Association of S100A4 and Osteopontin with Specific Prognostic Factors and Survival of Patients with Minimally Invasive Breast Cancer

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Abstract

Purpose: S100A4 and the estrogen-inducible osteopontin are alone capable of inducing angiogenesis and metastasis in rodent models for breast cancer. The present study assesses the relationship of S100A4 and osteopontin with vessel density and estrogen receptor α (ERα) in primary tumors and with survival of patients to ascertain their involvement in metastatic breast cancer.

Experimental Design: Primary tumors from 312 patients treated for minimally invasive human breast cancer were immunocytochemically stained and then assessed for the significance of their association with each other using Fisher’s exact test or with patient survival over 18 years of follow-up using Kaplan-Meier plots and Wilcoxon-Gehan statistics.

Results: Antibodies to S100A4 significantly stained endothelial cells of vessels adjacent to S100A4-staining groups of carcinoma cells, and antibodies to osteopontin significantly stained groups of carcinoma cells staining for ERα (P < 0.0001). There was a significant association of tumors staining for S100A4 with those with high vessel density (P = 0.021) and of tumors staining for osteopontin with those staining for ERα (P = 0.034). The association of staining for S100A4, osteopontin, or vessel density with patient death was significant (P < 0.0001, P = 0.005, and P = 0.014, respectively). The difference in cumulative proportion surviving between S100A4-positive patients with higher or lower vessel density increased up to about 12 years, but thereafter decreased to virtually zero after 18 years of follow-up. Patients with both S100A4-positive and osteopontin-positive primary tumors showed a statistically significant reduction in survival time over those with either one alone (P < 0.019), although in multivariate regression analysis, only staining for S100A4 was significant (P < 0.001).

Conclusions: It is suggested that in human breast cancer, S100A4 exerts some of its effects through angiogenesis, and that osteopontin is dependent on ERα for its expression.

The process of metastasis is ultimately responsible for the death of most patients suffering from the common carcinomas. Nonetheless, not all patients suffering from primary breast cancer necessarily develop metastases and die of the disease (1, 2). To identify those patients at high risk of dying from metastatic spread, a series of pathologic factors have been used. These factors include the size of the primary tumor, the histologic grade (3), the existence of tumor in their draining lymph nodes (4), and more recently the presence of estrogen receptor α (ERα; refs. 5–7) and levels of intratumoral microvessels (8–12). To identify additional genes that may be involved more specifically with metastasis of breast cancer, we have first identified molecules that can induce a nonmetastatic rat mammary cell line rat mammary 37 (Rama 37) to become metastatic in syngeneic rats (13, 14) and assessed their association with survival of a group of patients treated by mastectomy/radical mastectomy for breast cancer (7, 15). Thus far, two proteins have been identified in the rat mammary model system that can induce metastasis: the calcium-binding intracellular protein S100A4 (16) and the secreted glycoprophoprotein osteopontin (17).

Both S100A4 and osteopontin proteins and their mRNAs occur at higher levels in primary human carcinomas than in the corresponding nonmalignant tissues (18–22). Moreover, the immunocytochemical presence of S100A4 and osteopontin above a basal threshold in primary breast carcinomas is associated over a 19-year follow-up period with decreased survival for one group of patients treated solely by radical
mastectomy/mastectomy with no adjuvant therapy (23, 24). The association between immunocytochemical detection of S100A4 and osteopontin in the primary breast carcinomas and early patient death is consistent with some of the results obtained by others (25–27); however, this is not invariable, and lack of association has been reported over shorter periods of time (5-7 years) for patients treated by more modern adjuvant therapies (28, 29). We have now investigated, using immunocytochemical techniques, the levels of S100A4 and osteopontin in tumor specimens from a large group of patients with minimally invasive breast cancer treated by local excision with the usual adjuvant therapy (30) and their relationship with potential prognostic factors and patient death from metastatic breast disease.

Materials and Methods

Patients and specimens. The study was based on 312 patients who presented with stage I or II primary symptomatic breast cancer to the Breast Unit of the Royal Liverpool University Hospital between 1982 and 1991. Patients had primary unilateral breast cancer treated by local excision with or without radiotherapy and possessed no disseminated tumors at the time of diagnosis. The excised lymph nodes were recorded histologically as positive or negative for carcinoma. The patients’ ages varied from 17 to 89 years (mean age, 57 years), and all had invasive carcinomas. The distribution of tumor sizes (T1, <2 cm; T2, 2-5 cm; and T3, >5 cm in diameter; 312 patients), nodal status (200 patients), menopausal status (173 patients), and histologic grade (1-3; 312 patients) were recorded. Approximately half the patients have been described previously (30); the remainder have been assessed more recently.6 The patients were staged clinically according to the international tumor-node-metastasis system (23, 24), and only patients with operable cancer (T1-3,N 0-1, and M0) were included. Premenopausal and postmenopausal patients received adjuvant chemotherapy, and postmenopausal patients received tamoxifen, 20 mg once a day. Follow-up information was obtained from the Merseyside Cancer Registry updated for patient survival to July 1, 2000. Follow-up ranged from 9 to 18 years with a mean of 12.7 years. Data were subsequently verified from general practitioner records to confirm whether patients were alive, dead of cancer, or dead of other causes. The Local Ethics Committee approval was obtained, and patient data were anonymized. Archival formalin-fixed, paraffin-embedded specimens were obtained from the primary tumors; they were predominantly invasive ductal carcinomas of no special type (NOS) with only 5.5% of special type (colloid and medullary).

Serology. Rabbit polyclonal antibody to human recombinant S100A4 was purchased from DAKO Ltd. (Ely, United Kingdom), and the mouse monoclonal antibody (mAb) to rat recombinant osteopontin (MBIII B101) was from the Developmental Studies Hybridoma Bank, University of Iowa (31). These antibodies specifically recognise either S100A4 or osteopontin of apparent molecular weights of 20,000 and 50,000, respectively, when tested in Western blots of extracts of breast carcinomas (23, 24). Binding of both antibodies to their respective proteins was inhibited by prior incubation of anti-S100A4 with 10 μg/ml human recombinant (r)S100A4 (32) or anti-osteopontin with 10 μg/ml human recombinant osteopontin (CCl074; Chemicon International, Harrow, United Kingdom). Several batches of both the rabbit polyclonal and mouse mAbs were purchased giving the same results. Additionally, rabbit polyclonal antisera to rat S100A4 was prepared on a column of rS100A4-Sepharose (33) and that to human osteopontin (LF -123) was obtained from Dr. Larry Fisher (NIH, Bethesda, MD; refs. 34, 35) as documented previously (23, 24). mAb which recognizes endothelial cells, anti-CD34 (Q BEND/10), was purchased from Serotec (Oxford, United Kingdom; ref. 30) and that to human ERα (ID3) from DAKO (36). Both mAbs recognized the correct size antigen on Western blots from SDs-polyacrylamide gels of extracts from human breast tumor tissue (30, 36).

Immunocytochemistry. Histologic sections were cut at 4 μm from the formalin-fixed, paraffin-embedded tumor specimens and mounted on amino-propyltriethoxysilane–coated slides. Endogenous peroxidase activity was inhibited by treatment with H2O2 (23). A antigen retrieval using a microwave oven was undertaken for ERα. Indirect immunocytochemistry was undertaken with a commercial antibody biotin complex containing horseradish peroxidase (23, 24) for 312 patients for S100A4 and 291 patients for osteopontin with the following modifications. Antibodies to human rS100A4 and to rat recombinant osteopontin were diluted 1:400 and 1:30 fold, respectively, those to rat rS100A4 and to human osteopontin 1:500 and 1:300, respectively. Sections were incubated at room temperature for 16 hours with antibodies to human S100A4 and rat osteopontin and for 3 hours with the antibody to human osteopontin, and bound antibody was detected with 1:200 biotinylated donkey anti-rabbit immunoglobulin (Amersham International, Bucks, United Kingdom) for the polyclonal antibodies or with 1:200 biotinylated sheep antirabbit immunoglobulin (Amersham International) for the mAb followed by antibody biotin complex (DAKO). Bound complexes were visualized with 3,3′-diaminobenzidine (Sigma, Poole, United Kingdom) and 0.003% (v/v) H2O2, and cell nuclei were counterstained with Mayer’s hemalum. Immunocytochemical staining for CD34 (258 patients) and for ERα (257 patients) was conducted as above for mAbs using 1:100 and 1: 25 dilutions for 16 hours and 90 minutes, respectively, except that for anti-CD34, in which histologic sections were preincubated with 0.1% (w/v) trypsin, 0.1% (w/v) CaCl2 in Tris/HCl (pH 7.6) at 37°C for 15 minutes, as described previously (36–38). Photographs were recorded on a Reichert Polvar microscope fitted with a Wratten 44 blue-green filter (23).

Assessment of staining. Slides were analyzed independently by two observers using light microscopy. The percentage of carcinoma cells staining for S100A4, osteopontin, or ERα was recorded from two sections of each specimen, 10 fields per section at ×200 magnification. Staining for S100A4, osteopontin, and ERα was evaluated as negative (−), <1% and positive staining (+), >1% of the carcinoma cells stained (36). In all cases, positive immunocytochemical staining was abolished by prior incubation with the appropriate recombinant protein. For staining for S100A4 and osteopontin, there was agreement between the two observers in 96.2% and 93.7% of the slides corresponding to κ = 0.92 and 0.87, respectively, which represents a good degree of consistency. In 7.4% and 11.5% of pairs of histologic sections analyzed for staining for S100A4 and osteopontin, respectively, heterogeneous staining was sufficiently high to affect whether a section was classified as negatively or positively stained. In those cases, two additional sections were immunocytochemically stained and analyzed to obtain a consensus result. The same results for staining with commercial antibodies to S100A4 and to osteopontin were obtained with 20 specimens selected at random and stained with a laboratory-raised antiserum to the respective antigens. Microvessel densities of sections stained for CD34 were assessed as described previously (30, 38). Areas containing the greatest numbers of microvessels were first identified at low magnifications (<40 × 100) and then counted at ×200 magnification using a square grid graticule (field size = 0.68 mm2), 10 fields per section were scored. The highest number of microvessels per field was recorded. Patients were divided into two categorical groups of lower and higher vessel density usually by the median cutoff value of 109 vessels per field (30). This method yielded the most significant correlation with patient survival over other methods, which recorded the means of the highest 3, 5, or 10 fields in about half of these patients (30).

6 C. Roshanlall and J. Winstanley, unpublished results.
**Statistical methods.** The association of immunocytochemical staining for S100A4 and for osteopontin with other tumor variables was assessed using Fisher’s two-sided exact test (39). Tumor variables were converted into two categorical groups for tumor size (T1 versus T2 and T3), nodal status (0 versus ≥1), histologic grade (1 and 2 versus 3), menopausal status (− versus +), patient age (<50 versus >50 years), vessel density (below versus above median value), and ERα status (− versus +). Level of agreement between observers was assessed using the Kappa statistic; a value of >0.61 was taken to be satisfactory (39). The association of staining for S100A4, for osteopontin, or for other tumor variables with patient survival was evaluated with Kaplan-Meier plots and analyzed using generalized Wilcoxon-Gehan statistics (39); non–cancer-related deaths were excluded. To assess unadjusted relative risk (RR) for survival and 95% confidence interval (95% CI), the data were analyzed using Cox’s univariate method (39). To determine if the association of patient survival with S100A4 or with osteopontin was independent of other prognostic factors, a multivariate analysis was undertaken using the Cox proportional hazards model (40). Data processing and statistical analyses were done using Excel version 97 (Microsoft Corp., Redmond, WA) and Statistical Package for the Social Sciences version 11.01 (SPSS, Inc., Chicago, IL).

**Results**

**Immunocytochemical staining.** When 312 breast carcinomas were examined for immunocytochemical staining of the carcinoma cells for S100A4, 91 (29.1%) were unstained, and 221 (70.9%) were stained to varying degrees. The staining was mainly confined to the cytoplasm but with some nuclear staining (Fig. 1A). In addition, endothelial cells were also stained to varying degrees. When 291 of these breast carcinomas were subsequently immunocytochemically stained for osteopontin, 148 (50.9%) were unstained, and 143 (49.1%) were stained for this antigen. In this case, staining for osteopontin occurred both in the membrane and cytoplasm (Fig. 1B). Antibodies against two other potential prognostic indicators, that for the marker for endothelial cells, CD34, and that for ERα stained microvessels (Fig. 1C) or the nuclei of carcinoma cells (Fig. 1D), respectively. Antibodies to S100A4 stained significantly endothelial cells in microvessels adjacent to groups of S100A4-staining carcinoma cells (Fig. 1E). In contrast, antibodies to osteopontin stained significantly carcinomatous areas that expressed immunologically detectable ERα (Fig. 1F). The reverse associations were not the case (data not shown).

**Association of S100A4 and osteopontin with other tumor variables.** The presence of positive immunocytochemical staining for S100A4 and for osteopontin in the carcinoma cells was cross-tabulated for the primary tumors with some other tumor variables reported to be predictive of patient outcome (Table 1). Of these, only the presence of higher vessel density in the primary tumor showed a statistically significant association with carcinoma cells staining for S100A4, using a median cutoff of 109 counts per microscopic field (Table 1). This association was also significant if the highest quartile (100-75%) were compared with the rest (<75%) using a cutoff of 162 counts per microscopic field (P = 0.040; data not shown). In contrast, there was no association between positive immunocytochemical staining for osteopontin and higher vessel density at either the 50% (Table 1) or 75% cutoff levels (P = 0.43; data not shown). There was also a statistically significant association of carcinomas positively staining for osteopontin with positive staining for ERα, but this did not occur between carcinomas staining positively for both S100A4 and for ERα (Table 1).

In this group of patients, the association of tumor in the draining lymph nodes was of borderline significance with positive staining for S100A4 and of no significance with that for osteopontin (Table 1). There was a borderline association...
between staining for S100A4 and for osteopontin (Table 1). The remaining variables measured (tumor size, histologic grade, menopausal status, and age or other demographic difference) showed no significant association with positive staining for S100A4 or for osteopontin. However, higher vessel densities using the 50% cutoff showed good correlation with large tumor size ($P = 0.006$), with involved lymph nodes ($P = 0.038$), and a near significant association with staining for ERα ($P = 0.058$) but not with high-grade tumors ($P = 0.511$; data not shown).

**Association of S100A4 and osteopontin with patient survival.**

Out of 76 patients who were classified as S100A4 negative, 92% were alive at the census date compared with 48% of the 196 patients who were classified as S100A4 positive. The median survival time of the S100A4-negative patients was >204 months, highly significantly longer than the 186 months for the S100A4-positive patients over the 18-year follow-up period (Fig. 2A). Moreover, women with S100A4-negative carcinomas had an unadjusted RR for survival of 1.80 (95% CI, 1.10-2.94) compared with the osteopontin-positive group. If patients in the positively staining group for osteopontin were subdivided further as before into a weakly staining group (80 patients) and a strongly staining group (45 patients), the overall differences were highly significant (Wilcoxon test: $\chi^2 = 12.21, 2 df, P = 0.0022$). In that case, women with osteopontin-negative carcinomas had an unadjusted RR for survival of 1.4 (95% CI, 0.80-2.42) compared with those with weakly staining carcinomas, and a RR of 2.7 (95% CI, 1.5-4.9) compared with those with strongly staining carcinomas for osteopontin (data not shown). Only those patients treated with anti-hormonal therapy after surgery showed a significant difference in survival between those with osteopontin-positive and osteopontin-negative tumors (Wilcoxon statistic: $\chi^2 = 7.2, 1 df, P = 0.0073$; RR, 1.8; 95% CI, 1.03-3.15). Untreated patients showed no significant difference ($\chi^2 = 0.001, 1 df, P = 0.98$; RR, 1.1; 95% CI, 0.4-3.4). In contrast both anti-hormonally treated ($\chi^2 = 17.5, 1 df, P < 0.0001$; RR, 7.9; 95% CI, 2.4-25) and untreated patients ($\chi^2 = 4.0, 1 df, P = 0.045$; RR, 7.1; 95% CI, 0.96-53) showed significant differences with similar risk factors for survival between those with S100A4-positive tumors and those with S100A4-negative tumors.

**Association of other tumor variables with patient survival.**

The other tumor variables that showed a significant negative association with patient survival time were nodal status, tumor size, histologic grade, and vessel density (Table 2). The positive association of staining for ERα with survival time was significant, 66% were alive at the census date compared with 63% of the 125 patients who were classified as osteopontin positive. The median survival time of the osteopontin-negative patients was >216 months, significantly longer than the >204 months for those classified as positive over the same 18-year period (Fig. 2B). Women with osteopontin-negative carcinomas had an unadjusted RR for survival of 1.80 (95% CI, 1.10-2.94) compared with the osteopontin-positive group. If patients in the positively staining group for osteopontin were subdivided further as before into a weakly staining group (80 patients) and a strongly staining group (45 patients), the overall differences were highly significant (Wilcoxon test: $\chi^2 = 12.21, 2 df, P = 0.0022$). In that case, women with osteopontin-negative carcinomas had an unadjusted RR for survival of 1.4 (95% CI, 0.80-2.42) compared with those with weakly staining carcinomas, and a RR of 2.7 (95% CI, 1.5-4.9) compared with those with strongly staining carcinomas for osteopontin (data not shown). Only those patients treated with anti-hormonal therapy after surgery showed a significant difference in survival between those with osteopontin-positive and osteopontin-negative tumors (Wilcoxon statistic: $\chi^2 = 7.2, 1 df, P = 0.0073$; RR, 1.8; 95% CI, 1.03-3.15). Untreated patients showed no significant difference ($\chi^2 = 0.001, 1 df, P = 0.98$; RR, 1.1; 95% CI, 0.4-3.4). In contrast both anti-hormonally treated ($\chi^2 = 17.5, 1 df, P < 0.0001$; RR, 7.9; 95% CI, 2.4-25) and untreated patients ($\chi^2 = 4.0, 1 df, P = 0.045$; RR, 7.1; 95% CI, 0.96-53) showed significant differences with similar risk factors for survival between those with S100A4-positive tumors and those with S100A4-negative tumors.

### Table 1. Association of staining for S100A4 and osteopontin with other tumor variables

| Tumor variable* | S100A4 | | | | | OPN | | | |
|-----------------|-------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Negative, $n$ (%) | Positive, $n$ (%) | Statistical significance | | | Negative, $n$ (%) | Positive, $n$ (%) | Statistical significance | | | |
| LN–             | 38 (78) | 74 (61) | 0.050 | | | 54 (64) | 50 (65) | 1.00 | | | |
| LN+             | 11 (22) | 48 (39) | | | | 30 (36) | 27 (35) | | | | |
| Grade 1, 2     | 66 (79) | 151 (73) | 0.30 | | | 103 (76) | 100 (76) | 1.00 | | | |
| Grade 3        | 18 (21) | 57 (27) | 0.021 | | | 53 (49) | 65 (51) | 0.79 | | | |
| VD–            | 46 (60) | 81 (45) | | | | 55 (51) | 62 (49) | | | | |
| VD+            | 30 (40) | 101 (55) | 0.183 | | | 56 (43) | 34 (29) | 0.034 | | | |
| ERα–           | 21 (30) | 63 (40) | | | | 75 (57) | 82 (71) | 0.081 | | | |
| ERα+           | 48 (70) | 95 (60) | | | | 47 (37) | 34 (26) | | | | |
| S100A4–        | — | — | | | | 81 (63) | 97 (74) | | | | |
| S100A4+        | — | — | | | | — | — | | | | |
| OPN–           | 47 (58) | 81 (45) | 0.081 | | | — | — | | | | |
| OPN+           | 34 (42) | 97 (55) | | | | — | — | | | | |

Abbreviations: LN, lymph node; VD, vessel density; OPN, osteopontin.

*Number of patients with carcinomas either classified as staining (+) or not staining (−) for S100A4 or for OPN.

†Probability $P$ value between paired samples from Fisher’s exact test (two-sided values).
statistically significant after 8 years of follow-up ($\chi^2 = 4.42$, 1 df, $P = 0.036$; RR, 0.54; 95% CI, 0.30-0.97) but then lost significance over the entire 18-year period (Table 2).

Association of S100A4 and osteopontin in combination with other tumor variables and patient survival. The association of patients with carcinomas either staining or not staining for S100A4 or for osteopontin and their survival in subgroups defined by the different tumor variables were analyzed. In these subgroups, staining for S100A4 or for osteopontin was usually associated with poorer survival times (e.g., Fig. 3A for higher vessel density). Four of the tumor variables assessed were associated with a reduction in patient survival time over that with S100A4 or with osteopontin alone: lymph node status, tumor size, histologic grade, and vessel density (Table 3). However, unlike the other tumor variables (Table 3) and although significant, the difference in survivors between patients with higher or lower vessel density increased up to about 12 years and thereafter decreased to virtually nothing after 18 years of follow-up (Fig. 3A). Univariate analyses for survival of patients with S100A4-positive or with osteopontin-positive carcinomas showed that RRs for not having one of the other tumor variables were similar in most cases to those in the

Fig. 2. Association of immunocytochemical staining for (A) S100A4 and for (B) osteopontin (OPN) with overall survival of patients. A, the cumulative proportion of surviving patients as a percentage of the total for each year after presentation for either (a) patients with carcinomas classified as negatively staining (1% carcinoma cells stained) or (b) positively staining for S100A4. For S100A4-negative carcinomas, 100% corresponds to 76 patients and 100% for S100A4-positive carcinomas to 196 patients. There were 71 censored observations in (a) with 13 dead of other causes and 125 censored observations in (b) with 27 dead of other causes. The two curves are highly significantly different (Wilcoxon statistic: $\chi^2 = 21.9$, 1 df, $P < 0.0001$). B, the cumulative proportion of surviving patients as a percentage of the total for each year after presentation for either (a) patients with carcinomas classified as negatively staining or (b) positively staining for osteopontin. For osteopontin-negative carcinomas, 100% corresponds to 131 patients, and 100% for osteopontin-positive carcinomas corresponds to 125 patients. There were 105 censored observations in (a) with 24 dead of other causes and 84 in (b) with 15 dead of other causes. The two curves are highly significantly different (Wilcoxon statistic: $\chi^2 = 7.89$, 1 df, $P = 0.0050$).
unselected patients (Table 3). The survival times for patients with S100A4-negative or with osteopontin-negative tumors with or without any of the other tumor variables were usually not significantly different (e.g., Fig. 3A for vessel density). One tumor variable, ERα, was associated with an overall increase in patient survival; the RR was 0.72 or 0.65 for patients staining for S100A4 or for osteopontin, respectively, but neither value achieved significance (Table 3). Patients with both immunocytochemically detectable S100A4 and osteopontin in the primary tumors showed a statistically significant reduction in survival time over those with either one alone (Fig. 3B). However, although patients with osteopontin-negative tumors showed a significant difference in survival between those stained positively or negatively for S100A4, the converse was not true (Fig. 3B). Moreover, RR for patients with osteopontin-positive tumors with or without S100A4 (14.9) was much greater than that for patients with S100A4-positive tumors with or without osteopontin (1.92; Table 3).

**Analysis of tumor variables for independent association with patient survival.** All tumor variables were included in a multivariate regression analysis for the 105 patients available with full data sets. Following analysis, the first variable to emerge was large tumor size (T3; Cox's statistic: $\chi^2 = 13.69$, 1 df, $P < 0.001$) followed by staining for S100A4 ($\chi^2 = 7.89$, 1 df, $P = 0.005$). On controlling the data for tumor size and S100A4 (overall: $\chi^2 = 27.29$, 2 df, $P < 0.001$), there was no significant association between nodal status, histologic grade, osteopontin, vessel density, and patient survival (residual: $\chi^2 = 9.62$, 4 df, $P = 0.047$). None of the possible pairwise higher order interaction terms in the multivariate analysis were statistically significant, implying that the effect of staining for S100A4 and patient survival is similar over the prognostic group defined by tumor size. The corrected RR for survival of women with S100A4-negative carcinomas was 7.89 (95% CI, 1.87-33.3), much higher than that of all other factors, including large tumor size (RR, 4.04; 95% CI, 1.93-8.46). If data for S100A4 were eliminated, the most significant variable became lymph nodes ($P = 0.002$; RR, 2.93; 95% CI, 1.47-5.83) followed by tumor size ($P = 0.026$; RR, 2.16; 95% CI, 1.10-4.26) and then staining for osteopontin became of borderline significance ($P = 0.067$). If data for osteopontin were eliminated, the most significant variable was still S100A4 ($P = 0.005$; RR, 5.32; 95% CI, 1.64-17.3) followed then by lymph nodes ($P = 0.014$; RR, 2.28; 95% CI, 1.18-4.40) and tumor size ($P = 0.018$; RR, 2.21; 95% CI, 1.15-4.24).

**Discussion**

The novelty of our findings is that immunocytochemical staining for S100A4 or osteopontin in the primary tumors is separately associated with poorer patient survival in patients with minimally invasive breast cancers, as it is in advanced disease (23, 24). However, although the risk factors for staining for S100A4 are similar in both disease states (23), that for staining for osteopontin is considerably reduced from 21 in advanced disease (24) to 1.8 in minimally invasive breast cancer. Moreover, the level of immunocytochemical staining with the same antibody for osteopontin is also reduced from 86% for the advanced group to only 49% for the present minimally invasive group of patients using a 1% cutoff level (24). A similar reduction is maintained if a 5% cutoff level is used, then only 18% show positive staining for the previous group compared with 66% for the previous group of patients (data not shown). Thus, there is a relatively large group of 31% of patients with tumors with 1% to 5% of the carcinoma cells stained for osteopontin. Thus, the present group of patients with minimally invasive breast cancer contains both a lower percentage of tumors staining positively and a larger percentage of these contain low, weakly staining levels for osteopontin (1-5% carcinoma cells stained). This result suggests that immunoreactive osteopontin increases between the minimally invasive and invasive stage of breast cancer, whereas the level of immunoreactive S100A4 does not.

When the tumor variables that show a significant association with patient survival are tested for association with staining for S100A4 or for osteopontin in the primary tumors, only higher vessel density shows a significant association with S100A4 and only that for ERα with osteopontin. Coexpression of S100A4 and higher vessel density, including staining of endothelial cells, has also been observed in a different screen-detected group of breast cancer patients (41). These results further substantiate the results reported here. Coexpression of osteopontin and ERα has been reported previously in another group of breast cancer patients, but that association was only of borderline significance (24). The association between osteopontin and two other estrogen-inducible proteins pS2 and progesterone receptor is also significant (24). These associations suggest molecular links, either intracellularly or extracellularly, between S100A4 and higher vessel density and between osteopontin and estrogen/ERα, pS2, and progesterone receptor (42). Moreover, the presence (42) or absence (43) of estrogen-dependent transcriptional elements in the promotor may be a basis for the observed

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**Table 2. Association of tumor variable with patient survival times**

<table>
<thead>
<tr>
<th>Tumor variable</th>
<th>Patient (n)</th>
<th>$\chi^2$</th>
<th>$P^*$</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td>283</td>
<td>10.50</td>
<td>0.0012</td>
<td>2.01 (1.30-3.11)</td>
</tr>
<tr>
<td>Histologic grade</td>
<td>295</td>
<td>5.39</td>
<td>0.02</td>
<td>1.49 (0.95-2.34)</td>
</tr>
<tr>
<td>Nodal status</td>
<td>175</td>
<td>24.33</td>
<td>&lt;0.0001</td>
<td>3.52 (2.08-5.94)</td>
</tr>
<tr>
<td>S100A4</td>
<td>272</td>
<td>21.99</td>
<td>&lt;0.0001</td>
<td>6.82 (2.75-16.9)</td>
</tr>
<tr>
<td>OPN</td>
<td>256</td>
<td>7.89</td>
<td>0.0050</td>
<td>1.80 (1.10-2.94)</td>
</tr>
<tr>
<td>Vessel density</td>
<td>256</td>
<td>6.01</td>
<td>0.014</td>
<td>1.61 (1.03-2.51)</td>
</tr>
<tr>
<td>ERα</td>
<td>229</td>
<td>2.01</td>
<td>0.16</td>
<td>0.79 (0.47-1.31)</td>
</tr>
</tbody>
</table>

Abbreviation: OPN, osteopontin.

$^*$ Probability ($P$) was determined by the generalized Wilcoxon-Gehan test with 1 df (Materials and Methods).

$^\dagger$ RR and 95% CI were determined using a Cox univariate analysis with 1 df (Materials and Methods).

1. Tumor size: T1 vs T3; 2-cm diameter cutoff.

2. Histologic grade: 1 vs 2 + 3.

3. No nodes vs ≥1 nodes with tumor.

4. Negative vs positive staining.

5. Above vs below median vessel density of 109 vessels per field.

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S100A4 can act in an oligomeric form and stimulate positivity in the primary tumors. In animal model systems, dependent, at least in part, on S100A4 but not on osteopontin endothelial cells suggests that higher vessel density is a variable staining for S100A4 are associated with S100A4-staining within the same tumor, groups of carcinoma cells with positive the census date is virtually the same. Moreover, the fact that die significantly more rapidly, the overall percentage survival at the cumulative proportion of surviving patients with S100A4-negative, low vessel density carcinomas (100% = 40 patients); b, patients with S100A4-positive, low vessel density carcinomas (100% = 75 patients); c, patients with S100A4-negative, high vessel density carcinomas (100% = 24 patients); and d, patients with S100A4-positive, high vessel density carcinomas (100% = 85 patients). There were 36 censored observations in (a) with 7 dead of other causes, 51 in (b) with 9 dead of other causes, 23 in (c) with 3 dead of other causes, and 48 in (d) with 12 dead of other causes. In pairwise tests, (a) and (b) (Wilcoxon statistic: \( \chi^2 = 9.75, 1 \text{ df}, P = 0.0016 \)), (c) and (d) (\( \chi^2 = 11.06, 1 \text{ df}, P = 0.0009 \)), and (b) and (d) (\( \chi^2 = 6.11, 1 \text{ df}, P = 0.0424 \)) were significantly different, but (a) and (c) were not (\( \chi^2 = 0.42, 1 \text{ df}, P = 0.52 \)). Overall differences between patient groups are significant using Wilcoxon-Gehan statistics (\( \chi^2 = 25.08, 3 \text{ df}, P < 0.0001 \)). Data for staining for S100A4 and vessel densities were available for only 224 patients. B, the cumulative proportion of surviving patients is also shown for each year after presentation for the following: a, patients with S100A4-negative, osteopontin-negative carcinomas (100% = 41 patients); b, patients with S100A4-positive, osteopontin-negative carcinomas (100% = 75 patients); c, patients with S100A4-negative, osteopontin-positive carcinomas (100% = 28 patients); and d, patients with S100A4-positive, osteopontin-positive carcinomas (100% = 85 patients). There were 38 censored observations in (a) with 9 dead of other causes, 58 in (b) with 15 dead of other causes, 27 in (c) with 3 dead of other causes, and 51 in (d) with 10 dead of other causes. In pairwise tests, (a) and (b) (Wilcoxon statistic: \( \chi^2 = 9.68, 1 \text{ df}, P = 0.0024 \)), (c) and (d) (\( \chi^2 = 11.39, 1 \text{ df}, P = 0.0007 \)), and (b) and (d) (\( \chi^2 = 5.47, 1 \text{ df}, P = 0.019 \)) were significantly different, but (a) and (c) were not (\( \chi^2 = 0.24, 1 \text{ df}, P = 0.62 \)). Overall differences between patient groups were significant using Wilcoxon-Gehan statistics (\( \chi^2 = 25.08, 3 \text{ df}, P < 0.0001 \)). Data for staining for S100A4 and for osteopontin were available for only 229 patients.

Fig. 3. Association of immunocytochemical staining for S100A4 with survival of patients divided into groups by (A) vessel density status and (B) osteopontin (OPN) – staining status. A, the cumulative proportion of surviving patients as a proportion of the total is shown for each year after presentation for the following: a, patients with S100A4-negative, low vessel density carcinomas (100% = 40 patients); b, patients with S100A4-positive, low vessel density carcinomas (100% = 75 patients); c, patients with S100A4-negative, high vessel density carcinomas (100% = 24 patients); and d, patients with S100A4-positive, high vessel density carcinomas (100% = 85 patients). There were 36 censored observations in (a) with 7 dead of other causes, 51 in (b) with 9 dead of other causes, 23 in (c) with 3 dead of other causes, and 48 in (d) with 12 dead of other causes. In pairwise tests, (a) and (b) (Wilcoxon statistic: \( \chi^2 = 9.75, 1 \text{ df}, P = 0.0016 \)), (c) and (d) (\( \chi^2 = 11.06, 1 \text{ df}, P = 0.0009 \)), and (b) and (d) (\( \chi^2 = 6.11, 1 \text{ df}, P = 0.0244 \)) were significantly different, but (a) and (c) were not (\( \chi^2 = 0.42, 1 \text{ df}, P = 0.52 \)). Overall differences between patient groups are significant using Wilcoxon-Gehan statistics (\( \chi^2 = 25.08, 3 \text{ df}, P < 0.0001 \)). Data for staining for S100A4 and vessel densities were available for only 224 patients. B, the cumulative proportion of surviving patients is also shown for each year after presentation for the following: a, patients with S100A4-negative, osteopontin-negative carcinomas (100% = 41 patients); b, patients with S100A4-positive, osteopontin-negative carcinomas (100% = 75 patients); c, patients with S100A4-negative, osteopontin-positive carcinomas (100% = 28 patients); and d, patients with S100A4-positive, osteopontin-positive carcinomas (100% = 85 patients). There were 38 censored observations in (a) with 9 dead of other causes, 58 in (b) with 15 dead of other causes, 27 in (c) with 3 dead of other causes, and 51 in (d) with 10 dead of other causes. In pairwise tests, (a) and (b) (Wilcoxon statistic: \( \chi^2 = 9.68, 1 \text{ df}, P = 0.0024 \)), (c) and (d) (\( \chi^2 = 11.39, 1 \text{ df}, P = 0.0007 \)), and (b) and (d) (\( \chi^2 = 5.47, 1 \text{ df}, P = 0.019 \)) were significantly different, but (a) and (c) were not (\( \chi^2 = 0.24, 1 \text{ df}, P = 0.62 \)). Overall differences between patient groups were significant using Wilcoxon-Gehan statistics (\( \chi^2 = 25.08, 3 \text{ df}, P < 0.0001 \)). Data for staining for S100A4 and for osteopontin were available for only 229 patients.

Differences in association of ERα with osteopontin-staining tumors but not with S100A4-staining tumors, and the differences in association of osteopontin and of S100A4 with patient survival in the Tamoxifen-untreated group of patients. Although when higher rather than lower vessel densities were present in the tumors, patients with S100A4-positive tumors die significantly more rapidly, the overall percentage survival at the census date is virtually the same. Moreover, the fact that within the same tumor, groups of carcinoma cells with positive staining for S100A4 are associated with S100A4-staining endothelial cells suggests that higher vessel density is a variable dependent, at least in part, on S100A4 but not on osteopontin positivity in the primary tumors. In animal model systems, S100A4 can act in an oligomeric form and stimulate angiogenesis by promoting chemotactic motility of endothelial cells (41) and the formation of invasive capillary-like structures (43). Thus, the association between S100A4 positivity and higher vessel density found in human breast carcinomas is consistent with cellular motility/invasion found in rodent model systems (26, 44–46), in contrast to that of osteopontin in predominantly stimulating cellular adhesion (47, 48). That the functions of S100A4 and osteopontin are predominantly different in promoting metastasis of breast cancer cells in vitro (46, 48) is also consistent with the results obtained here on their additive effects on survival in human breast cancer (Table 3). This does not imply that S100A4 or osteopontin separately can induce metastasis alone in animals, but these genes have to be overexpressed with others [e.g., H-ras] in the rat
injected into the left cardiac ventricle of female and CXCR4 or CTGF endow MDA-MB-231 breast cancer cells

3. References

cytoskeleton (51, 52) and, like osteopontin, with cellular many of which are associated, like S100A4, with the cellular led to the identification of other metastasis-associated genes, Similarly, recent advances in DNA microarray technology have significantly raises the RR from about 8 to about 15 overall.

Tumor size

Histologic grade

Nodal status

Vessel density

ERα

S100A4d

OPN

Abbreviations: TV, tumor variable; OPN, osteopontin.

* Median survival in months and cumulative survival (%) of patients with S100A4-positive or OPN-positive tumors and either not expressing (TV−) or expressing (TV+) one of the other tumor variables.

† Probability ρ for the significance of the difference of the other tumor variable for the S100A4-positive or OPN-positive patients (Wilcoxon statistics, 1 df).

Cost: less likely to metastasize to bones (50).

The finding that involved lymph nodes is excluded as an independent variable in relation to survival times only when staining for both S100A4 and osteopontin are included in the analyses suggests that involved lymph nodes are confounded by staining for both S100A4 and osteopontin. This result suggests that both S100A4 and osteopontin independently contribute to the appearance of tumor in the lymph nodes of patients with breast cancer. Moreover, although staining for osteopontin is not independently associated with patient death when assessed with that for S100A4, additional staining for osteopontin significantly raises the RR from about 8 to about 15 overall. Similarly, recent advances in DNA microarray technology have led to the identification of other metastasis-associated genes, many of which are associated, like S100A4, with the cellular cytoskeleton (51, 52) and, like osteopontin, with cellular adhesion (53). Furthermore, a profile of 70 genes differentially expressed between primary tumors of node-negative patients with either a good or a poor prognosis has been established (54, 55). However, the split in the overall survival for both node-negative and node-positive patients lacking any clinically detectable metastases is 94.5% in the good prognosis group and 54.6% in the poor prognosis group. These survival figures obtained with 70 genes are comparable with those obtained in this study with just two specific gene products. In the future, stratification of patients according to the expression of a relatively few proteins that can cause metastasis, rather than the many molecular changes associated with the malignant/metastatic process, opens the way to target such early groups with agents specifically against those gene products that can cause metastasis in experimental situations.

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Table 3. Summary of the survival of S100A4-positive or osteopontin-positive patients divided into groups by the other tumor variables

<table>
<thead>
<tr>
<th>Tumor variable</th>
<th>S100A4-positive tumors</th>
<th>OPN-positive tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median survival (mo)*</td>
<td>Cumulative survival (%)*</td>
</tr>
<tr>
<td></td>
<td>TV−</td>
<td>TV+</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td>185</td>
<td>165</td>
</tr>
<tr>
<td>Nodal status</td>
<td>≥180</td>
<td>8</td>
</tr>
<tr>
<td>Vessel density</td>
<td>186</td>
<td>173</td>
</tr>
<tr>
<td>ERα</td>
<td>≥216</td>
<td>120</td>
</tr>
<tr>
<td>S100A4d</td>
<td>≥216</td>
<td>120</td>
</tr>
<tr>
<td>OPNd</td>
<td>≥216</td>
<td>120</td>
</tr>
</tbody>
</table>

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