Advances in Brief

Tissue Microarray Analysis of Signal Transducers and Activators of Transcription 3 (Stat3) and Phospho-Stat3 (Tyr705) in Node-negative Breast Cancer Shows Nuclear Localization Is Associated with a Better Prognosis[1]

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Abstract

Purpose: **Although a high frequency of tumors contain constitutively activated signal transducers and activators of transcription 3 (Stat3), its relationship to breast cancer and patient survival has not been determined in a large retrospective study of node-negative tumors. To further elucidate the role of Stat3 in breast cancers, the expression patterns of Stat3 and Phospho-tyrosine residue 705 (Tyr705) Stat3 were correlated with survival outcome and clinicopathological parameters in a large cohort of node-negative breast cancer tumors.**

Experimental Design: **Immunohistochemical analysis of Stat3 and Phospho-Stat3 was performed on a breast cancer tissue microarray of 346 node-negative breast cancer specimens. These results were correlated with overall survival and other clinicopathological data.**

***Results*: Positive Stat3 cytoplasmic expression was seen in 69.2% of tumors, and positive Phospho-Stat3 (Tyr705) cytoplasmic expression was seen in 19.6% of tumors. Neither cytoplasmic expression showed significant association with survival or other clinical parameters. However, 23.1% of tumors had positive Stat3 nuclear expression, and those patients had a significantly improved short-term survival ($P = 0.0332$) at 5 years of follow-up. Upon analysis of positive Phospho-Stat3 (Tyr705) nuclear expression, seen in 43.5% of tumors, positive tumors had a significantly improved survival at both short-term 5-year survival ($P = 0.0054$) and long-term 20-year ($P = 0.0376$) survival analysis. Additionally, positive Phospho-Stat3 (Tyr705) nuclear expression is an independent prognostic marker of better overall survival node-negative breast cancer by multivariate analyses that included the variables of nuclear grade, Ki-67, estrogen receptor staining, progesterone receptor staining, Her2 staining, age, and tumor size.**

Conclusions: **These findings support a role for Stat3 and Phospho-Stat3 (Tyr705) overexpression in node-negative breast cancer and provide initial evidence that Phospho-Stat3 (Tyr705) may be a marker for improved overall survival independent of other prognostic markers.**

Introduction

Stat[3] proteins are transcription factors that are activated by a wide range of cytokine receptor-associated kinases, growth factor receptor tyrosine kinases, and nonreceptor tyrosine kinases originally defined as the signaling mechanism for IFN receptors (1). Stat3 has posed a special challenge because, unlike other Stat proteins, it shows an embryonic lethal phenotype (2). This has complicated characterization of its function and, in part, led to some controversy regarding the role of Stat3 in tumors. Although there is some evidence that it is an oncogene, there is also evidence that it behaves as a tumor suppressor (3).

Stat3, like other Stat proteins, contains an SH2 domain, which is a common motif found in signaling molecules that mediate protein-protein interactions by binding directly to specific phosphotyrosines. Stat3 is activated by phosphorylation of Tyr705 by c-Jun NH_2-terminal kinases, growth factor tyrosine kinases, or other mechanisms (4). Phosphorylation precipitates dimerization, which is stabilized by reciprocal phospho-tyrosine SH2 interactions. Stat3 dimers then move to the nucleus, where they bind to specific DNA response elements in target gene promoters and enable gene transcription. Some target genes of Stat3 include those involved in apoptosis, cell cycle regulation, and induction of growth arrest such as Bcl-xL, cyclin D1, p21, WAF1/CIP1, and c-myc (5).

Because Stat3 plays such a pleomorphic role in signal transduction, its role as an oncogene or a tumor suppressor may be a function of the setting. In the context of the mouse mammary gland, Stat3 is activated both during apoptotic involution and during the highly proliferative phase of early pregnancy (6). Subsequent conditional knockout studies in mice have shown that Stat3 is essential in mammary gland epithelial cell apoptosis

Received 5/13/02; revised 9/19/02; accepted 9/30/02.

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[1] Supported by grants from the Patrick and Catherine Weldon Donaghue Foundation for Medical Research, the United States Army (DAMD17-01-1-0463), and grants from the NIH, including RO-1 GM57604.

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[3] The abbreviations used are: Stat, signal transducers and activators of transcription; Tyr705, tyrosine residue 705; ER, estrogen receptor; PR, progesterone receptor; TBS, Tris-buffered saline.

and involution (7, 8). In humans, Stat3 is activated in several mammary epithelial cells and breast carcinoma cell lines (9–13). There is evidence of increased Stat binding in the nuclei of breast cancer tumors compared with normal breast tissue or benign lesions (14, 15). An immunohistochemical study of 62 cases of invasive malignant breast cancer tumors by Berclaz *et al.* (16) found that Stat3 was expressed only in the cytoplasm of nontumor regions of breast cancer specimens but was expressed in both the cytoplasm and nuclei of malignant regions of the specimens. However, they found no correlation between Stat3 subcellular localization expression and survival. Recently, an antibody specific for the activated (phospho-Tyr705) form of Stat3 has become available (17). In prostate tissue, the activated form of Stat3 localized predominantly to the nuclei of malignant glands (18). This activated form may be a better probe for function than total Stat3, but it has not yet been evaluated in breast cancer tissues.

Tissue microarray technology (19, 20) is a highly efficient and economical way to evaluate hundreds of tumors (21). Breast cancer is a common application of this technology. Breast cancer tissue microarray cores have been demonstrated to be representative of the conventional tumor specimens because as they have very high concordance for common biomarkers as well as reproducible prognostic associations (22–24) and recently reviewed (29). Results from validation studies have shown that in the majority of the cases, one tissue microarray core alone could adequately represent the antigen expression of the corresponding whole section and be representative of the association between the staining level and clinical end point (24). The use of archival tissue for a retrospective study allows for protein expression to be analyzed with the benefit of long-term followup (survival) information. Here, we evaluate the expression and subcellular localization of both Stat3 and Phospho-Stat3 (Tyr705) by immunohistochemistry in a tissue microarray containing a cohort of 346 node-negative archival-infiltrating breast cancer tumors. The expression and localization information were correlated with standard clinicopathological factors and with overall patient survival.

Materials and Methods

Tissue Microarray Construction. The tissue microarrays were constructed as previously described (20), with 0.6-mm diameter cores spaced 0.8 mm apart. Expression was evaluated on two arrays, including a validation array and a cohort array. The validation array, as previously described (22), was constructed with three replicate cores for each of the breast cancer cases. The validation array consisted of the following number of cases from each decade: 1930s (14 cases), 1940s (8 cases), 1950s (12 cases), 1960s (13 cases), 1970s (11 cases), 1980s (9 cases), and 1990s (15 cases). The cohort node-negative breast cancer array was constructed from paraffin-embedded formalin-fixed tissue blocks from the Yale University Department of Pathology archives. The specimens were resected between 1962 and 1980, with a follow-up range of patient breast cancer history between 4 months and 53.8 years, with a mean follow-up time of 15.6 years. Time between tumor resection and fixation was not available for the tumors in this cohort. Representative tumor regions were selected for coring and placement in the tissue microarray using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD). The tissue microarrays were then cut to 5-μm sections and placed onto slides by use of an adhesive tape-transfer system (Instrumedics, Inc., Hackensack, NJ) and UV cross-linking.

Immunohistochemistry. The tissue microarray slides were deparaffinized with xylene rinses and then transferred through two changes of 100% ethanol. Endogenous peroxidase activity was blocked by a 30-min incubation in a 2.5% hydrogen peroxide/methanol buffer. Antigen retrieval was performed by boiling the slides in a pressure cooker filled with a sodium citrate buffer (pH 6.0). After antigen retrieval, the slides were incubated with 0.3%BSA/1× TBS for 1 h at room temperature to reduce nonspecific background staining, followed by a series of 2-min rinses in 1× TBS, 1× TBS/0.01% Triton, and 1× TBS. Primary antibody was applied for 1 h at room temperature (1:100 dilution of Phospho-Stat (Tyr705) antibody (Cell Signaling Technology, Beverly, MA) in 0.3%BSA/1× TBS or 1:150 dilution of Stat3 antibody (Cell Signaling Technology) in 0.3%BSA/1× TBS). After a series of TBS rinses as described above, bound antibody was detected by using an antirabbit horseradish peroxidase-labeled polymer secondary antibody from the Dako Envision TM + System (Dako, Carpinteria, CA). The slides were rinsed in the TBS series, visualized with a 10-min incubation of liquid 3,3′-diaminobenzidine in buffered substrate (Dako) for 10 min. Finally, the slides were counterstained with hematoxylin and mounted with Immunomount (Shandon, Pittsburgh, PA). Immunohistochemical staining was also done for ER, PR, and Her2 as described previously (22). Ki-67 expression was assessed using purified antihuman monoclonal antibody (1:200, overnight incubation; PharMingen, San Diego, CA).

Evaluation of Immunohistochemical Staining. For each spot, the regions of most intense and/or predominant staining pattern were scored by eye. Traditionally, immunohistochemistry scoring of stain intensity includes a variable for the area percentage stained with the specimen, but because of the small size of the spot (0.6 mm in diameter), no area variable is included. The nuclear and cytoplasmic staining was determined separately for each specimen. The staining intensity was graded on the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. For specimens that were uninterpretable or were not infiltrating carcinoma, a score of not applicable (N/A) was given. Scoring of the tissue microarray was completed by two independent observers (M. D. F. and D. K.), with very high correlation between scorers ($P < 0.0001$) for both of the Stat3 cytoplasmic and nuclear localization scores and for both of the Phospho-Stat3 (Tyr705) cytoplasmic and nuclear localization scores. Discrepant scores between the two observers were averaged to arrive at a single final score. In order for a tumor core to be considered positive for Stat3 or Phospho-Stat3 (Tyr705) expression in either the cytoplasm or nucleus, it had to have a score of one (1) or greater from both observers.

Statistical Analysis. All analyses were completed using Statview 5.0.1 (SAS Institute Inc., Cary, NC). The correlation between the scores of both scorers and the relationships of Stat3 expression or Phospho-Stat3 (Tyr705) expression and clinicopathological parameters were measured using the χ^2 test. The prognostic significance of the parameters was assessed for pre-

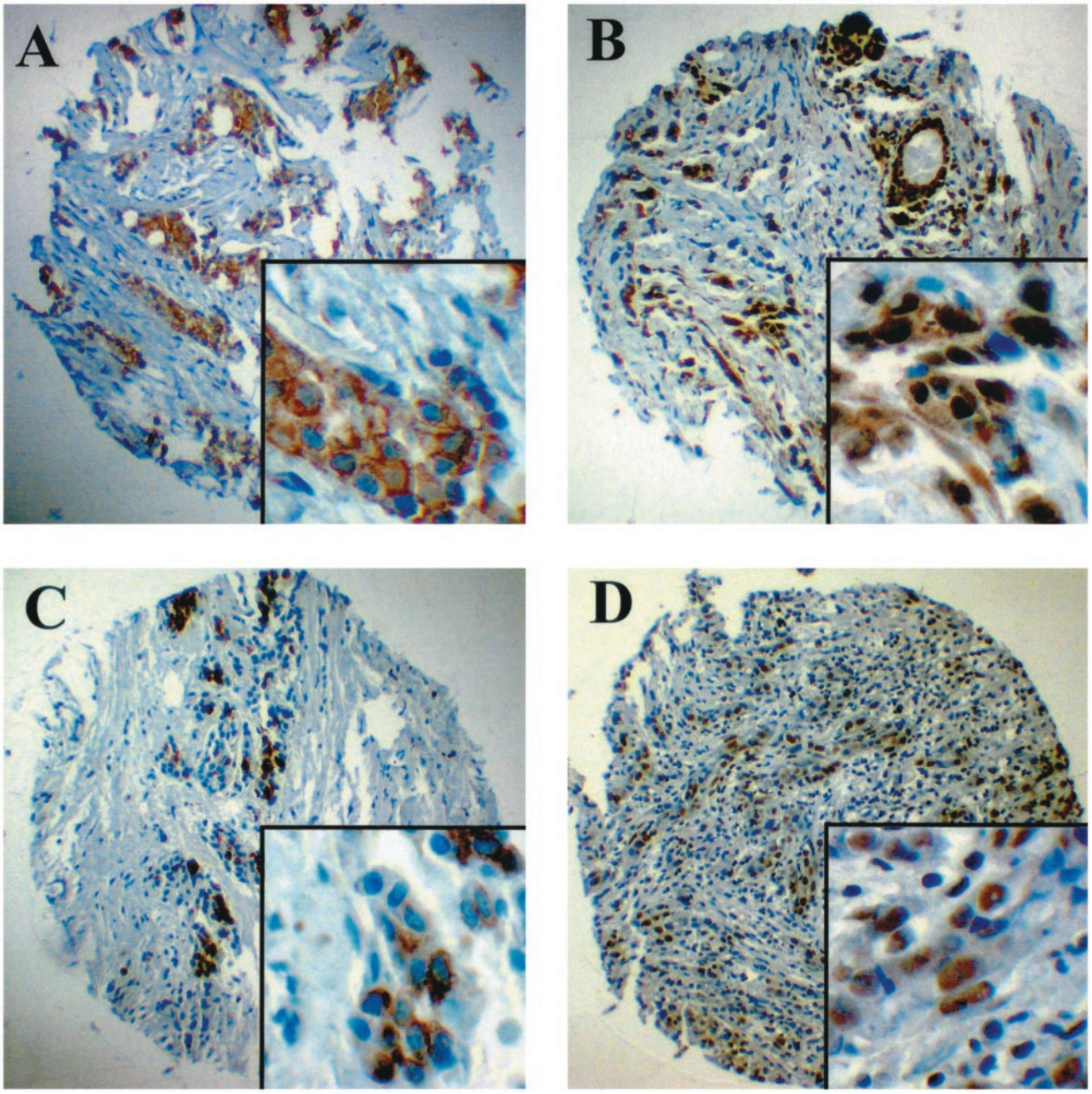

Fig. 1 Stat3 and Phospho-Stat3 (Tyr705) expression on representative node negative breast cancer tissue microarray spots as follows: *A*, strong Stat3 cytoplasmic staining; *B*, strong Stat3 nuclear staining shown with some cytoplasmic staining; *C*, strong Phospho-Stat3 (Tyr705) cytoplasmic staining; *D*, strong Phospho-Stat3 (Tyr705) nuclear staining. Figures are at ×100 magnification, inset at ×400 magnification.

dictive value using the Cox proportional hazards model with overall survival as an end point. Survival curves were calculated using the Kaplan-Meier method, with the significance evaluated using the Mantel-Cox long-rank test.

Results

Validation of the Antibodies on Archival Tissues. Because this study was done entirely using tissue microarrays with specimens from the 1960s to 1980s, immunohistochemical analysis of a validation tissue microarray was performed to study the preservation of antigenicity in older breast cancer tissue specimens. Although the validation was done for both the Stat3 antibody and the Phospho-Stat3 (Tyr705), we were particularly concerned that the phospho-specific antibody would be affected by long-term storage of the tissue in paraffin. Pathologist-based evaluation of the validation array showed representative staining patterns for both antibodies throughout the last 60 years. The number of patients sampled from each time period is insufficient

for statistical analysis, but no progressive or systematic loss of staining was seen for either antibody.

Immunohistochemical Staining of Node-negative Breast Cancer Tissue Microarray. Of the 346 node-negative breast cancer tumors on the tissue microarray, 286 tumor cores (82.7%) were interpretable for Stat3 staining, of which 258 of them (90.2%) had associated survival information. Uninterpretable spots were because of either loss of tissue on the tissue microarray or no tumor cells in the spot. The immunohistochemical staining of the breast cancer tissue microarray with Stat3 showed cytoplasmic (Fig. 1*A*) and/or nuclear (Fig. 1*B*) localization. There were 198 tumors (69.2%) that were positive for Stat3 cytoplasmic staining and 66 tumors (23.1%) positive for Stat3 nuclear staining. The distribution of Stat3 cytoplasmic staining levels and Stat3 nuclear staining levels are shown in Fig. 2, *A* and *B*, respectively. A subset of 64 tumors was positive for both Stat3 cytoplasmic staining and Stat3 nuclear staining (22.4%).

Staining with Phospho-Stat3 (Tyr705) also showed cytoplasmic (Fig. 1*C*) and/or nuclear localization (Fig. 1*D*). There were 285 tumor cores (82.4%) interpretable for Phosho-Stat3 (Tyr705) staining, of which 255 (89.5%) had associated survival information. Uninterpretable spots were because of either loss of tissue on the tissue microarray or no tumor cells in the spot. There were 56 tumors (19.6%) positive for Phospho-Stat3 (Tyr705) cytoplasmic staining and 124 tumors (43.5%) positive for Phospho-Stat3 (Tyr705) nuclear staining. A subset of tumors was positive for both Phospho-Stat3 (Tyr705) nuclear and cytoplasmic staining (44 of 285, 15.4%). The distribution of Phospho-Stat3 (Tyr705) cytoplasmic and nuclear staining are respectively shown in Fig. 2, *C* and *D*.

Survival Analyses. The expression of Stat3 and Phospho-Stat3 (Tyr705) as evaluated by immunohistochemical staining were correlated with overall survival of the patients at both 5- and 20-year follow-up times. To determine whether Stat3 or Phospho-Stat3 (Tyr705) expression level is correlated with outcome, Kaplan-Meier survival curves were generated for each antibody and subcellular localization. There was no significant difference in overall survival for cytoplasmic Stat3 staining at either 5 years (Fig. 3*A*) or 20 years (Fig. 3*E*) of follow-up or for Phospho-Stat3 (Tyr705) cytoplasmic staining at follow-up of 5 years (Fig. 3*C*) or 20 years (Fig. 3*G*). However, significant survival differences were seen with Stat3 and Phospho-Stat3 (Tyr705) nuclear staining.

Positive Stat3 nuclear staining was significantly correlated with better outcome at 5 years of follow-up ($P = 0.0332$; Fig. 3*B*) but was not correlated with survival long term at 20 years (Fig. 3*F*). A significant correlation between Phospho-Stat3 (Tyr705) positive nuclear staining and better outcome was seen at both 5 and 20 years of follow-up (Fig. 3*D*; $P = 0.0054$) and (Fig. 3*H*; $P = 0.0376$) respectively. Sixty-four of 66 tumors positive for Stat3 nuclear staining were also positive for Stat3 cytoplasmic staining (97%) and, therefore, had virtually identical survival outcomes as Stat3 nuclear staining alone (5-year follow-up, $P = 0.0375$; 20-year follow-up, $P = 0.2140$; survival curves not shown). There was no significant difference in survival between tumors positive for both PhosphoStat3 (Tyr705) nuclear and cytoplasmic staining at either a 5-year survival cutoff ($P = 0.1098$) or 20 years ($P = 0.0829$; survival curves not shown).

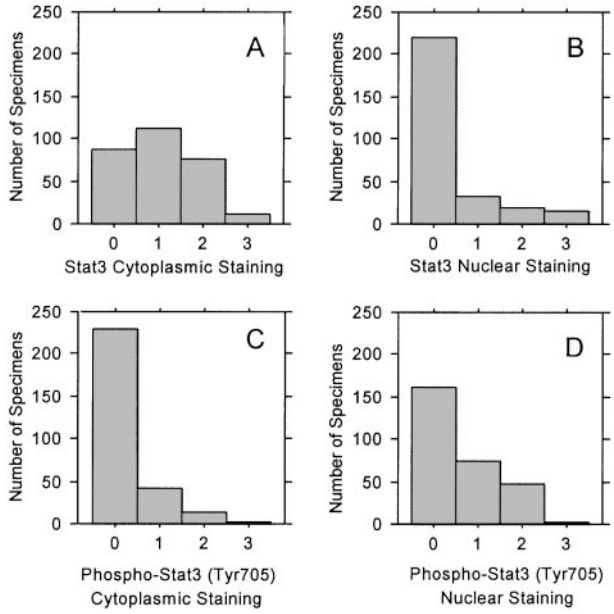

Fig. 2 Distribution of Stat3 and Phospho-Stat3 (Tyr705) immunohistochemical staining scores in node-negative breast cancer. *A*, Stat3 cytoplasmic staining scores; *B*, Stat3 nuclear staining scores; *C*, Phospho-Stat3 (Tyr705) cytoplasmic staining scores; *D*, Phospho-Stat3 (Tyr705) nuclear staining scores.

Clinicopathological Correlations and Multivariate Analyses. Multivariate analysis using the Cox proportional hazards model was done to assess the independent predictive value of Stat3 nuclear and Phospho-Stat3 (Tyr705) nuclear expression. The classic prognostic variables used to assess independence included tumor size, patient age at diagnosis, nuclear grade, Ki-67 nuclear staining as an index of proliferation rate, ER expression, PR expression, and HER2 expression. Stat3 nuclear staining was not a statistically significant independent predictor of overall survival ($P = 0.0556$; Table 1). However, Phospho-Stat3 (Tyr705) nuclear staining was independently predictive of overall survival ($P = 0.0469$) with a relative risk of 2.35 (Table 2). Tumor size of >2 cm was the only other variable with independent prognostic value in the multivariate analyses shown in Tables 1 and 2. Statistically significant variables in the multivariate analyses tables are highlighted in bold.

Discussion

To our knowledge, this is the first study to examine the expression of Stat3 and tyrosine phosphorylated Stat3 in breast cancer. We used standard immunohistochemical methods to assay activated Stat3 by both assessment of subcellular localization and phosphorylation status. Because activation of Stat3 signaling involves both tyrosine phosphorylation and translocation to the nucleus, we hypothesized that either or both of these parameters could provide a representation of tumors in which a Stat3-mediated signaling pathway had been activated. Our findings show activation of Stat3 signaling as assessed by either nuclear Stat3 or Phospho-Stat3 (Tyr705) is predictive of signif-

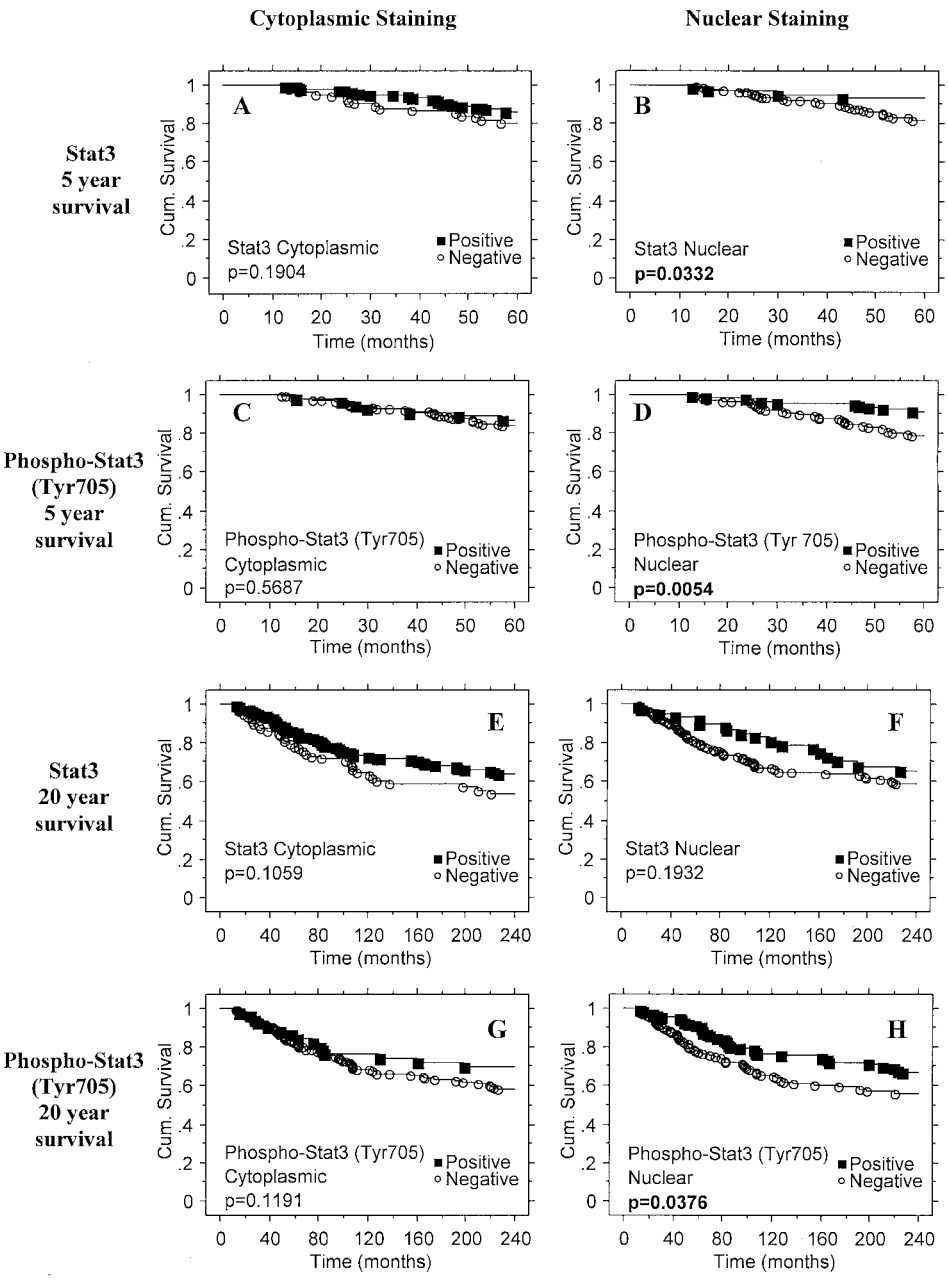

Fig. 3 Stat3 and Phospho-Stat3 (Tyr705) Kaplan-Meier survival curves in node-negative breast cancer. *A*, Stat3 cytoplasmic staining 5 year survival curve; *B*, Stat3 nuclear staining 5-year survival curve; *C*, Phospho-Stat3 (Tyr705) cytoplasmic staining 5-year survival curve; *D*, Phospho-Stat3 (Tyr705) nuclear staining 5-year survival curve; *E*, Stat3 cytoplasmic staining 20-year survival curve; *F*, Stat3 nuclear staining 20-year survival curve; *G*, Phospho-Stat3 (Tyr705) cytoplasmic staining 20-year survival curve; *H*, Phospho-Stat3 (Tyr705) nuclear staining 20-year survival curve.

icantly better clinical outcome. Furthermore, nuclear Phospho-Stat3 (Tyr705) is independent of all other commonly used prognostic markers and pathological parameters, except for tumor size.

The finding that activated Stat3 is associated with better outcome in breast cancer is subject to numerous interpretations. Because Stat3 is known to be persistently activated in src-transformed lines (25, 26), it is not surprising to find it activated in a large fraction of the tumors. The fact that it is associated with better outcome may simply mean that tumors that activate these pathways are less aggressive than tumors that progress even in the absence of Stat3 activation. Alternatively, it may be that Stat3 plays a role as a tumor suppressor protein. Evidence that Stat3 plays a role in cellular differentiation and apoptosis (8) may be consistent with better outcome in breast cancer if nuclear Phospho-Stat3 expression is selecting a group of well-differentiated tumors.

Constitutive Stat3 activation has been found in many types of cancers, including prostate (18), ovary (27), leukemia (28), and breast (16), however, there is very little data on its affect on outcome. We hope the availability of the Phospho-Stat3 antibody and our data will stimulate others to test for correlation with improved patient survival in other tumor types. Although additional investigation is needed to dissect

Table 1 Multivariate analysis of clinicopathological parameters and nuclear Stat3 expression

Multivariate analysis of Stat3 and prognostic factors with 5 year overall survival with 198 tumors (performed using a Cox proportional hazards model).

Variable	OS[a] *P*	Hazard ratio (95% CI)
Nuclear grade (high)	0.1958	2.25 (0.66–7.72)
Ki-67 (high)	0.4821	1.33 (0.60–2.93)
ER (neg)	0.9330	1.04 (0.44–2.45)
PR (neg)	0.4910	1.29 (0.62–2.70)
Her2 (pos)	0.9973	0.99 (0.38–2.55)
Age (young)	0.7682	0.81 (0.41–1.93)
Tumor size (>2 cm)	**0.0341**	**2.29 (1.06–4.93)**
Stat3 nuclear staining (neg)	0.0556	2.85 (0.97–8.33)

[a] OS, overall survival; CI, confidence interval; neg, negative; pos, positive.

Table 2 Multivariate analysis of clinicopathological parameters and nuclear Phospho-Stat3 (Tyr705) expression

Multivariate analysis of Phospho-Stat3 (Tyr705) and prognostic factors with 5-year overall survival with 195 tumors (performed using a Cox proportional hazards model).

Variable	OS *P*	Hazard ratio (95% CI)
Nuclear grade (high)	0.0674	3.96 (0.91–16.82)
Ki-67 (high)	0.5400	1.28 (0.56–2.78)
ER (neg)	0.6935	0.84 (0.35–2.03)
PR (neg)	0.7972	1.10 (0.53–2.30)
Her2 (pos)	0.8310	1.11 (0.43–2.84)
Age (young)	0.3764	1.42 (0.65–3.10)
Tumor size (>2 cm)	**0.0412**	**2.21 (1.03–4.74)**
Phospho-Stat3 (Tyr705) nuclear staining (neg)	**0.0469**	**2.35 (1.01–5.46)**

OS, overall survival; CI, confidence interval; neg, negative; pos, positive.

the different mechanisms of Stat3 signaling and its role in breast cancer development, many downstream targets have been identified, including cyclin D1, c-myc, p21 WAF1/CIP1, and Bcl-xL. The protein products of these targets are potential candidates for future investigations using our node-negative breast cancer cohort or other similar cohorts.

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