A, Adsay NV, Shen-Ong GL, Berg K, Hollingsworth MA, Cameron JL, Yeo CJ, Kern SE, Goggins M, Hruban RH. Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. *Cancer Res.* 2003; 63:8614–8622. [PubMed: 14695172]
- [13]. Ito K, Suzuki T, Akahira J, Sakuma M, Saitou S, Okamoto S, Niikura H, Okamura K, Yaegashi N, Sasano H, Inoue S. 14-3-3sigma in endometrial cancer--a possible prognostic marker in early-stage cancer. *Clin Cancer Res.* 2005; 11:7384–7391. [PubMed: 16243811]
- [14]. Iwata N, Yamamoto H, Sasaki S, Itoh F, Suzuki H, Kikuchi T, Kaneto H, Iku S, Ozeki I, Karino Y, Satoh T, Toyota J, Satoh M, Endo T, Imai K. Frequent hypermethylation of CpG islands and

- loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma. *Oncogene*. 2000; 19:5298–5302. [PubMed: 11077447]
- [15]. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin*. 2007; 57:43–66. [PubMed: 17237035]
- [16]. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med*. 1998; 4:844–847. [PubMed: 9662379]
- [17]. Laronga C, Yang HY, Neal C, Lee MH. Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. *J Biol Chem*. 2000; 275:23106–23112. [PubMed: 10767298]
- [18]. Lee MH, Lozano G. Regulation of the p53-MDM2 pathway by 14-3-3 sigma and other proteins. *Semin Cancer Biol*. 2006; 16:225–234. [PubMed: 16697215]
- [19]. Leffers H, Madsen P, Rasmussen HH, Honore B, Andersen AH, Walbum E, Vandekerckhove J, Celis JE. Molecular cloning and expression of the transformation sensitive epithelial marker stratifin. A member of a protein family that has been involved in the protein kinase C signalling pathway. *J Mol Biol*. 1993; 231:982–998. [PubMed: 8515476]
- [20]. Liu X, Minin V, Huang Y, Seligson DB, Horvath S. Statistical methods for analyzing tissue microarray data. *J Biopharm Stat*. 2004; 14:671–685. [PubMed: 15468758]
- [21]. Liu Y, Chen Q, Zhang JT. Tumor suppressor gene 14-3-3sigma is down-regulated whereas the proto-oncogene translation elongation factor 1delta is up-regulated in non-small cell lung cancers as identified by proteomic profiling. *J Proteome Res*. 2004; 3:728–735. [PubMed: 15359725]
- [22]. Lodygin D, Diebold J, Hermeking H. Prostate cancer is characterized by epigenetic silencing of 14-3-3sigma expression. *Oncogene*. 2004; 23:9034–9041. [PubMed: 15489902]
- [23]. Mhawech P, Benz A, Cerato C, Greloz V, Assaly M, Desmond JC, Koeffler HP, Lodygin D, Hermeking H, Herrmann F, Schwaller J. Downregulation of 14-3-3sigma in ovary, prostate and endometrial carcinomas is associated with CpG island methylation. *Mod Pathol*. 2005; 18:340–348. [PubMed: 15257317]
- [24]. Moreira JM, Ohlsson G, Rank FE, Celis JE. Down-regulation of the tumor suppressor protein 14-3-3sigma is a sporadic event in cancer of the breast. *Mol Cell Proteomics*. 2005; 4:555–569. [PubMed: 15644556]
- [25]. Muslin AJ, Tanner JW, Allen PM, Shaw AS. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*. 1996; 84:889–897. [PubMed: 8601312]
- [26]. Nakajima T, Shimooka H, Weixa P, Segawa A, Motegi A, Jian Z, Masuda N, Ide M, Sano T, Oyama T, Tsukagoshi H, Hamanaka K, Maeda M. Immunohistochemical demonstration of 14-3-3 sigma protein in normal human tissues and lung cancers, and the preponderance of its strong expression in epithelial cells of squamous cell lineage. *Pathol Int*. 2003; 53:353–360. [PubMed: 12787309]
- [27]. Nakayama H, Sano T, Motegi A, Oyama T, Nakajima T. Increasing 14-3-3 sigma expression with declining estrogen receptor alpha and estrogen-responsive finger protein expression defines malignant progression of endometrial carcinoma. *Pathol Int*. 2005; 55:707–715. [PubMed: 16271083]
- [28]. Osada H, Tatematsu Y, Yatabe Y, Nakagawa T, Konishi H, Harano T, Tezel E, Takada M, Takahashi T. Frequent and histological type-specific inactivation of 14-3-3sigma in human lung cancers. *Oncogene*. 2002; 21:2418–2424. [PubMed: 11948426]
- [29]. Pantuck AJ, Seligson DB, Klatte T, Yu H, Leppert JT, Moore L, O'Toole T, Gibbons J, Belldgrun AS, Figlin RA. Prognostic relevance of the mTOR pathway in renal cell carcinoma: implications for molecular patient selection for targeted therapy. *Cancer*. 2007; 109:2257–2267. [PubMed: 17440983]
- [30]. Papa V, Gliozzo B, Clark GM, McGuire WL, Moore D, Fujita-Yamaguchi Y, Vigneri R, Goldfine ID, Pezzino V. Insulin-like growth factor-I receptors are overexpressed and predict a low risk in human breast cancer. *Cancer Res*. 1993; 53:3736–3740. [PubMed: 8339284]
- [31]. Perathoner A, Pirkebner D, Brandacher G, Spizzo G, Stadlmann S, Obrist P, Margreiter R, Amberger A. 14-3-3sigma expression is an independent prognostic parameter for poor survival in colorectal carcinoma patients. *Clin Cancer Res*. 2005; 11:3274–3279. [PubMed: 15867223]

- [32]. Pezzino V, Papa V, Milazzo G, Gliozzo B, Russo P, Scalia PL. Insulin-like growth factor-I (IGF-I) receptors in breast cancer. *Ann N Y Acad Sci.* 1996; 784:189–201. [PubMed: 8651569]
- [33]. Samuel T, Weber HO, Rauch P, Verdoodt B, Eppel JT, McShea A, Hermeking H, Funk JO. The G2/M regulator 14-3-3sigma prevents apoptosis through sequestration of Bax. *J Biol Chem.* 2001; 276:45201–45206. [PubMed: 11574543]
- [34]. Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M, Kurdistani SK. Global histone modification patterns predict risk of prostate cancer recurrence. *Nature.* 2005; 435:1262–1266. [PubMed: 15988529]
- [35]. Shen D, Nooraie F, Elshimali Y, Lonsberry V, He J, Bose S, Chia D, Seligson D, Chang HR, Goodlick L. Decreased expression of annexin A1 is correlated with breast cancer development and progression as determined by a tissue microarray analysis. *Hum Pathol.* 2006; 37:1583–1591. [PubMed: 16949910]
- [36]. Shvarts O, Seligson D, Lam J, Shi T, Horvath S, Figlin R, Belldegrun A, Pantuck AJ. p53 is an independent predictor of tumor recurrence and progression after nephrectomy in patients with localized renal cell carcinoma. *J Urol.* 2005; 173:725–728. [PubMed: 15711252]
- [37]. Simooka H, Oyama T, Sano T, Horiguchi J, Nakajima T. Immunohistochemical analysis of 14-3-3 sigma and related proteins in hyperplastic and neoplastic breast lesions, with particular reference to early carcinogenesis. *Pathol Int.* 2004; 54:595–602. [PubMed: 15260850]
- [38]. Surmacz E. Function of the IGF-I receptor in breast cancer. *J Mammary Gland Biol Neoplasia.* 2000; 5:95–105. [PubMed: 10791772]
- [39]. Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H, Imai K. Inactivation of the 14-3-3 sigma gene is associated with 5' CpG island hypermethylation in human cancers. *Cancer Res.* 2000; 60:4353–4357. [PubMed: 10969776]
- [40]. Tzivion G, Shen YH, Zhu J. 14-3-3 proteins; bringing new definitions to scaffolding. *Oncogene.* 2001; 20:6331–6338. [PubMed: 11607836]
- [41]. Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S. Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer. *Oncogene.* 2001; 20:3348–3353. [PubMed: 11423985]
- [42]. Urano T, Saito T, Tsukui T, Fujita M, Hosoi T, Muramatsu M, Ouchi Y, Inoue S. Efp targets 14-3-3 sigma for proteolysis and promotes breast tumour growth. *Nature.* 2002; 417:871–875. [PubMed: 12075357]
- [43]. Urano T, Takahashi S, Suzuki T, Fujimura T, Fujita M, Kumagai J, Horie-Inoue K, Sasano H, Kitamura T, Ouchi Y, Inoue S. 14-3-3sigma is down-regulated in human prostate cancer. *Biochem Biophys Res Commun.* 2004; 319:795–800. [PubMed: 15184053]
- [44]. Vercoutter-Edouart AS, Lemoine J, Le Bourhis X, Louis H, Boilly B, Nurcombe V, Revillion F, Peyrat JP, Hondermarck H. Proteomic analysis reveals that 14-3-3sigma is down-regulated in human breast cancer cells. *Cancer Res.* 2001; 61:76–80. [PubMed: 11196201]
- [45]. Yang H, Wen YY, Zhao R, Lin YL, Fournier K, Yang HY, Qiu Y, Diaz J, Laronga C, Lee MH. DNA damage-induced protein 14-3-3 sigma inhibits protein kinase B/Akt activation and suppresses Akt-activated cancer. *Cancer Res.* 2006; 66:3096–3105. [PubMed: 16540659]
- [46]. Yang HY, Wen YY, Chen CH, Lozano G, Lee MH. 14-3-3 sigma positively regulates p53 and suppresses tumor growth. *Mol Cell Biol.* 2003; 23:7096–7107. [PubMed: 14517281]
- [47]. Zhang Y, Karas M, Zhao H, Yakar S, LeRoith D. 14-3-3sigma mediation of cell cycle progression is p53-independent in response to insulin-like growth factor-I receptor activation. *J Biol Chem.* 2004; 279:34353–34360. [PubMed: 15187095]

Figure 1. 14-3-3 σ protein expression in breast tissues

Immunohistochemical staining for 14-3-3 σ protein is seen on representative breast tissue samples. (A) Normal tissue showing weak cytoplasmic epithelial staining of glandular cells. (B) Ductal hyperplasia showing moderate staining (C) DCIS showing strong staining and (D) IDC showing moderate to strong staining. All pictures were taken with a 20 $\times$ objective.

Figure 2. 14-3-3 σ protein expression on the breast TMA stratified by histological category
 The integrated intensity of 14-3-3 σ cytoplasmic protein expression in cells staining by immunohistochemistry is shown in these box plots. N is the number of spots in each histologic group. The histologies shown are normal breast glandular epithelial cells from breast reduction surgeries (BR), morphologically normal cells adjacent to cancerous lesions (NL), ductal hyperplasia (DH), ductal carcinoma in situ (DCIS), invasive ductal carcinoma from primary cancers (IDC), and metastatic cells (MET). The middle line of the box plot represents the median; the upper and lower ends of the box are the 75th and 25th percentiles, respectively; the whiskers represent the 95th percentile. The Mann-Whitney *U* test was used for two-group comparisons. Expression in normal cells was significantly lower than DH ($P = 0.0066$), DCIS, ($P < 0.0001$), IDC ($P < 0.0001$) and MET ($P = 0.0026$).

Figure 3. 14-3-3 σ protein expression in IDC and DCIS predicts the probability of survival in individuals with breast cancer

The graphs show Kaplan-Meier disease-specific survival curves dichotomized by higher versus lower 14-3-3 σ protein expression in glandular epithelium. N is the number of patients in each group. **A) 14-3-3 σ expression in IDC.** Dashed line shows higher 14-3-3 σ expression in IDC (≥ 0.93 integrated intensity) and solid line shows lower 14-3-3 σ expression in IDC (< 0.93 integrated intensity). Individuals with higher levels of 14-3-3 σ tend to have a slightly higher increased probability of survival compared to those with lower 14-3-3 σ ($P = 0.056$). **B) 14-3-3 σ expression in DCIS.** Dashed line shows the top quartile of patients with the highest 14-3-3 σ expression in DCIS lesions (≥ 0.83 integrated intensity) and solid line shows lower 14-3-3 σ expression in DCIS (< 0.83 integrated intensity). Individuals with lower levels of 14-3-3 σ has a significantly higher increased probability of survival compared to those with higher 14-3-3 σ ($P=0.0002$).

Figure 4. The change in 14-3-3 σ protein expression between IDC and DCIS ($\Delta 14-3-3\sigma$) predicts the probability of survival in individuals with breast cancer

The integrated expression of 14-3-3 σ in glandular epithelium from DCIS lesions ($DCIS_{\text{expression}}$) was subtracted from the integrated expression values from glandular epithelium in IDC lesions ($IDC_{\text{expression}}$) to produce a new variable ($\Delta 14-3-3\sigma$). The graphs show Kaplan-Meier disease-specific survival curves dichotomized by $\Delta 14-3-3\sigma \geq 0$ (solid line) versus $\Delta 14-3-3\sigma < 0$ (dashed line). N is the number of patients in each group. Individuals with $\Delta 14-3-3\sigma < 0$ (i.e., ($IDC_{\text{expression}} < DCIS_{\text{expression}}$)) has a significantly higher increased probability of death due to disease compared to those with $\Delta 14-3-3\sigma \geq 0$ ($P=0.0017$).

Table 1
Clinicopathologic parameters and 14-3-3 σ expression

All variables were tested using univariate Cox-model regression analysis as either a continuous or dichotomized variable, as noted. Abbreviations: CI, confidence interval; LVI, lymphovascular invasion; ER, estrogen receptor; PR, progesterone receptor; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ.

Variables	Descriptive Statistics	Hazard Ratio (95% CI)	P-Value
Age at Diagnosis	Continuous		
Median (Range)	51 (30 – 89)	0.97 (0.93 – 1.01)	0.16
25th to 75th Quartile	44 – 64		
Clinical Stage	Continuous		
I	33 (46%)	3.77 (1.87 – 7.60)	0.00022
II	25 (35%)		
III	12 (17%)		
IV	1 (1%)		
Tumor Grade	Continuous		
1	16 (22%)	1.99 (0.902 – 4.37)	0.088
2	21 (30%)		
3	34 (48%)		
Metastasis	Dichotomized		
Absent	34 (48%)	6.43 (1.79 – 23.2)	0.0044
Present	25 (35%)		
Unknown	12 (17%)		
Tumor Size (cm)	Continuous		
Median (Range)	1.90 (0.1 – 9.0)	1.26 (0.995 – 1.61)	0.055
25th to 75th Quartile	1.00 – 2.75		
LVI	Dichotomized		
Absent	26 (37%)	2.34 (0.782 – 6.98)	0.13
Present	24 (34%)		
Unknown	21 (29%)		
ER Status	Dichotomized		
Positive	48 (68%)	0.509 (0.181 – 1.43)	0.20
Negative	18 (25%)		
Unknown	5 (7%)		
PR Status	Dichotomized		
Positive	44 (62%)	0.533 (0.193 – 1.47)	0.23
Negative	24 (34%)		
Unknown	3 (4%)		
HER-2/new Status	Dichotomized		0.052

Variables	Descriptive Statistics	Hazard Ratio (95% CI)	P-Value
Positive	18 (25%)	2.95 (0.991 – 8.79)	
Negative	45 (64%)		
Unknown	8 (11%)		
14-3-3 σ in IDC	Continuous	0.628 (0.265 – 1.49)	0.29
Median (Range)	0.61 (0 – 3.0)		
25th to 75th Quartile	0.05 – 1.18		
14-3-3 σ in DCIS	Continuous	4.70 (1.52 – 14.6)	0.0073
Median (Range)	0.50 (0 – 2.2)		
25th to 75th Quartile	0.00 – 0.84		

Table 2**Multivariate Cox Model**

Grade was dichotomized as low (grade 1 and 2) vs. high (grade 3). Stage was dichotomized as low (stage I and II) vs. high (stage III and IV).

Variable	Hazard Ratio	95% Confidence Interval	P-Value
14-3-3 σ (Continuous)	7.98	1.08 – 59.01	0.042
Grade	0.36	0.058 – 2.29	0.280
Stage	8.78	0.99 – 77.86	0.051
ER Status	0.12	0.0092 – 1.53	0.100
Age	1.06	0.97 – 1.16	0.210