Stanniocalcin-1: A Novel Molecular Blood and Bone Marrow Marker for Human Breast Cancer

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ABSTRACT

Purpose: Very few tumor molecular markers have been identified that are highly specific for breast cancer when applied to blood and bone marrow (BM). Stanniocalcin (STC)-1 is a recently discovered human gene that has been implicated in cellular calcium homeostasis and resistance to hypoxia and is located on chromosome 8p in a region associated with amplification in breast cancer. We investigated STC-1 mRNA as a potential molecular marker for detection of breast cancer metastasis in the blood and BM.

Experimental Design: Using the reverse transcriptase-PCR and electrochemiluminescence detection assay to assess for STC-1 mRNA expression, we evaluated 7 breast cancer cell lines, 34 primary breast cancer tumors, and the BM of 71 patients and the blood of 58 patients with American Joint Committee on Cancer stage 0–IV breast cancer.

Results: In this cohort of primarily early-stage breast cancer patients, the detection of STC-1 mRNA in the BM and blood significantly correlated with multiple histopathological prognostic factors, including primary tumor size, number of positive lymph nodes, T stage, M stage, N stage, and overall American Joint Committee on Cancer stage. STC-1 mRNA was not detected in the blood or BM of volunteers without cancer. In situ hybridization studies with a STC-1 antisense RNA probe also confirmed STC-1 mRNA expression in breast cancer cell lines and primary breast tumors.

Conclusions: STC-1 is proposed as a novel, specific, and clinically useful molecular marker for detecting occult breast cancer cells in the BM and blood.

INTRODUCTION

AJCC stage I and II breast cancer patients who have undergone curative surgery and show no evidence of LN or distant metastasis still have a long-term recurrence rate of 30–50% (1–3). The BM and cortical bone are the most common sites of breast cancer metastasis, followed by visceral and soft tissue sites (4). Breast cancer patients who ultimately develop systemic metastases after complete surgical resection may remain clinically free of disease for years before disease recurrence finally reaches the threshold of current clinical and radiographic methods of detection. The earliest possible diagnosis of breast cancer, both primary and recurrent, is of considerable clinical importance, and it can be used to make treatment decisions while tumor burden is low, and when patients are most likely to respond to adjuvant therapy. Breast cancer is known to hematogenously spread to the BM and other distant sites. However, a clinically useful molecular marker has not yet been validated for the detection of occult breast cancer metastases in the blood and BM.

A highly conserved homologue of the fish glycoprotein STC has recently been identified in humans (5, 6). In fish, the product of this gene is secreted by the organ of Stanniarius in response to hypercalcaemia, resulting in the inhibition of calcium absorption from the gills and gut and increased resorption of phosphorus by the kidney (7). STC-1 has been mapped to 8p11.2-p21, a region of gene amplification described in breast cancer (8–11). For this reason, we evaluated STC-1 as a potential molecular tumor marker for breast cancer. The human STC gene (STC-1) consists of 4 exons and spans 13 kb, with an open reading frame of 741 bp. The 4-kb human STC-1 mRNA transcript codes for a 247-amino acid glycoprotein (Mₐ ~30,000). The STC-1 protein shares 70–80% amino acid sequence similarity with the homologous STC proteins of several bony fish species and 98% amino acid sequence similarity to mouse STC protein (6, 12–14).

Although its physiological function in humans is unclear at this time, recombinant human STC-1 is physiologically active in both mammals and fish, causing decreased calcium uptake and increased phosphate absorption when applied to the duodenum...
of rats and swine and decreased renal phosphate excretion after injection into goldfish and rats (13–15). Limited expression of the STC-1 gene in humans has been identified in the renal tubules, ovary, prostate, pancreas, small intestine, and thyroid (5, 16). In contrast to its effects in fish, in which STC appears to act as an endocrine hormone, STC-1 appears to function in an autocrine/paracrine fashion to regulate calcium and phosphate uptake and excretion in mammals (13–15). However, STC-1 has no significant amino acid or nucleotide homology to the major mammalian calcium regulation endocrine hormones, calcitonin and parathyroid hormone (17).

The RT-PCR assay has been used to detect occult metastatic tumor cells in breast cancer patients, based on its ability to detect tumor-associated mRNA as a surrogate of tumor cells (18). At the present time, breast cancer mRNA tumor markers are relatively few in number, and many of them are also expressed by normal cells found in the blood, BM, and LNs (19–22). The detection of occult circulating tumor cells in the blood and BM of patients who are clinically free of residual breast cancer is important in identifying patients with subclinical metastatic disease. Breast cancer patients often experience a prolonged clinical disease-free interval between initial diagnosis/treatment and clinical recurrence. The ability to detect early recurrence in the blood or BM may allow for adjuvant treatment to be instituted when the patient’s tumor burden is still quite minimal. Monitoring of patient response to systemic adjuvant therapy using specific tumor mRNA markers would be similarly useful, allowing for the early modification of adjuvant treatment to an alternative regimen if persistent occult tumor cells are detected in the blood or BM. Therefore, the identification of new and specific RT-PCR markers for detecting occult metastatic breast cancer cells in the blood and BM is necessary.

Based on its location in a region known to contain breast cancer-related genes and its apparent role in cellular calcium homeostasis, we investigated STC-1 mRNA as a potential molecular marker for the detection of breast cancer cells in the blood and BM using RT-PCR and quantitative ECL analysis and real-time quantitative RT-PCR. Preliminary evaluation of STC-1 in our laboratory revealed no expression of STC-1 mRNA in the blood or BM of healthy volunteers without cancer.4 Therefore, we assessed the blood and BM of patients with breast cancer for evidence of STC-1 mRNA expression in this study and correlated STC-1 expression with known clinicopathological prognostic factors. ISH was also used to validate STC-1 mRNA expression in breast cancer cell lines and in paraffin-embedded breast cancer tumor sections.

MATERIALS AND METHODS

Cell Lines. The breast cancer cell lines BT-20, MCF-7, HBL-100, T-47D, MDA-MB-231, 734B, JM992Br, PM277Br, and the choriocarcinoma cell line JAR were used. The cell lines were grown in RPMI 1640 (Gemini Bioproducts, Calabasas, CA) plus 10% heat-inactivated FCS, penicillin (Life Technologies, Inc., Grand Island, NY), and streptomycin (Life Technologies, Inc.) in 75-cm² flasks.

Patient Specimens. Breast tumor surgical specimens and blood were obtained after obtaining informed patient consent, and in consultation with the surgeon and pathologist. All tissues were collected and dissected under stringent sterile conditions to prevent RNA contamination. Institutional review board approval was obtained for the use of human subject blood, BM, and tumor specimens from breast cancer patients and normal healthy donors. Blood was collected from 58 female patients with AJCC stage 0–IV breast cancer. Bilateral percutaneous needle iliac crest BM aspirations were performed on an additional 71 AJCC stage I–III breast cancer patients under general anesthesia after obtaining informed consent and just before resection of their primary breast tumors and/or axillary LNs. Blood specimens obtained from 44 healthy donors with no history of cancer were used as negative controls in the blood RT-PCR/ECL assay. Frozen mononuclear cells isolated from adult normal healthy donor BM (n = 3) were purchased from BioWhittaker, Inc. (Walkersville, MD) and used as negative controls in the BM RT-PCR/ECL assay. All patient specimens were coded before being assessed, and individuals performing RT-PCR assays were blinded regarding all patients’ clinical status.

Ten ml of blood were collected in sodium citrate-containing tubes from breast cancer patients and healthy volunteer donors, as described previously (19). PBLs from whole blood and mononuclear cells from BM were isolated using Purescript RBC lysis buffer (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer’s instructions. TRI Reagent (Molecular Research Center, Cincinnati, OH) was used to isolate total RNA from the cell lines, surgical tumor specimens, BM, and PBLs as described previously (19). The concentration, purity, and amount of total RNA were determined by UV spectrophotometry. Purified RNA was then treated with RQ1 RNase-free DNase (Promega, Madison, WI) to eliminate any genomic DNA contamination.

RT-PCR Primer and Probe Synthesis. Biotinylated oligonucleotide primers for PCR and a TBR chelate-labeled hybridization probe for use in an ECL PCR cDNA product detection assay were synthesized by Gemini Biotech (Alachua, FL) and Midland Certified Reagent Company (Midland, TX), respectively, as described previously (23, 24). To avoid amplification and detection of genomic DNA, primer and probe sequences were designed to span 1 intron and designed for optimal use in the PCR and ECL assay systems. STC-1 primer sequences used were as follows: 5′ primer, 5′-TGAGGTGGTCCAGCT-GGCAATC-3′ (exon 3); and 3′ primer, 5′-GCCCAATCGCAGGATGATG-3′ (exon 4); with a resulting cDNA product of 172 bp (Fig. 1).
All PCR products were then detected using a quantitative ECL probe-based assay (igen International, Inc., Gaithersburg, MD), as described previously (24, 25). The TBR-labeled ECL probe was designed in our laboratory and synthesized by Midland Certified Reagent Company. The sequence for the STC-1 oligonucleotide probe was 5′-TCCAGCAGGCTTCGGA-CAAG-3′.

PCR primers and a TaqMan fluorescent probe for a new real-time quantitative PCR assay were also designed in our laboratory and were used to evaluate STC-1 mRNA expression levels in breast cancer cell lines. The STC-1 real-time PCR primer sequences used were as follows: 5′ primer, 5′-CAC-TTCTCCACAGATACT-3′ (exon 3); and 3′ primer, 5′-CATGTTAGGCCCCAATTTC-3′ (exon 4). The probe was conjugated with 6-carboxyfluorescein at the 5′ end and hexachloro-6-carboxyfluorescein at the 3′ end. Probe sequence was 5′-CCTGCTGGAATGTGATGAAGACAC-3′. The real-time PCR primers and TaqMan probe were synthesized by MWG-Biotec (High Point, NC) and Biosource International (Camarillo, CA), respectively. A reference standard for STC-1 mRNA copy number quantification was developed in our laboratory. Briefly, STC-1 PCR product was generated from breast cancer cell lines known to express STC-1. After confirmation of the expected PCR product size (111 bp) by agarose gel electrophoresis, the STC-1 bands were excised from the gel and purified (Qiagen, Inc., Valencia, CA). The purified STC-1 PCR product was ligated into a plasmid vector, which was then used to transform vector-competent Escherichia coli cells according to the manufacturer’s instructions for chemical transformation (Topo TA Cloning kit; Invitrogen Life Technologies, Inc., Carlsbad, CA). Confirmation of successful STC-1 PCR product ligation into the plasmid vector was obtained after restriction enzyme digestion. Serial 10-fold dilutions of the STC-1-ligated plasmids were then performed using molecular biology grade water and were used as copy number standards during all real-time PCR reactions assays.

RT-PCR/ECL Assay. The integrity of all RNA specimens was verified by performing RT-PCR/ECL to detect the mRNA of the housekeeping gene coding for porphobilinogen deaminase. Primer and probe sequences and PCR conditions for porphobilinogen deaminase have been described previously (26). Tissue processing, RNA extraction, RT-PCR assay set-up, and post-PCR product analysis were carried out in separate designated rooms to avoid contamination. Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s instructions (Promega). All reverse transcription reactions were carried out with oligo(dT) priming, as described previously (27). The same amount of RNA was used in all reactions for all samples in the study, including the control samples. Hybridization (Middlesex, United Kingdom) thermocyclers were used to perform all PCR reactions. The optimized PCR conditions for STC-1 analysis consisted of 1 cycle of denaturing at 95°C for 5 min; followed by 35 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min; and a final product extension step at 72°C for 10 min.

In each experiment set-up, samples of RT-PCR reagents without mRNA, H2O alone, and normal healthy donor PBL mRNA were used in all RT-PCR steps and in ECL analysis as negative controls. Tumor cell lines known to be RT-PCR for STC-1 expression were used in all assays as positive controls.

ECL detection assays with the Origen Analyzer (igen International, Inc.) were used for detection of STC-1 PCR cDNA products using a TBR-labeled hybridization probe, as described previously (24, 26). Negative controls used with the ECL assay included the H2O controls for the reverse transcription and PCR reactions as well as ECL assay bead, probe, and buffer solutions without cDNA products. The STC-1 RT-PCR assay was considered positive when the ECL assay results (in ECL U) were greater than 3 SDs above the mean ECL values of BM and blood from healthy volunteer donors. Assays were repeated at least twice to verify results.

Real-Time Quantitative PCR Assay. All RNA samples were isolated from breast cancer cell lines and quantified as described above. The synthesis of cDNA from total cellular RNA was performed as described above. The integrity of all RNA specimens was verified by performing quantitative real-time PCR to detect the mRNA of the housekeeping gene coding for glyceraldehyde-3-phosphate dehydrogenase.

A total of 1 μg of RNA was used for all samples in the study, including the positive control samples. The iCycler thermocycler (Bio-Rad, Hercules, CA) was used to perform all real-time quantitative PCRs. The optimized PCR conditions for real-time STC-1 analysis consisted of 1 cycle of denaturing at 95°C for 10 min, followed by 40 cycles at 54°C for 1 min and 72°C for 1 min, and a final product extension step at 72°C for 10 min. Threshold cycle numbers were calculated during PCR amplification for each STC-1 plasmid standard, and a standard curve was then created using the iCycler data analysis software. Based on this standard curve, absolute copy numbers were then calculated for all experimental PCR samples after 40 cycles of amplification.

ISH Assay. MCF-7 and BT-20 breast cancer cell lines were grown to 30–40% confluence on culture well glass slides, using the culture conditions described above, and then rapidly fixed with methanol and acetone washes. Paraffin-embedded archived primary breast cancer tissues, both IDCs and ILCs, were cut into 5-μm sections, deparaffinized, rehydrated, and prepared for ISH. RNA antisense probes against STC-1 were constructed using the STC-1 PCR cDNA product from a cell line known to express STC-1 (Ambion, Inc., Austin, TX).

ISH with the STC-1 antisense probe was then performed on both tumor cell lines and human breast tumor specimens (Ambion, Inc.). A digoxigenin-labeled β-actin antisense RNA probe (Ambion, Inc.) was used as a positive control, and hybridization buffer without RNA probe was used as a negative control. After ISH, all slides were then rinsed with Tris-buffered saline buffer [50 mM Tris-HCl (pH 7.5) and 150 mM NaCl] and developed with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt solution at 30°C (Boehringer Mannheim, Mannheim, Germany) until adequate colorization was obtained. The color reaction was then stopped with nuclease-free water, and the slides were mounted with an aqueous mounting solution. All slides were then viewed with a microscope and photographed with a digital camera.

Statistical Analysis. To investigate the association between established breast cancer clinicopathological characteristics and the presence of tumor-associated STC-1 mRNA, χ2 and
Wilcoxon rank-sum tests were used. P ≤ 0.05 was considered statistically significant.

RESULTS

RT-PCR/ECL Assay Detection Sensitivity. To assess the potential sensitivity of the STC-1 RT-PCR/ECL assay for detection of tumor cells in blood and BM under established optimal assay conditions, an in vitro model was set up by serially diluting tumor cells in healthy donor PBLs. The breast cancer cell lines MCF-7 and BT-20, both known to express STC-1 mRNA, were serially diluted in healthy donor PBLs to determine the detection sensitivity of the RT-PCR assay. Tumor cells could be detected at concentrations of 1–5 tumor cells in 10^7 PBLs with the STC-1 RT-PCR/ECL assay.

RT-PCR Analysis of Cell Lines and Primary Breast Tumors. The expression of STC-1 in cell lines and primary breast tumors was evaluated by RT-PCR. The cell lines MCF-7, 734B, BT-20, T-47D, MDA-MB-231, JM992Br, PM277Br, and JAR were assessed for STC-1 mRNA marker expression by non-real-time RT-PCR/ECL analysis. All seven breast cancer cell lines and JAR expressed STC-1 mRNA. In addition, 34 primary breast tumor specimens from patients with AJCC stage I–III breast cancer were tested for STC-1 mRNA expression. A total of 31 of these 34 primary tumor specimens (91%) were found to be positive for the STC-1 mRNA breast tumor marker (Table 1). The mean ECLU value for the 31 STC-1-positive breast tumors was 183,381, whereas the mean ECLU value for the three STC-1-negative tumors was 7,550.

Quantitative real-time RT-PCR was performed to determine STC-1 mRNA copy level in the breast cancer cell lines JM992Br, T-47D, BT-20, MCF-7, and HBL-100. These cell lines were found to express variable STC-1 mRNA copy levels, ranging from 219 to 31,200 copies per 250 ng of cellular RNA (Table 2).

STC-1 Expression in BM. Breast cancer cell detection in BM was evaluated using the STC-1 RT-PCR/ECL assay because occult metastasis in the BM has been shown to be an independent and negative prognostic factor in breast cancer (28–34). BM specimens obtained from three healthy donors with no history of cancer were negative for STC-1 expression under optimal assay conditions. Patient clinical status was classified according to AJCC stage at the time of BM collection as follows: 38 of 71 patients (54%) had AJCC stage I breast cancer; 30 of 71 patients (42%) had AJCC stage II disease, and 3 of 71 patients (4%) had AJCC stage III disease (Table 3). The histopathological status of the axillary LNs, based on H&E staining results, was also evaluated as a component of patient clinical status. A single patient with a LN that was negative by H&E stain but positive for cytokeratin-positive cells by immunohistochemistry alone was therefore not included in the LN status analysis. The mean ECLU value for the three BM specimens from healthy donors was 1,800 ECLU. Overall, 18 of 71 patients (25%) had detectable STC-1 mRNA in their BM. The mean ECLU value for the 18 STC-1-positive BM specimens was 19,400, whereas the mean ECLU value for the 53 STC-1-negative BM specimens was 7,880. Five of 38 stage I patients (13%), 11 of 30 stage II patients (37%), and 2 of 3 stage III patients (67%) had detectable STC-1 mRNA in the BM.

STC-1 Expression in BM and Clinical Correlation. Clinicopathological data were reviewed and analyzed for the 71 breast cancer patients who underwent RT-PCR of their BM. Age, tumor grade, tumor type, Bloom-Richardson grading score, size of primary tumor, TNM stage, overall AJCC stage, number of involved axillary LNs, and estrogen/progesterone receptor status were evaluated relative to STC-1 expression using χ² and Wilcoxon rank-sum tests in all 71 patients. The presence of STC-1 mRNA in the BM significantly correlated with the number of involved LNs (P < 0.002, Wilcoxon rank-sum test), size of the primary tumor (P = 0.05, Wilcoxon rank-sum test), T stage (P < 0.01, Wilcoxon rank-sum test), N stage (P < 0.01, χ² test), and overall AJCC stage (P < 0.001,
Table 4  Correlation between STC-1 RT-PCR blood marker status and patient characteristics

<table>
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<th>Clinical characteristics</th>
<th>STC-1 blood marker status*</th>
<th>P*</th>
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<tr>
<td></td>
<td>Negative (n = 46)</td>
<td>Positive (n = 12)</td>
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<tr>
<td>No. of LNs</td>
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<td>4.3</td>
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<tr>
<td>Mean</td>
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<tr>
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* Patient numbers do not equal 58 for all clinical characteristics due to incomplete patient data from referring institution.

* T stage and N stage not included (not significant). Significant values shown in bold.

* Wilcoxon rank-sum test.

* χ² test.

Wilcoxon rank-sum test; Table 3). The number of involved axillary LNs, TNM stage, and overall AJCC stage are all primary clinicopathological determinants of breast cancer prognosis and are surrogates of disease outcome. All other clinicopathological parameters failed to reach significance with respect to STC-1 marker status of the BM. Due to the small number of clinical disease-related events in this group of predominantly early-stage patients, correlation of STC-1 BM marker status with disease recurrence and death was not feasible. Long-term follow-up will be required to further define the precise prognostic significance of detection of STC-1 mRNA in the blood.

**STC-1 Expression in Blood.** The detection of occult circulating breast tumor cells in the peripheral blood has been demonstrated previously using molecular assays (35, 36). Although the prognostic significance of this finding remains unclear, we have shown previously that the detection of breast tumor mRNA markers in the blood correlates with AJCC stage of disease (37).

Blood was obtained from 44 healthy donors with no history of cancer. STC-1 mRNA was not detected in any of the normal donor blood specimens under the optimal assay conditions. The mean STC-1 RT-PCR assay value for the blood of the 44 healthy donors without cancer was 5,934 ECL U.

Blood was obtained from 58 breast cancer patients. Patient clinical status was classified according to the AJCC stage at the time of blood collection as follows: 1 of 58 patients had ductal carcinoma in situ (AJCC stage 0); 24 of 58 patients (41%) had AJCC stage I breast cancer; 19 of 58 patients (33%) had stage II disease; 8 of 58 patients (14%) had stage III disease; and 6 of 58 patients (10%) had stage IV disease (Table 4). Overall, 12 of 58 patients (21%) had detectable STC-1 mRNA in their blood. The mean value for the 12 STC-1-positive blood specimens was 46,709 ECL U, whereas the mean value for the 46 STC-1-negative blood specimens was 6,394 ECLU. Three of 24 stage I patients (13%) had detectable STC-1 mRNA in the blood, whereas 3 of 19 stage II patients (16%), 2 of 8 stage III patients (25%), and 4 of 6 stage IV patients (67%) were positive for STC-1 mRNA.

**STC-1 Expression in Blood and Clinical Correlation.** Clinicopathological data were reviewed and analyzed for the 58 breast cancer patients who underwent RT-PCR analysis of their blood. Age, tumor grade, tumor type, Bloom-Richardson grading score, size of primary tumor, TNM stage, overall AJCC stage, number of involved axillary LNs, and estrogen/progesterone receptor status were evaluated relative to STC-1 expression using χ² and Wilcoxon rank-sum tests in all 58 patients. The presence of STC-1 mRNA in the blood significantly correlated with AJCC stage (P = 0.03, Wilcoxon rank-sum test), size of the primary tumor (P < 0.03, Wilcoxon rank-sum test), and the presence of distant metastases (P = 0.003, χ² test), all of which are established prognostic factors for breast cancer (Table 4). All other clinicopathological parameters failed to reach significance with respect to STC-1 marker status. The number of involved axillary LNs approached significance (P = 0.06, Wilcoxon rank-sum test) with respect to RT-PCR status of the blood. Due to the small number of clinical disease-related events in this group of predominantly early-stage patients, correlation of STC-1 blood marker status with disease recurrence and death was not feasible. Long-term follow-up will be required to further define the precise prognostic significance of detection of STC-1 mRNA in the blood.

**STC-1 ISH of Cell Lines and Primary Tumors.** A chromogenic ISH assay was performed to confirm and localize the presence of STC-1 mRNA in paraffin-embedded breast cancer primary tumors. The breast cancer cell lines BT-20 and MCF-7 and paraffin-embedded ILC and IDC tumors were assessed for STC-1 mRNA expression using a STC-1 antisense RNA probe. Both breast cancer cell lines (Fig. 2) and breast tumor cells (Figs. 3 and 4) were found to express STC-1 mRNA. The STC-1 ISH assay revealed dense and homogenous cytoplasmic staining in all breast cancer tumor cells, but no staining was seen in the surrounding nonductal mammary cells. Negative control ISH assays lacking the RNA probe did not stain breast cancer cell lines or primary tumors.

**DISCUSSION**

To date, the study of the mechanisms early breast cancer metastasis has been focused primarily on tumor cell spread to regional LNs. However, the presence of occult circulating metastatic tumor cells in the blood and BM may actually represent the earliest sign of systemic spread, and there is growing evidence that even this very early phase of systemic disease spread may be of prognostic importance. A significant correlation between prognosis and the detection of occult circulating breast tumor cells in the BM or peripheral blood has been confirmed in a number of studies to date (28–38). In terms of primary tumor characteristics, the detection of isolated tumor cells in the BM of breast cancer patients has been shown by other laboratories to correlate with increased primary tumor stage, increased nodal stage, the presence of vascular invasion, HER-2/neu overexpression, and estrogen/progesterone receptor expression (39). Although metastasis to the regional LNs has generally been thought to be the most significant prognostic factor with respect
to predicting later recurrence and death (40), there is evidence that BM micrometastasis may be an even more significant prognostic factor than micrometastasis to the regional LNs (31, 41). Thus, the early detection of occult circulating tumor cells in the BM and blood may be more informative than the detection of early metastatic disease in the regional axillary LNs, including the sentinel LN(s).

The optimal detection of occult breast cancer tumor cells requires a sensitive assay, and one that is highly specific. The STC-1 RT-PCR assay appears to fulfill these requirements when applied to the blood and BM of patients with breast cancer. Several clinically important applications of this assay can be considered. In addition to its potential value in ultrastaging patients without clinical evidence of systemic metastatic disease, another potentially important application of the STC-1 RT-PCR assay might be to follow tumor progression and/or response to therapy when metastatic disease is not detectable by other means. Yet another potential application of the STC-1 RT-PCR breast cancer assay is the evaluation of patients with suspected metastatic breast tumors that cannot be safely or
adequately biopsied by surgical means. The identification of subclinical metastatic breast cancer disease may also allow for the earlier application of adjuvant therapy, at a time when the patient’s low level of tumor burden may result in the improved efficacy of such treatments in terms of disease progression and, possibly, survival. The early use of bisphosphonates or other osteoclast inhibitors in patients with occult BM disease might also significantly retard the development of clinically symptomatic skeletal metastases in patients without other clinical evidence of systemic disease. Such an assay could also prove useful to more accurately stage and stratify early-stage patients into clinical trials.

The STC-1 RT-PCR assay appears to provide a highly sensitive method for detecting occult breast cancer tumor cells in the blood and BM and is therefore a promising candidate tumor marker for a single or multiple marker RT-PCR breast cancer molecular diagnostic assay. Moreover, in this study, the detection of circulating tumor cells in the BM and blood of patients with breast cancer by the STC-1 RT-PCR assay was significantly correlated with known clinicopathological prognostic factors. Relapse of disease and death due to disease are prolonged in breast cancer relative to other tumor types, making it difficult to identify early correlation between the presence of occult circulating tumor cells and clinical outcomes such as disease-free survival and overall survival. However, the strong correlation in this study between the detection of occult circulating tumor cells and known clinicopathological prognostic factors further adds to the available evidence that subclinical disease in the blood, and particularly in the BM, is likely to be clinically important in breast cancer. Breast cancer has a well-known propensity to metastasize to the skeleton, subjecting patients to pain and fractures due to the resulting osteolytic lesions. Because skeletal metastases most likely arise from circulating tumor cells in the blood and BM, the ability to detect these clinically and radiographically invisible metastatic tumor cells may allow for the earlier application of adjuvant therapy, at a time when the patient metastatic tumor burden is still minimal. The adjuvant treatment armamentarium available for breast cancer patients rivals that of most other cancers. Thus, for patients with subclinical circulating tumor cells in the BM and/or blood as their only site(s) of disease, the early and aggressive application of hormonal, chemotherapeutic, or osteoclast inhibitor therapies may have an important role in slowing disease progression beyond that which is currently obtained by using these agents only after gross metastatic disease is detected. Indeed, the prognostic impact of circulating occult tumor cells and the effects of treating patients with such minimal disease deserve further study within a clinical trial setting.

Additional study of the physiological role of STC-1 protein in both normal and cancer cells is also needed. In our study, considerable variability in STC-1 mRNA copy levels was identified among different human breast cancer cell lines. Although the prognostic significance of STC-1 gene expression/overexpression in breast cancer, if any, is unknown at this time, there is recent evidence of up-regulation of STC-1 gene expression in human breast cancer cells subjected to hypoxic conditions in vitro (42). This finding suggests that STC-1 might potentially confer a phenotypic resistance to the relatively hypoxic environment present within both tumors and bone.

In summary, we assessed STC-1 as a potential molecular tumor marker for occult breast cancer in the BM and blood of patients with AJCC stage 0–IV breast cancer. STC-1 mRNA, as detected by RT-PCR, appears to be highly sensitive and specific to occult breast cancer cells and is not expressed in normal blood and BM cells. Although few disease-related clinical events have occurred in the relatively early-stage patients evaluated in this study to date, the presence of breast cancer–associated STC-1 mRNA in the BM and blood correlated significantly with the primary clinicopathological determinants of disease outcome (43). These findings, in addition to previous

Fig. 4 Representative ISH of primary breast cancer tumor with STC-1 antisense RNA probe. ILC labeled with STC-1 RNA probe (left panel) and negative control (no RNA; right panel).
studies demonstrating the prognostic significance of occult circulating breast tumor cells in the blood and BM, suggest that STC-1 may be a useful and highly sensitive new molecular marker for breast cancer.

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