Shc Proteins Are Strong, Independent Prognostic Markers for Both Node-Negative and Node-Positive Primary Breast Cancer

Pamela A. Davol, Robert Bagdasaryan, Gerald J. Elfenbein, Abby L. Maizel, and A. Raymond Frackelton, Jr.

ABSTRACT

Activated Shc signaling proteins are implicated in many pathways associated with aggressive disease, and many breast cancer cell lines derived from highly aggressive tumors contain high levels of activated, tyrosine phosphorylated (PY)-Shc (the M, 46,000 and M, 52,000 isoforms) relative to levels of an inhibitory M, 66,000 Shc isoform. It was, therefore, hypothesized that high amounts of PY-Shc relative to the M, 66,000 Shc isoform would serve as a marker for aggressive neoplasms. Semi-quantitative immunohistochemical analyses of PY-Shc and p66 Shc were performed on archival primary breast tumor specimens from 116 women, 17 of whom experienced relapse (6.1 years median follow-up of nonrelapsed patients). Consistent with our hypothesis, staining intensities demonstrated that increased amounts of PY-Shc (P < 0.01) and decreased expression of p66-Shc protein (P = 0.028) correlated with disease recurrence. Modeled as the ratio of PY-Shc to p66 Shc, the Shc ratio correlated strongly with nodal status (P = 0.003), tumor stage (P = 0.0025), and disease stage (P = 0.002) and was 2-fold higher in primary tumors of patients who subsequently relapsed (P < 0.001). Univariate Cox proportional hazards analyses of relapse-free survival demonstrated the prognostic value of PY-Shc (P = 0.01), p66 Shc (P = 0.04), and the Shc ratio (P = 0.004) as continuous variables, with a hazard ratio (HR) of 10 (P = 0.007) for the Shc ratio. Shc ratio cut points of <0.35 and >0.65 were identified and independently validated to maximize negative predictive value and positive predictive value. Patients with low Shc ratios (n = 36) had a 0.08 HR of relapse (P = 0.007) compared with patients with high Shc ratios, experiencing an 8-year cumulative 2.9% and 55% relapse hazard, respectively, compared with a 22% relapse hazard in the total cohort. The Shc ratio had similar prognostic value for disease-specific survival. In multivariate models, the Shc ratio, both as a continuous variable and as a cut point-categorized variable, was independent of all measured covariates (including nodal status, tumor stage, disease stage, grade, estrogen receptor status, and adjuvant therapy) and was a stronger prognostic marker than all but nodal status. All relapsed node-positive patients had very high Shc ratios (>0.80; P = 0.006) in their primary tumors.

Furthermore, the Shc ratio was a strong, independent prognostic indicator in node-negative patients (79 patients, 10 recurrences), with a HR of 0.086 (P = 0.02) that was independent of clinical markers and adjuvant therapy. Patients with low and high Shc ratios experienced a 3.6% and 64% relapse hazard, respectively, compared with 20% in the total node-negative cohort.

INTRODUCTION

Nearly 200,000 women in the United States are diagnosed with invasive breast cancer yearly, most presenting with early stage, node-negative disease (1). Although only a small percentage of these patients have aggressive disease that will recur after removal of the primary tumor (2), most relapsed patients die of their disease. Unfortunately, prognostic markers currently accepted for clinical use, such as nodal status, disease stage, tumor stage, histological grade, and steroid receptor status, (3) do not adequately identify which early stage, node-negative patients are at low or high risk for disease recurrence. For this reason, most patients choose to receive adjuvant therapies (4–6). One urgent need, therefore, is for better prognostic indicators for early stage, node-negative patients. Additionally, patients with node-positive, nonmetastatic disease comprise another important group whose clinical management could benefit from additional prognostic markers that are independent of those currently in use.

Numerous growth factors and their receptors have been implicated in breast cancer development and aggressiveness (7–13). These include Her-2/neu, other members of the EGF1 receptor family, insulin-like growth factor-I, and many others (reviewed in Refs. 14–18). We reasoned that the level of activation of a downstream signaling protein common to all of the receptors would provide an indicator of the total of growth factor receptor signaling in tumor cells and, therefore, a measure of their aggressiveness.

One such second messenger is the adapter protein Shc, which becomes tyrosine phosphorylated in response to signaling from all of these receptors, from many G protein-coupled receptors, and in response to cellular interactions with the extracellular matrix (19–22). Shc is involved in responses to stimuli that activate cell proliferation, invasion, and motility and control anchorage-independent growth (23–35). Although tyrosine phosphorylation of the M, 52,000 and M, 46,000 isoforms of Shc seems to drive these reactions forward, an alternative M, 66,000 Shc isoform seems to inhibit some of these processes (36, 37); additionally, p66 Shc is an apoptotic sensitizer to oxidative stress (38, 39). Such stress may be generated by chronic activation of growth factor pathways, by infiltrating neutrophils and macrophages, and by neovascularization of hypoxic tumors (40, 41). Previously, we reported that many cell lines isolated from aggressive breast cancers harbor high levels of the activated PY-Shc (M, 52,000 and M, 46,000 isoforms) but express little of the counterposed M, 66,000 Shc isoform (42). We hypothesized that aggressive primary breast tumors destined to recur would display relatively high levels of PY-Shc and low levels of its inhibitory M, 66,000 isoform. Here, we report an affirmative test of this hypothesis in patients with early stage, node-negative breast cancer, as well as in patients with more advanced, node-positive disease.

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3 The abbreviations used are: EGF, epidermal growth factor; PY, phosphotyrosine; Gst, glutathione-s-transferase; dn, dominant-negative; IHC, immunohistochemical; AJCC, American Joint Committee on Cancer; ER, estrogen receptor status; PR, progesterone receptor status; RFS, relapse-free survival; DFS, disease-specific survival; HR, hazard ratio; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; d.f., degree(s) of freedom; wt, wild type; uPA, urokinase-type plasminogen activator; PAI, uPA inhibitor.
MATERIALS AND METHODS

Patients and Patient Primary Breast Tumor Samples

Patient specimens in this study were obtained from the archives of the Department of Pathology, Roger Williams Medical Center (Providence, RI), and consisted of 116 consecutive cases (17 relapsed) available in paraffin blocks of formalin-fixed primary tumors surgically removed from patients with invasive, nonmetastatic breast carcinoma between 1989 and 1995. Detailed clinicopathological characteristics of these patients are presented in Table 1. Of the 116 patients, 99 (85%) were diagnosed with invasive ductal carcinoma, 5 (4%) with invasive lobular carcinoma, and 3 (3%) with other tumor subtypes. All 116 patients had histologically confirmed diagnoses, and the tumor content of chosen archival blocks and sections was verified for each case. Additionally, 79 patients (11 relapsed) were negative for nodal involvement by axillary lymph node dissection (mean nodes examined, 18 in the stage 1 and 2 patients), 25 patients were node positive (5 relapsed), and 11 patients did not have nodal dissection (1 relapsed). Patients in this study were treated with breast-conserving surgery (n = 8; 2 relapsed) or mastectomy only (n = 17; 1 relapsed), or with surgery and local radiation (n = 10; 3 relapsed), or with surgery and chemotherapy or hormonal adjuvant therapy, with or without local radiation (n = 81; 11 relapsed). Nonrelapsing patients had a median follow-up of 6.1 years; relapsed patients, 3.1 years to relapse. Relapse was defined as local (in residual breast tissue or ipsilateral chest wall; n = 1), a single node-positive patient who received radiation and hormonal adjuvant therapy after initial partial mastectomy), regional (to ipsilateral lymph node; n = 4), or distant (n = 12). Patient confidentiality was maintained according to the guidelines established by the hospital’s Institutional Review Board.

Antibodies

Phosphospecific Shc Antibody. A New Zealand White rabbit was immunized with an N-acetylated tyrosine-phosphorylated Shc peptide, N-acetyl-LeuPheAspAspProSer([P]Tyr)ValAsnValGlnAsnLeuCyS (corresponding to human Shc amino acids 311–323, coupled to Keyhole limpet hemocyanin). Antibodies were immuno-affinity purified from serum collected 10 weeks later, after two booster immunizations (custom prepared by Research Genetics, Huntsville, AL). Antibody specificity was assessed by immunoprecipitation of Shc from EGF-stimulated stable clones of the HBL-100 epithelial cell line (American Type Culture Collection, Manassas, VA) expressing a Gst-wt-Shc fusion protein or a Gst-Shc[Y317]F dn-fusion protein that has lost the immunizing Y[317] phosphorylation site (although it still contains the Y[239] secondary phosphorylation site; Fig. 1A; Refs. 43 and 44). EGF stimulated strong tyrosine phosphorylation of wt-Shc (at both the 239Y and 317Y phosphorylation sites) and weaker phosphorylation of the dn-Shc (at only the 239Y

Table 1

<table>
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<tr>
<th>Characteristics</th>
<th>All patients</th>
<th>Stage I or II node negative</th>
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<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
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<tr>
<td>All patients</td>
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<tr>
<td>Other</td>
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$^a$ ER and PR status of <10 fmol/mg were defined as negative; ≥10 fmol/mg, positive. Treatments were grouped as surgery only, surgery followed by radiation, or surgery with either hormonal therapy (tamoxifen), chemotherapy, or both, and with or without local radiation (adjuvant). Tumor subtypes: ductal, invasive ductal carcinoma; lobular, invasive lobular carcinoma; other, mostly mixed invasive ductal and lobular carcinoma. Shc ratio cutpoints: low, <0.35; intermediate, ≥0.35 and ≤0.65; high, >0.65.

$^b$ No. 17/116 indicates that there were 17 recurrences of 116 patients with known values for the indicated clinical characteristic.

$^c$ RFS and DSS from Kaplan-Meier functions are given in percentage, 8 years after initial surgery.

$^d$ Of the eight patients with more than three positive nodes, five recurred and the longest follow-up was 7.0 yr. For grade, RFS and DSS are reported at 7 yr. For patients with invasive lobular carcinoma and other, there was no recurring disease; the longest follow-up was 7.1 yr and 6.1 yr, respectively.
Fig. 1. Specificity of the phosphospecific antibodies for PY[317]Shc. A, the antibody immunoprecipitates Shc only when Shc contains tyrosine-phosphorylated Y[317]. Transfected, stable subclones of the epithelial cell line HBL-100, carrying either the pEBG empty expression vector (v), the vector containing wild-type Gst-Shc (wt), or the dominant-negative mutant of Shc, Gst-ShcY[317]F (dn), were stimulated where indicated for 10 min with 100 ng/ml EGF (Collaborative Research, Bedford, MA), then extracted in Triton-based buffer containing a kinase and phosphatase inhibitors and immunoprecipitated with pan-Shc rabbit polyclonal antibody directed to the Shc protein backbone (RαShc, top left). Upstate Biotechnology Inc., Lake Placid, NY) or with the antibody generated to the PY[317] 15-mer peptide from Shc (PShc, top right) or preimmune immunoglobulin isolated from that same rabbit (Pl, top right). The immunoprecipitated proteins were resolved on 7.5% SDS-PAGE and probed with monoclonal antibody to phosphotyrosine (clone 4G10; Upstate Biotechnology Inc.; bottom). Expression of either wt-Shc or dn-Shc markedly inhibited EGF-stimulated tyrosine phosphorylation of the endogenous M, 46,000, 52,000, and 60,000 Shc isoforms (Fig. 1A). The antibody to phos-tyrosine in the presence of 10 mM phenylphosphate (PY−Shc) was completely inhibited by the potent PY haptenic analogue phenylphosphate (Fig. 1C). In contrast, pan-Shc antibody (directed to the protein backbone) staining was not inhibited by the PY peptide (Fig. 1, C4 and C5), whereas pure anti-PY staining was completely inhibited by the potent PY haptenic analogue phenylphosphate (Fig. 1, C6 and C7).

The specificity and usefulness of these antibodies was evaluated further in IHC staining of human breast tissue (Fig. 1C). Anti-PY-Shc stained the glandular tissue strongly (Fig. 1C1), and, as expected, staining was markedly inhibited by the immunizing PY peptide (Fig. 1C2) but only slightly inhibited by a partial antagonist, the PY analogue phenylphosphate (Fig. 1C3). Antibodies were immunofluorescence purified from serum collected 10 weeks later, after two booster immunizations (custom prepared by Research Genetics). Antibody specificity has been demonstrated by: (a) its high titer against the CH2 domain by ELISA assay (data not shown); (b) its ability to immunoprecipitate p56 Shc but not other Shc isoforms. To test this, we took advantage of our observations that the epithelial cell line HBL-100 contains normal levels of p66 Shc, comparable with levels of p52 Shc (Fig. 1A), whereas the breast cancer cell line MDA-MB-453 lacks detectable p66 Shc (42). The rabbit antibody immunoprecipitated only the p66 Shc isoform (Fig. 1D); (c) the ability of the affinity-purified antibody, but not the preimmune immunoglobulin from the same rabbit, to stain tumor cells in breast cancer specimens (Fig. 1E); and (d) the inability of the antibody to stain tumor-infiltrating lymphocytes. Lymphocytes lack p66 Shc expression (19). Fine specificity analysis suggests that

Positions of the three endogenous Shc isoforms, p46, p52, and p66 are indicated, along with the position of the expressed recombinant Gst fusion protein with wt-Shc or dn-Shc. B, immunocytochemical staining of EGF-stimulated HBL-100 cells expressing wt-Shc but not dn-ShcY[317]F. HBL-100 clones expressing either the wt-Shc (wt) or the dn-ShcY[317]F (dn) as in A, and growing in tissue culture on Falcon chambered slides, were exposed to 100 ng/ml EGF for 15 min (+) or not (−) and then fixed in neutral buffered formalin and processed for immunostaining, as usual (see “Materials and Methods”), except that the primary antibodies were either the preimmune immunoglobulin (Pre), the anti-PY[317] antibody (aPY−Shc), or the monoclonal antibody specific for phosphotyrosine (aPP), each as described in A, Bar, 40 μm. C, IHC staining of Shc in breast tissue. Sections from paraaffin-embedded, formalin-fixed breast tissue were immunostained as described in “Materials and Methods,” with anti-phospho-Shc (1), anti-phospho-Shc in the presence of 10 μM homologous 15-mer phospho-peptide (2), anti-phospho-Shc in the presence of 10 μM phenylphosphate, the phosphotyrosine haptenic analogue (3), antibody directed to the Shc protein backbone (4), the antibody in 4, but in the presence of 10 μM phospho-peptide (5), antibody to phosphotyrosine (6), and antibody to phosphotyrosine in the presence of 10 μM phenylphosphate (7). Magnification ×100. D, specificity of antibodies to p66 Shc in immunoprecipitation. Cultures of HBL-100 epithelial cells and MDA-MB-453 breast cancer cells were extracted and immunoprecipitated with affinity-purified antibodies generated in rabbits by immunization with a peptide unique to p66 Shc (aPP66) or with the pan-Shc rabbit antibody (RαShc) used in the previous panels. The immunoprecipitated proteins were resolved by SDS-PAGE and detected by probing with the pan-Shc antibody (RαShc). E, specificity of antibodies to p66 Shc in IHC staining of breast cancer. Sections from paraaffin-embedded, formalin-fixed breast tissue were stained with preimmune sera or rabbit antibody specific for p66 Shc (anti-p66 Shc). Tissue was counterstained with hematoxylin. Magnification ×100.
Subsequently relapse, showing strong immunostaining of PY-Shc (left) relative to p66 Shc (right). Bottom, adjacent sections from a primary tumor of a patient with stage I disease who did not subsequently relapse, showing very weak immunostaining for PY-Shc (left) compared with p66 Shc (right). Immunostained slides were counterstained with hematoxylin. Bar, 100 μm.

Fig. 2. Scoring of Shc IHC staining in breast cancers. A, scoring system for intensity patterns of IHC staining. PY-Shc staining of selected fields of six different breast tumors that were chosen to represent the six levels of staining patterns: 0, background staining; 1, a low level, punctate, and nonuniform cellular staining; 2, a low level, punctate, and uniform cellular staining; 3, a low level, evenly distributed cellular staining; 4, a moderate level, evenly distributed cellular staining; 5, a high level, evenly distributed cellular staining. Magnification, ×100. B, IHC staining of PY-Shc and p66 Shc in a low Shc ratio primary tumor and in a high Shc ratio primary tumor. Top, adjacent sections from a primary tumor of a patient with AJCC stage I disease who did not subsequently relapse, showing very weak immunostaining for PY-Shc (left) compared with p66 Shc (right). Bottom, adjacent sections from a primary tumor of a patient with stage I disease who did subsequently relapse, showing strong immunostaining of PY-Shc (left) relative to p66 Shc (right). Immunostained slides were counterstained with hematoxylin. Bar, 100 μm.

these antibodies preferentially recognize the dephospho (serine 36) form of this peptide.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized, and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then immunohistochemically stained according to the manufacturer using the CSA Peroxidase System (DAKO, Carpinteria, CA) after target retrieval and endogenous peroxidase quenching with the CSA Ancillary System (DAKO). Affinity-purified phosphospecific Shc antibody (2.5 μg/ml) or p66-Shc antibody (2.5 μg/ml) was diluted in Background Reducing Components (CSA Ancillary System) and incubated with tissue samples for 1 h at room temperature. Primary antibodies were detected by incubating for 15 min with biotinylated goat antirabbit immunoglobulins, and the signal was amplified and visualized by dianaminobenzidine precipitation at the antigen site. Samples were counterstained with hematoxylin.

IHC Scoring

An average staining intensity for all tumor cells in a section was determined (46, 47). Staining intensities of tumor cells were scored on a 0–5 scale (Fig. 2A) by a scorer (P. A. D.) blinded to the patients’ outcomes and clinicopathological characteristics. These intensities were multiplied by the proportion of tumor cells at each intensity to accurately reflect the heterogeneous intensities of tumor staining. The results were then summed and multiplied by 20 to achieve a scale of 0 (no tumor cells stained) to 100 (all tumor cells stained maximally). A random subset of 48 slides from 24 patients was scored independently by a second scorer (A. L. M.) blinded both to patient outcome and to the other scorer’s results. The Pearson correlation coefficient for the combined PY-Shc and p66 Shc scores was 0.80 \( (P < 0.0001) \) and for the ratio of PY-Shc to p66 Shc was 0.95 \( (P < 0.0001) \). The two scorers had a 96% concordance (23 of 24) in assignment of patients to high versus low ratios of PY-Shc to p66 Shc staining.

Statistical Analysis

The Shc ratio was correlated with various clinicopathological parameters using Kendall’s rank correlation tests, Wilcoxon’s rank-sum tests, or Kruskal-Wallis tests, as appropriate. PY-Shc, p66-Shc, and Shc ratio values in primary tumors from patients who remained relapse free and in primary tumors from patients whose disease later clinically recurred were compared using Student’s \( t \) tests or the Wilcoxon’s rank-sum test, as appropriate. RFS was taken as the interval between surgical removal of the primary tumor and first clinical recurrence, or to last follow-up; DSS was calculated as the years elapsed between initial diagnosis and death with relapsed breast cancer. RFS and DSS functions were estimated by Kaplan-Meier analysis, and differences between RFS (and DSS) curves generated by Shc ratio cut points and other dichotomous or categorical markers (nodal and steroid receptor status, disease and tumor grade, histological grade, and so on) were tested by log-rank analysis. Prognostic significance and HR for PY-Shc, p66 Shc, the Shc ratio (both as a continuous and as a categorized variable), and the other potential variables were analyzed in univariate, stratified, and multivariate Cox proportional hazards models, with RFS or DSS as end points. Patients who died free of breast cancer or were otherwise lost to follow-up were considered as censored observations at the time of their deaths or loss to follow-up. For Cox analysis, categorical clinical markers and the categorized Shc ratios were each expanded into \( m \)-1 indicator variables for each of the \( m \) subcategories shown in Table 3. These design indicator variables were kept intact in the Cox models. The relationship between the Shc variables and AJCC stage, which is a composite index primarily reflecting tumor stage and nodal status, was assessed in Cox models that did not include nodal status or tumor stage. A multivariate model was developed by purposeful selection of covariates (48). The base model initially included all variables that were significant at \( < 0.25 \) (Table 1), retaining those that remained significant at \( < 0.20 \). The Shc ratio, as indexed categorical or as continuous variables, was then added, resulting in the expulsion of tumor stage with significance \( > 0.4 \). Additional single variables from Table 1 were added back individually and retained in the provisional models (see Table 6) if significant at \( < 0.1 \). Proportional hazards assumptions for Cox models were assessed using Schoenfeld residuals, and the goodness of fit of the model was graphically estimated using Cox-Snell residuals. Wald tests and partial LR tests were used to assess the significance of coefficients (HRs) from Cox regressions. Significant differences between models were tested by partial LR analysis. Statistical calculations were performed using the Stata statistical package (Stata versions 7.0 and 8.0; StataCorp., College Station, TX). All statistical tests were two sided, except when testing our initial, one-sided hypothesis that the levels of PY-Shc compared with levels of p66 Shc protein (the Shc ratio) would be relatively higher in tumors from patients with aggressive disease (Tables 2 and 4).

Table 2. Comparison of Shc levels in primary breast cancers of nonrelapsed and relapsed patients

<table>
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<tr>
<th>Marker</th>
<th>Nonrelapsed (n = 99)</th>
<th>Relapsed (n = 17)</th>
<th>( P )</th>
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<tr>
<td>PY-Shc</td>
<td>30 ± 2</td>
<td>41 ± 5</td>
<td>0.011*</td>
</tr>
<tr>
<td>p66 Shc</td>
<td>60 ± 2</td>
<td>50 ± 6</td>
<td>0.028*</td>
</tr>
<tr>
<td>Shc ratio</td>
<td>0.59 ± 0.06</td>
<td>1.20 ± 0.24</td>
<td>&lt;0.001*</td>
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</table>

*P using the Student’s \( t \) test. Statistically significant values \( (P < 0.05) \) are in bold. 

\( ^b \) \text{P using the Wilcoxon’s rank-sum test.}
Shc Proteins in Breast Cancer Prognosis

RESULTS

Association of PY-Shc and p66-Shc IHC Staining with Clinicopathological Characteristics of Breast Cancer Patients

Clinicopathological characteristics of the 116 female patients with invasive, nonmetastatic breast cancer are shown on the left in Table 1. Of these patients, 17 (15%) relapsed and 10 (9%) died with their recurrent disease. Log-rank analysis of relapse demonstrated a significant predictive value for the standard clinical prognostic variables: nodal status, AJCC disease stage, tumor size/stage (tumor-node-metastasis), and histopathological grade. Patients younger than 50 years at diagnosis showed a poorer disease-free survival and DSS, but neither age at diagnosis nor steroid receptor status attained significance in this relatively small population. Therapeutic regimen approached significance in the node-negative patients (Table 1).

Blinded to clinicopathological characteristics and patient outcome, we located archival paraffin blocks from each primary tumor, verified their tumor content, and then sectioned and immunohistochemically analyzed the tumor sections for expression of PY-Shc and p66 Shc. Consistent with our hypothesis, tumors from patients that subsequently relapsed demonstrated a significantly higher mean IHC staining score for PY-Shc compared with nonrelapsed patients and a significantly lower mean IHC staining score for p66 Shc (Table 2 and Figs. 2 and 3). Because of the counterpoised roles of PY-Shc and p66 Shc, our original hypothesis predicted that the relative levels of PY-Shc compared with p66 Shc would more effectively reflect tumor aggressiveness than the absolute level of either one alone. Accordingly, we modeled this as a simple ratio of PY-Shc to p66-Shc scores. The Shc ratio for the patients who later relapsed was twice as high as that for nonrelapsing patients (P < 0.001; Table 2).

We evaluated the relationships between the Shc ratio and clinicopathological characteristics of the breast cancer patients. Shc ratio was strongly associated with the accepted prognostic markers: nodal status, disease stage, and tumor stage (each P < 0.004; Table 3). Primary tumors from patients who had more than three positive nodes, disease stage III, or tumor stage T2 or T4 exhibited a 2-fold higher Shc ratio than, respectively, tumors from patients who were node negative, had stage I disease, or had stage T1 tumors. ER-negative tumors showed a trend toward higher Shc ratios compared with ER-positive tumors, approaching statistical significance. No association with a significance <0.3 was detected between the Shc ratio and tumor grade, PR status, histological subtype, patient age at diagnosis, or treatment regimen.

Univariate Analysis of RFS

Higher levels of PY-Shc and lower levels of p66 Shc were associated with increased risk of relapse, with independent HRs of 8 and 5, respectively (Table 4). When mutually adjusted in a bivariate Cox model, the prognostic values of PY-Shc and p66 Shc were not only retained but appeared to be strengthened (70% increase in PY-Shc HR...
of the Shc ratio to identify these women is its PPV, which is the fraction of patients with a high Shc ratio who later relapsed (again, assuming no confounding by therapy; see below). Graphical analysis of all potential cut points showed a major PPV peak of 0.29 (7 of 24 patients relapsed) at a Shc ratio cut point of 0.65 (Fig. 4 and scatter plot in Fig. 3A), giving by total incidence a 2.4 relative risk of relapse for the high Shc ratio subgroup compared with the total Instructional group. Consistent with the relative risk by incidence, log-rank analysis of the 0.35 and 0.65 cut points showed that patients with low Shc ratios had a decreased risk of relapse (P = 0.027 by log-rank trend analysis). The more favorable outcomes of patients with Shc ratios <0.35 compared with patients with Shc ratios ≥0.65 are visually apparent from graphs of the Kaplan-Meier functions (Fig. 3B). However, because the NPV and PPV analyses might have over-optimized the model, it was important to test the validity of these cut points using an independent set of patients, the Validating group.

Graphical analysis of all potential cut points for the Validating group showed a major NPV peak of 1.00 (no false negatives of 17 patients), again at a Shc ratio cut point of <0.35, and a major PPV peak of 0.30 (6 relapsed patients of 20), again at a Shc ratio cut point of ≥0.65 (Fig. 4 and scatter plot in Fig. 3A). By log-rank analysis of this Validating group, patients with Shc ratios less than the 0.35 cut point had a decreased risk of relapse compared with patients with Shc ratios higher than the 0.65 cut point (P = 0.008), as is apparent from graphs of their Kaplan-Meier RFS functions (Fig. 3B), validating the determined cut points.

Relapse incidence in the combined group of 116 patients (Fig. 4 and

and a 40% increase in p66-Shc HR, along with higher statistical significance, indicating that PY-Shc and p66 Shc are mutually independent prognostic indicators that may have additional mutual synergistic value (Table 4). Consistent with this interpretation, the bivariate model was a significant improvement over the univariate PY-Shc or p66 Shc models (LR, Δχ² = 6.73, Δd.f. = 1, P < 0.01; and LR, Δχ² = 4.82, Δd.f. = 1, P = 0.028, respectively). Further confirming our initial hypothesis, an increased Shc ratio was associated with increased hazard (HR = 10; P = 0.004; Table 4).

Selection and Validation of Cut Points for the Shc Ratio

For success as a clinical prognostic marker, the Shc ratio would need to accurately identify women who may be currently overtreated (i.e., women at low risk of disease recurrence). The accuracy of identifying such women (assuming no confounding by therapy; see below) can be measured by the NPV of the Shc ratio, the fraction of patients with a low Shc ratio who did not relapse. To choose an optimal Shc ratio NPV, the 116 tumor samples, blinded to clinicopathological characteristics, patient outcome, and Shc ratio, were randomized into Instructing and Validating groups of 58 patients each (using the pseudo-random number generator in the Stata statistical package). The Instructing group consisted of 30 patients with stage I, 24 with stage II, and 4 with stage III disease and contained a total of 10 patients who later relapsed, whereas the Validating group consisted of 29 patients with stage I, 28 patients with stage II, and 1 patient with stage III disease and contained a total of 7 patients who later relapsed. No significant difference in overall disease outcome (P > 0.49 by univariate log-rank analyses) was found between the Instructing and Validating groups or between these groups and the combined population, suggesting that outcome of each sample group was representative of the total population. Graphical analysis of NPV calculated for each of many potential Shc ratio cut points showed a major NPV peak of 0.95 (one false negative of 19 patients) at a Shc ratio cut point of <0.35 (Fig. 4 and scatter plot in Fig. 3A), giving by total incidence a relative risk of relapse of 0.31 for the low Shc ratio subgroup compared with the total Instructional group.

The Shc ratio would have additional clinical use if it could accurately identify a subpopulation of women who have an increased risk of relapse compared with the total population. A measure of the ability
scatter plot in Fig. 3A3) was only 2.8% (1 of 36) in patients with Shc ratios 0.35 (NPV, 0.97) but 30% (13 of 44) in patients with Shc ratios 0.65 (PPV, 0.30), compared with 15% (17 of 116) in patients in the total population. Kaplan-Meier analysis of RFS as a function of the cut point-categorized Shc ratio confirmed the association of lower Shc ratios with very favorable RFS (Fig. 3B3; P < 0.001 by log rank and log-rank trend analysis; Table 1). Patients with low Shc ratios had <8% the hazard of relapse as patients with high Shc ratios (Table 4) and 13% the hazard of the total, unpartitioned patient cohort (P = 0.003 by log-rank analysis). By Nelson-Aalen cumulative hazard analysis, 8 years after diagnosis, the unpartitioned total population had experienced a 22% risk of relapse compared with patients with low, intermediate, and high Shc ratios experiencing a 2.9, 9, and 55% risk of relapse, respectively. By comparison, patients with low Shc ratios had a much more favorable RFS than seen for all of the clinical markers in Table 1, except grade 1. The Shc ratio and grade 1 (in which no relapses occurred; see below) had equivalent RFS, yet the low Shc ratio group still comprised a substantial portion (>30%) of the total patient population (Table 1). Conversely, patients with high Shc ratios had nearly as poor a RFS as patients with T4 tumors, yet the Shc ratio identified a much larger number of patients at risk (44 with high Shc ratios compared with only 3 with T4 tumors).

**Univariate Analysis of DSS**

In addition to its value as a predictor of relapse, the Shc ratio also predicted DSS. Univariate Cox analysis indicated significantly increased hazard of death from relapsed breast cancer with decreasing p66 Shc levels (HR, 14; P = 0.03) and increasing Shc ratio (HR, 14; P = 0.005; Table 4). Hazard analysis of the patients grouped by cut points indicated that patients with low Shc ratios had less than one-sixth the hazard of death with relapsed disease compared with patients with high Shc ratios (Table 4). Compared with the total, unpartitioned population of patients, patients with high Shc ratios had a 2-fold increased hazard, whereas patients with low Shc ratios had only 30% as much hazard of dying from their disease. By incidence, 7 of 44 patients who had high Shc ratios, but only 2 of 36 with intermediate and 1 of 36 with low Shc ratios, died with relapsed disease (P = 0.016; Table 1). Similar to what was seen for RFS above, patients with low Shc ratios had a much more favorable DSS than that provided by most of the clinical indicators in Table 1, except that the DSS was approximately equal for low Shc ratios, ER/PR positive, and grade of one.

**Bivariate Analysis of RFS and DSS**

**Shc Ratio as a Categorical Variable.** To examine possible relationships between the prognostic ability of the Shc ratio and the known clinically useful prognostic markers, we first examined the prognostic value of the Shc ratio in bivariate Cox models, pairwise with each potential covariate: nodal status, tumor stage, disease stage, histological grade, ER and PR status, histological subtype, age at diagnosis, and therapeutic regimen. As can be seen in Table 5, the Shc ratio, categorized by cut points, remained a significant independent prognostic indicator when examined in the bivariate Cox models, with the HR of relapse for low Shc ratio compared with high Shc ratio patients remaining close to 0.08. By partial LR analysis, the addition of the Shc ratio not only significantly improved the model fit for all covariates tested but was actually a much stronger contributor to model fit than all but nodal status (Table 5). For example, in the bivariate model with AJCC stage, the contribution of the Shc ratio to model fit was significantly greater (LR: \( \Delta \chi^2 = 11.8, \Delta df. = 2, P = 0.0027 \)) than that of AJCC stage (LR: \( \Delta \chi^2 = 3.15, \Delta df. = 2, P = 0.207 \)). The bivariate model with histological grade and the Shc ratio was unusual because the single patient with a false negative Shc ratio (low Shc ratio, but disease recurred) had no value available for grade. Thus, in the 99 patients for whom grade values were available, there were no relapses among patients with low Shc ratios (NPV, 1.0), and the relapse HR comparing patients with a low Shc ratio with those with high Shc ratios, thus, approached 0 (Table 5). Comparison of the Shc ratio by log-rank analysis either unstratified (P = 0.0001) or stratified on grade (P = 0.0001) suggested that the prognostic value of the categorized Shc ratio was independent of grade. This was strongly confirmed by LR tests, in which the contribution of the Shc ratio to the fit of the bivariate model (LR: \( \Delta \chi^2 = 22.24, \Delta df. = 2, P < 0.0001 \)) was much stronger than the contribution of the grade (LR: \( \Delta \chi^2 = 7.36, \Delta df. = 2, P = 0.025 \); Table 5).

In contrast to the strong prognostic independence of the categorical Shc ratio, AJCC stage and tumor stage seemed to lose much of their prognostic strength when mutually adjusted with the categorical Shc ratio (Table 5). By LR analysis, the addition of either AJCC stage or tumor stage failed to improve the Shc ratio univariate model. However, although nodal status seemed to lose some of its own HR and Wald test significance, the addition of nodal status very significantly improved the Shc ratio univariate model (LR: \( \Delta \chi^2 = 11.16, \Delta df. = 2, P = 0.0038 \)). Histological grade seemed to retain all of its prognostic value when mutually adjusted for the categorical Shc ratio and contributed significantly to the fit of the bivariate model (Table 5).

Univariate Cox analyses reciprocally stratified on the categorized Shc ratio and the clinical markers provided relative hazards and levels of significance indistinguishable from those shown for the bivariate analyses in Table 5. Univariate log-rank analyses reciprocally stratified on the categorized Shc ratio and the clinical markers led to the same conclusions, except that P values for the unstratified and stratified Shc ratios were typically significant at an order of magnitude or more lower value for P. Additionally, the significance of histological grade (P = 0.04) did not decrease when stratified on the Shc ratio (Pgrade = 0.024) in log-rank analysis, suggesting again that the prognostic value of grade was independent of the categorical Shc ratio. To reduce Table 5 complexity, HR was omitted for patients with intermediate Shc ratios. Their values generally were HRs of 0.20–0.25, with Wald values for P < 0.03, and these were similarly unaffected by potential covariate clinical markers (Tables 4 and 6). All of the other clinical markers listed in Tables 1 and 3 (ER status, therapy, and so on) failed to confound the prognostic value of the Shc ratio or improve model fit and themselves failed to maintain levels of significance <0.3 when they were similarly tested in bivariate Cox models and in stratified Cox and log-rank models with the Shc ratio (data not shown).

**Shc Ratio as a Continuous Variable.** As a continuous variable, the Shc ratio retained its prognostic value when mutually adjusted in the pairwise, bivariate Cox models for nodal status, disease stage, tumor stage, grade (Table 5), and all of the other, weaker clinical markers (data not shown), including therapy (see below). The largest observed decreases in HR for the Shc ratio were 15% when adjusting for tumor stage and 23% when adjusting for grade. Neither of these HR decreases were significant (LR: \( \Delta \chi^2 = 0.95, \Delta df. = 1, P > 0.05 \)) adjusting for grade). Just as for the Shc ratio as a categorical variable, the addition of the Shc ratio as a continuous variable improved model fit (LR, P < 0.05) for all potential covariates, except for tumor stage, in which a significant model improvement was nearly attained. Nodal status, disease stage, tumor stage, and grade retained much of their prognostic value as well, both in HR magnitude and significance. Even though only nodal status significantly improved model fit of the Shc ratio (for nodal status; LR: \( \Delta \chi^2 = 13.8, \Delta df. = 2, P = 0.0001 \)), the contributions of both grade (P = 0.077) and AJCC stage (P = 0.082) approached significance (Table 5).

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The major conclusions, then, from bivariate analysis are that as a prognostic marker for RFS, the Shc ratio (as both a cut point categorical variable and as a continuous variable) is not only largely independent of all of the other clinical markers but is a stronger prognostic marker than all but nodal status.

Multivariate Modeling of RFS and DSS. The unusual strength of the Shc ratio encouraged us to explore its potential relationships to other markers in multivariate models, although sample size dictated caution. Development of provisional Cox multivariate models for RFS by the method of purposeful covariate selection (Ref. 48 and see “Materials and Methods”) resulted in a base model dominated by the strong prognostic marker, nodal status, and also containing tumor stage and therapeutic modality (Table 6). Adding the Shc ratio improved the model fit (as a continuous variable: $\Delta \chi^2 = 4.55$, d.f. = 1, $P = 0.03$).
Con- firming this impression, the difference between the RFS functions for the low Shc ratio group showed greatly improved rank-sum test). This notion was reinforced by Kaplan-Meier graphical analysis (Fig. 5). Prognostic Value of Shc in Early Stage, Node-Negative Breast Cancer. Although strong prognostic markers such as nodal status, disease stage, and tumor stage exist for breast cancer, their usefulness is primarily confined to patients with node-positive or advanced (stage III) node-negative disease (3). Consistent with this, in our total study population, each of these characteristics was a strong prognostic marker ($P < 0.003$ by log-rank analysis (Table 1); $P < 0.003$ in univariate Cox models with HR of 1.5–2.2 (Table 5)). In our relatively small stage I and II, node-negative subpopulation (79 patients), however, neither disease stage nor tumor stage attained significant prognostic value ($P > 0.7$ by log rank; $P > 0.4$ in Cox models).

In contrast, the scatter plots for Shc ratios of nonrelapsed versus relapsed patients with early stage, node-negative breast cancer suggested that the Shc ratio might have prognostic value in this clinically important patient subgroup (Fig. 5A1). Consistent with this, primary tumors from patients who did not relapse had lower Shc ratios ($0.53 \pm 0.08$ compared with $1.06 \pm 0.34$; $P = 0.034$ by Wilcoxon’s rank-sum test). This notion was reinforced by Kaplan-Meier graphical analysis of RFS: the low Shc ratio group showed greatly improved outcome compared with the high Shc ratio group (Fig. 5B1). Confirming this impression, the difference between the RFS functions for low versus high Shc ratio patients was highly significant by univariate log rank analysis ($\chi^2 = 13.9, P = 0.0010$). On univariate Cox proportional hazards analysis, low Shc ratio patients had only 0.086 as much hazard of relapse as high Shc ratio patients (Wald test, $P = 0.022$; LR: $\chi^2 = 9.0, d.f. = 2, P = 0.011$; Table 5), and the assumption of proportional hazards was not violated ($P > 0.70$). By incidence, of the 11 of 79 node-negative patients who relapsed, 1 of 30 had low, 3 of 28 had intermediate, and 7 of 21 had high Shc ratios (Pearson $\chi^2 = 9.65, P = 0.008$). By Nelson-Aalen cumulative hazard analysis, 8 years after diagnosis, the unpartitioned node-negative population had experienced a 20% hazard of relapse compared with patients with low, intermediate, and high Shc ratios who experienced a 3.6, 11, and 64% hazard of relapse, respectively. Of the 11 relapsed patients, 6 died (1 of 30 with low, 2 of 28 with intermediate, and 3 of 21 with high Shc ratios), suggesting that the prognostic value of the categorized Shc ratio for DSS will be similar to or only slightly less than that observed for the total study population (Table 4).

Although none of the standard clinical markers useful in advanced breast cancer had RFS prognostic significance at $P < 0.15$ for node-negative patients (Table 1), we tested their ability to confound or interact with the prognostic value of the Shc ratio. The categorized Shc ratio retained its full prognostic value when mutually adjusted in pairwise, bivariate Cox analyses with disease stage, tumor stage, and each of the other potential covariates (Table 5). The HR of relapse of the Shc ratio was essentially unaltered and retained two-sided statistical significance ($P < 0.05$). The largest change in HR, <20%, occurred when mutually adjusting for tumor stage (Table 5). This effect was insignificant ($\Delta \chi^2 = 0.36, \Delta d.f. = 2, P > 0.05$). Stratified log-rank analyses of Shc ratios led to the same conclusions, yielding both unstratified and stratified values for $P < 0.01$. As might be expected from these results, preliminary multivariate modeling similar to that described for the total patient cohort resulted in the retention of only the Shc ratio, reducing to the univariate model examined previously in Table 5.

Prognostic Value of Shc in Node-Positive Breast Cancer. Preliminary examination of the cohort of node-positive patients suggested that the Shc ratio might have prognostic value in this clinically important patient subgroup as well. Shc ratios were clearly higher in primary tumors of node-positive patients who subsequently relapsed (Fig. 5A2; mean Shc ratio of 1.57 $\pm$ 0.31 compared with 0.68 $\pm$ 0.11; $P = 0.008$ by Wilcoxon’s rank-sum test). In fact, no relapses occurred in either the low or intermediate Shc ratio groups (Fig. 5B2). Cox regression analysis of the Shc ratio as a continuous variable indicated a HR of 5.5 (95% confidence interval, 1.6–18; $P = 0.006$) for an increase in the Shc ratio of 1.0 and HR $>100$ for the Shc ratio full range; as a cut point-categorized variable, the hazard of the low and intermediate Shc ratio groups compared with the high Shc ratio group, of course, approached 0. Inspection of the Shc ratio scattergrams in Fig. 5A2 suggests that separate, higher cut points should be explored for node-positive patients; however, this apparent increase in Shc ratio required for relapse may reflect the benefits of more prevalent systemic adjuvant therapy in the node-positive compared with the node-negative patients.

Shc Ratio: Prognostic or Predictive? Effects of Therapy. There were no significant differences in therapy received by patients in the low, intermediate, or high Shc ratio groups ($P = 0.53$), suggesting that therapeutic differences did not confound assessment of the prognostic value of the Shc variables. Consistent with this, the Shc variables retained their full independent prognostic value when adjusted for therapy in a bivariate Cox proportional hazards model or stratified model, and in the multivariate model discussed above, therapy approached significance but did not alter the prognostic value of the Shc ratio either as a categorical or continuous variable (Table 6).

Useful markers may be prognostic independent of therapy, predictive of response to therapy, or a mixture of both (4). For breast cancer, it would be important to know whether a marker can accurately assign risk to patients whose only treatment has been removal of the primary tumor; a small cohort of 25 patients met this criterion. Even though...
the Shc ratios of these patients were very evenly distributed (nine low, eight intermediate, and eight high), all three patients who relapsed had high Shc ratios; all had stage I disease. By log rank analysis, the categorical Shc ratio was a significant predictor of relapse ($P = 0.018$), and by LR analysis of Cox regression, the Shc ratio as a continuous variable also had significant prognostic strength ($\chi^2 = 4.74$, d.f. = 1, $P = 0.029$). By Nelson-Aalen analysis, the 7-year cumulative relapse hazard of patients with high Shc ratios was 0.77. Thus, although this is a very small group of patients, their data suggest strongly that the Shc ratio has prognostic value in the absence of systemic adjuvant therapy.

The large majority of patients (81 patients; 11 relapses) received systemic adjuvant treatment (tamoxifen, chemotherapy, or both). Again, although the Shc ratios were rather evenly distributed (24 low, 23 intermediate, and 34 high), 9 of the 11 relapses occurred in patients with high Shc ratios, and none occurred in patients with low Shc ratios ($\chi^2 = 9.12$, log-rank, $P = 0.0025$; Cox regression LR: $\chi^2 = 11.87$, d.f. = 2, $P = 0.0027$ for the Shc ratio as a categorical variable; $P = 0.004$; LR: $\chi^2 = 5.78$, d.f. = 1, $P = 0.016$ for the Shc ratio as continuous variable). Thus, the Shc ratio is clearly prognostic in the presence of systemic adjuvant treatment as well. However, for these adjuvant-treated patients with high Shc ratios, the 7-year cumulative relapse hazard by Nelson-Aalen analysis was 0.33, considerably less than the 0.77 seen in the small cohort receiving only surgical therapy. If this difference is maintained in a large, well-controlled study, it would mean that the high Shc ratio identifies patients who respond well to systemic adjuvant therapy. This conclusion is consistent with the observation that the Shc ratio is a stronger prognostic indicator for RFS than for DSS (Table 4).

**DISCUSSION**

New prognostic markers are needed to help guide the clinical management of patients with early stage, node-negative disease and patients with more advanced, node-positive disease. Although many molecular markers have been proposed and tested, most, such as Her2/neu and p53, are usually responsible for a limited aspect of the aggressive tumor phenotype, seem to be involved in subsets of breast cancers, or are manifested and important often only in advanced stages of the disease. In contrast, the Shc signaling proteins are activated in multiple cellular pathways that play important roles in tumor aggressiveness: proliferation, motility, interactions with extracellular matrix, and survival. Thus, the levels of Shc activation may reflect and report overall tumor aggressiveness. These biological considerations, coupled with our observation that most breast cancer cell lines express high levels of activated, tyrosine-phosphorylated p52 Shc but relatively low levels of the inhibitory p66 Shc isoform, led us to hypothesize that primary breast cancers with high levels of PY-Shc compared with p66 Shc would exhibit a more aggressive phenotype and, hence, an increased risk of recurrence.

Affirming this hypothesis, we have shown here that semiquantitative IHC analysis of PY-Shc and p66 Shc in primary tumors may be used to predict disease outcome both in breast cancer patients with early stage, node-negative disease and in patients with more advanced, nonmetastatic disease. Although primary tumors that subsequently recurred generally contained higher levels of tyrosine-phosphorylated Shc and lower levels of p66 Shc protein than primary tumors that did not recur, as hypothesized, the relative rather than the absolute levels of PY-Shc and p66 Shc, modeled as the ratio of PY-Shc to p66 Shc, correlated much more strongly with RFS (Tables 2 and 4) and DSS (Table 4). This is consistent with a model in which PY-Shc and p66 Shc subserve at least partially opposing molecular functions.

Interestingly, in the total patient cohort, the Shc ratio correlated strongly with the clinically established prognostic markers, nodal status, disease stage, and tumor stage and showed a correlation with ER-negative status that approached significance (Table 3). These correlations notwithstanding, the Shc ratio (both as a continuous variable and as a cut point-derived categorical variable) retained nearly all of its prognostic value when mutually adjusted for each of these covariates individually or together in Cox models (in terms of HR, Wald test significance, and LR significance) or stratified log-rank analyses (Tables 5 and 6). Conversely, the Shc ratio appeared to weaken the prognostic value of other covariates (except histological grade), although the weakening did not reach significance on partial LR analysis. Nevertheless, nodal status significantly improved the bivariate models with the Shc ratio as either a continuous or categorical variable (Table 5). In contrast, the Shc ratio and tumor grade seemed to be totally independent, and grade made a significant contribution to the fit of the bivariate model with the categorical Shc ratio and nearly so with the Shc ratio as a continuous variable. The multivariate models (Table 6) suggest that the Shc ratio (continuous or categorical), together with therapy, is sufficiently powerful to account for all of the hazard attributable to tumor stage. However, the patient cohort was not large enough to adequately model all potential covariates and confounders simultaneously, nor to properly consider interaction terms, and, thus, the multivariate models in Table 6 should be considered provisional and interpreted cautiously. Expanded, well-controlled, and designed trials will allow better modeling of some of the weaker covariates (such as tumor stage and ER status). Clearly, however, from the univariate, bivariate, and provisional multivariate studies presented here, the Shc ratio is not only largely independent of all of the other tested clinical markers but, much like the thoroughly studied marker uPA/PAI-1 (49, 50), is a stronger prognostic marker than all but nodal status.

Expanded trials will also allow us to explore the potential relationships between the Shc ratio and additional prognostic biomarkers such as uPA/PAI-1 (49, 50), proliferation markers (3), matrispase and c-Met (51), elastase (52), p53 status (3, 4), Her2/neu (53), and cyclin E (54). Functions of several of these prognostic markers are known to involve tyrosine phosphorylation of Shc: uPA signaling through its receptor requires Shc tyrosine phosphorylation for its ability to stimulate cell motility (33); activated c-Met tyrosine phosphorylates Shc (55), and Her2/neu stimulates Shc tyrosine phosphorylation (56). Her2/neu overexpression in primary tumors seems to be a prognostic marker in node-positive disease, but its value in node-negative disease is not clear (4, 53). Approximately 20–30% of primary tumors overexpress Her2/neu as measured by fluorescence in situ hybridization or IHC (4, 53), and less than one-third of these actually have activated Her2/neu (57). We would expect that all patients whose primary tumors displayed overexpressed active Her2/neu would have high levels of PY-Shc and a high Shc ratio. Therefore, on mutually adjusting the Shc ratio pairwise with Her2/neu (and similarly with uPA or matrispase/c-Met) in Cox bivariate models, we would expect to find a small fraction of the prognostic value of the Shc ratio to be dependent on each of these covariates. In fact, this was the essence of our original hypothesis. Because Shc is downstream of many different signaling pathways important in an aggressive tumor phenotype, the level of PY-Shc (relative to p66 Shc) would reflect the integrated total of all of these signals. Indeed, we would expect that the prognostic value of the Shc ratio might be completely defined by the universe of molecular prognostic markers that alter the relative levels of PY-Shc and p66 Shc. Future patient studies of the Shc ratio and these other markers will begin to test this prediction.
and identical cut points were obtained by plotting HR as a function of cut point. The bimodal nature of these curves is particularly interesting (Fig. 4) and is caused in part by the lone relapsed patient with a very low Shc ratio and the tight cluster of three relapsed patients with a Shc ratio near the 0.35 cut point. Relapse in these patients may be attributable to non-Shc-involved biochemical lesions driving aggressive tumor behavior (such as activation of the Ras to mitogen-activated protein kinase pathway epistatic to Shc), to nonoptimal specimen fixation resulting in artificial tyrosine dephosphorylation of Shc, or to both. The stability of Shc tyrosine phosphorylation during routine formalin fixation of surgical specimens is currently being evaluated. Whereas cut points are customarily used for simplicity and to increase one’s ability to categorize patients’ clinicopathological and molecular characteristics, establishing cut points is, by design, an optimization process that requires subsequent validation. For this reason, we randomized the patients into cut point Instructing and Validating groups. Certainly, some over-optimization may remain, and we expect that these cut points will be refined and redefined as more experience is gained. However, allaying concern about the cut points, the Shc ratio as a continuous variable had very strong prognostic value that, like the cut point-categorized Shc ratio, was independent of all clinical markers available for the patients in this study.

The RFS prognostic ability of the Shc ratio was strong and independent of potential covariates in both the total patient cohort (that combined patients with node-negative and node-positive disease) and in the cohort of patients with early stage, node-negative disease. The RFS HRs of patients with low Shc ratios compared with those with high Shc ratios were 0.078 unadjusted (Tables 4 and 5) and 0.088 fully adjusted (Table 6) in the total cohort and 0.086 both unadjusted (Table 5) and fully adjusted for potential covariates in the node-negative cohort. Compared with the unpartitioned node-negative cohort, the patients with low Shc ratios experienced only 18% as much cumulative relapse hazard 8 years after diagnosis (3.6% compared with 20% hazard). Conversely, the high Shc ratio cohort experienced 2.9 times the hazard of the unpartitioned cohort (64% compared with 20% hazard). Furthermore, it seems that the Shc ratio identifies patients at high risk of relapse, independent of whether the relapsed patients go on to die from their disease. Of the seven relapsed node-negative patients who had high Shc ratios, only three died of their disease (6.4-year mean follow-up for the surviving relapsed patients), suggesting again that the high Shc ratio relapsing patients benefited from subsequent therapies. This seems to be in contrast to a new node-negative marker, cyclin E, in which all stage I patients with high cyclin E died from their disease (see below and Ref. 54).

These results compare favorably with recently reported prognostic markers in breast cancer, as well (4, 51, 58–60). In one recent study, a combined group of node-negative and node-positive patients with high levels of activated mitogen-activated protein kinases in their primary tumors had a higher incidence of recurrent disease (17 of 32) compared with patients with low levels (23 of 99; Ref. 59). In a gene expression array study by van’t Veer et al. (60), the expression levels of 70 marker genes had unprecedented prognostic value but still permitted an actuarial 10% rate of false negatives. Additionally, that assay requires many tumor sections, with each section containing at least 50% tumor cells, whereas the simple Shc IHC assays can be performed on limited surgical material containing relatively few tumor cells. In another study, components of the hepatocyte growth factor pathway seem to effectively identify a subpopulation of node-negative patients at relatively high risk of relapse and death (HR of 1.8 for cytoplasmic c-Met receptor and 1.8 for matrispase inhibitor), yet still leave a major portion of node-negative risk in the low HGF pathway risk group (51). Similarly, the excellent, conclusive meta-study of uPA/PAI-1 has proven their independent prognostic value in node-negative breast cancer (50). The HRs comparing the highest to the lowest of scores for uPA and PAI-1 in five fractional ranks was approximately 3.4 and 2.8 when modeled separately, or 2.4 and 1.9 when modeled together. Thus, although this is of unprecedented value, it still leaves substantial numbers of women at risk, with a NPV of 0.87 in the 20% of patients in even this lowest quintile of uPA/PAI-1 risk. Finally, very striking data have been reported on the DSS prognostic ability of cyclin E, especially in node-negative stage I disease, in which of 114 patients, all 12 with high cyclin E levels died of their disease within 7 years of diagnosis (NPV, 1; PPV, 1; Ref. 54).

After additional retrospective and prospective studies to further validate the Shc ratio assay and understand its relationships to other markers such as those discussed above, the Shc ratio assay, especially when used in concert with uPA/PAI-1, cyclin E and other emerging markers, may continue to sufficiently separate high versus low risk patients to affect patient management (4–6). For example, patients whose primary tumors have high Shc ratios but low cyclin E levels may define nearly all patients who can be cured by conventional adjuvant therapies. Furthermore, the assay should help allow the identification of a minimal therapeutic intervention that is sufficient to prevent relapse in the low risk group and, at the same time, markedly improve the efficiency with which new experimental strategies can be tested on the small group of patients otherwise destined to experience recurrent disease. To the extent that the Shc proteins are reporters or integral components of aggressive disease, therapeutic strategies that alter their activity or amount might be especially effective.

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Announcements

(Requests for announcements must be received at least three months before publication.)

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2005 April 16–20, Anaheim, CA

AACR SPECIAL CONFERENCES IN CANCER RESEARCH

A number of meetings are now being organized in the AACR’s series of smaller scientific meetings. Following are the topics, dates, locations, and program committees for these meetings. When full details of each meeting are available, AACR members will be the first to receive complete brochures and application forms for participation in these important conferences. Nonmembers may receive this information by sending their names and addresses to Meetings Mailing List, American Association for Cancer Research, 615 Chestnut Street, 17th Floor, Philadelphia, PA 19106-4404. Up-to-date program information is also available via the Internet at the AACR’s website (http://www.aacr.org).

SIXTH JOINT CONFERENCE OF THE AACR AND JCA, ADVANCES IN CANCER RESEARCH

January 25–29, 2004
Hilton Wai Koloa Village, Wai Koloa, Hawaii

Chairpersons
Waun Ki Hong, Houston, TX
Takahashi Tsuruo, Tokyo, Japan

RADIATION BIOLOGY AND CANCER: FROM MOLECULAR RESPONSES TO THE CLINIC

February 18–24, 2004
Laguna Cliffs Marriott Resort, Dana Point, CA

Chairpersons
Susan S. Wallace, Burlington, VT
Michael B. Kastan, Memphis, TN
George Iliakis, Essen, Germany

CALENDAR OF EVENTS

Third International Conference and 9th Annual Meeting of the International Society of Cancer Chemoprevention (ISCaC): Controversies in Tumor Prevention and Genetics, February 12–14, 2004, University of St. Gallen, Switzerland. E-mail: info@oncoconferences.ch; Website: www.oncoconferences.ch.


11th Conference on Advances in Neuroblastoma Research, June 16–19, 2004, Genoa, Italy. E-mail: anr2004@neuroblastoma.org; Website: www.anr2004.org.

6th International Conference on Head and Neck Cancer, August 7–11, 2004, Marriott Wardman Park, Washington, DC. Contact: Concepts in Meeting & Events, 1805 Ardmore Boulevard, Pittsburgh, PA 15221. Phone: 412.243.5156; Fax: 412.243.5160; E-mail: ssteighnercme@aol.com.

Molecular Targets for Cancer Therapy: 3rd Biennial Meeting, October 1–5, 2004, Don Cesar Beach Resort & Spa, St. Petersburg Beach, FL. Contact: Ann Gordon. Phone: 813.903.4975; E-mail: gordonac@moffitt.usf.edu.
Corrections

In the article by P. A. Davol et al., titled “Shc proteins are strong, independent prognostic markers for both node-negative and node-positive primary breast cancer,” which appeared in the October 15, 2003 issue of Cancer Research (pp. 6772–6783), Table 6 was omitted. Table 6 appears below.

Table 6 Multivariate cox models of RFS and DSS

<table>
<thead>
<tr>
<th>Models</th>
<th>RFS</th>
<th>DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>P</td>
</tr>
<tr>
<td>Base Model</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodal status</td>
<td>6.4</td>
<td>0.006</td>
</tr>
<tr>
<td>Tumor stage</td>
<td>5.4</td>
<td>0.095</td>
</tr>
<tr>
<td>Therapy</td>
<td>6.1</td>
<td>0.068</td>
</tr>
<tr>
<td>with SRcont</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shc Ratio</td>
<td>12.7</td>
<td>0.012</td>
</tr>
<tr>
<td>Nodal status</td>
<td>8.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Therapy</td>
<td>6.7</td>
<td>0.053</td>
</tr>
<tr>
<td>with SRcat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shc Ratio_low/high</td>
<td>0.088</td>
<td>0.024</td>
</tr>
<tr>
<td>Shc Ratio_inter/high</td>
<td>0.20</td>
<td>0.029</td>
</tr>
<tr>
<td>Nodal status</td>
<td>3.6</td>
<td>0.042</td>
</tr>
<tr>
<td>Therapy</td>
<td>5.7</td>
<td>0.074</td>
</tr>
</tbody>
</table>

* Model development is described in Methods. Wald-test values of P < 0.05 are indicated in bold type. Models use the Shc Ratio as an indexed categorical variable (SRcat) (see Table 4), and also as a continuous variable (SRcont).

* HR for SRcat compares the hazard of low to high and intermediate (inter) to high, as in Table 4. HR for SRcont reflects the full observed range of Shc Ratios. HR compares >3 positive nodes to 0 positive nodes: Stage III to Stage I; T4 to T1; for Therapy, surgery with radiation to surgery alone.

In the article by H. M. Sowter et al., titled “Predominant role of hypoxia-inducible transcription factor (Hif)-1α versus Hif-2α in regulation of the transcriptional response to hypoxia,” which appeared in the October 1, 2003 issue of Cancer Research (pp. 6130–6134), Raju Raval and John Moore’s middle initials were omitted. The correct author list is as follows: Heidi M. Sowter, Raju R. Raval, John W. Moore, Peter J. Ratcliffe, and Adrian L. Harris.
Shc Proteins Are Strong, Independent Prognostic Markers for Both Node-Negative and Node-Positive Primary Breast Cancer


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