Prognostic Significance of the Metastasis-inducing Protein S100A4 (p9Ka) in Human Breast Cancer

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ABSTRACT

The calcium-binding protein S100A4 is capable of inducing metastasis in rodent models for breast cancer. We now show that rabbit antibodies to recombinant rat S100A4 recognize specifically human S100A4 using Western blotting techniques and use them to assess the prognostic signif-

INTRODUCTION

Breast cancer is the most frequently encountered cancer in women of the Western world, with approximately half of these dying from this disease (1). The major cause of death is the metastatic spread of the disease from the primary tumor to distant sites in the body (2, 3), and therefore, the need for prognostic factors to indicate which patients are more likely to die of metastatic disease is important. Research into breast cancer has highlighted the prognostic significance of a number of pathological factors (3); these include the size of the primary tumor, the histological grade (4), and most importantly, the involvement of the draining lymph nodes of the tumor (5). Gene products involved in controlling cell proliferation, e.g., c-erbB-2 (6, 7), c-erbB-3 (8, 9), particularly those concerned with estrogen action [e.g., ER (10–12), PgR (13), and PgR (14, 15)], cell death [e.g., p53 (16, 17)], and invasion [e.g., cathepsin D (14, 18)] in tissue cultured systems have been of more limited value in predicting patient death from metastatic disease (19). This may be due in part to the fact that very few of these gene products have been shown to be capable of causing metastasis directly in experimental systems. However, one gene product, p9Ka, has recently been described with this property (20, 21).

p9Ka, now renamed S100A4, is a member of the S100 family of calcium-binding proteins (22). S100A4 or its mRNA is found at higher levels in metastatic relative to nonmetastatic rat (23) and mouse (24) tumor cell lines and benign relative to malignant human breast tumors (25). Elevation of the levels of rat (20) or human (26) S100A4 in benign rat mammary tumor cells by DNA transfection results in the induction of metastatic capability in some of the cells when they are injected into the mammary fat pads of syngeneic rats. In independent transgenic mouse models of breast cancer overexpression of S100A4 in neu oncogene-induced (27) or with mouse mammary tumor virus-induced (28) benign mammary tumors yields metastatic tumors. Moreover, in pilot studies on human colorectal adenocarcinoma specimens, elevated levels of immunocytochemically detected S100A4 are associated with the more malignant carcinoma-7ous regions of the primary tumors and with liver metastases (29). We now investigate, using immunocytochemical techniques, the presence of S100A4 in specimens of primary breast carcinomas from a comparatively large group of patients with sufficient follow-up time to assess whether its presence at time of diagnosis is significantly associated with patient death from metastatic disease.

MATERIALS AND METHODS

Patients, Specimens, and Serology. Archival formalin-fixed paraffin-embedded specimens were obtained from primary tumors of 349 unselected patients who presented with operable breast cancer between the years 1976 and 1982 to general surgery clinics in the Merseyside Region of the North West of England, as reported previously (7, 12, 18). The vast majority of the patients were Caucasian, and because health care is freely available in the United Kingdom, the patients were a fair reflection of the population, with 99.4% of the women in the area over 35 returned as Caucasian at the 1991 United Kingdom census. Treatment was either modified radical mastectomy in 85% of patients or simple mastectomy with sampling of axillary lymph nodes in 17% of patients. The lymph nodes were recorded as containing or not containing carcinoma on histological examinations, with no further breakdown of the

Received 7/19/99; accepted 1/17/00.

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1 Supported by the Cancer and Polio Research Fund, the North West Cancer Research Fund, and the Cancer Tissue Bank Research Centre.

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4 The abbreviations used are: ER, estrogen receptor; d.f., degrees of freedom; PgR, progesterone receptor; S100A4, recombinant S100A4 protein; RR, relative risk; CI, confidence interval.
The absence of metastatic disease at the time of presentation was 12 years. The patients were staged clinically according to the international TNM staging system. The number of nodes involved, patient age, menopausal status, and some histological grades were included in the study. No patients received any adjuvant systemic therapy. The rabbit polyclonal antisera to rat S100A4 protein was purified and used for immunohistological study. Normal and a transgenic strain of mice carrying S100A4 (11) were used. Immunocytochemistry. Histological sections on 3-aminopropyltriethoxysilane-coated slides (31) were cut from the paraffin-embedded sections (32), endogenous peroxidase activity was blocked (33), and indirect immunocytochemistry was carried out using a commercially available antibody complex containing horseradish peroxidase (34). The anti-S100A4 serum was diluted to 1/500, the sections were incubated at room temperature overnight, and bound antibody was detected with biotinylated donkey antirabbit immunoglobulin (Amersham, Bucks, United Kingdom), followed by ABCComplex (Dako Ltd.). The complex was visualized with 3, 3'-diaminobenzidine (Sigma, Dorset), 0.003% (v/v) H2O2 (35). The sections were then counterstained in Mayers' hemalum and mounted in DPX (Merck Ltd, Dorset, UK).

Slides were read independently by two observers using light microscopy. The percentage of carcinoma cells with cytoplasmic staining was recorded from two sections of each specimen, 10 fields per section at 200× magnification. Staining was evaluated initially in three groups: positive (+), >5%; borderline (±), 1–5%; and negative (−), <1% of the carcinoma cells stained. The borderline and negative staining groups were usually combined. Photographs were recorded on a Reichart Polvvar microscope fitted with a Wratten 44 blue green filter (36). Increasing the concentration of antibody 10-fold or using commercially supplied rabbit antihuman rat S100A4 protein gave identical results. Immunocytochemical staining for ER (340 patients), PgR (330 patients), pS2 (344 patients), p53 (348 patients), and c-erbB-3 (335 patients) was accomplished by similar standard procedures (37) to those described previously for c-erbB-2 (342 patients) and cathepsin D (270 patients) (7, 18).

**Protein Samples and Western Blotting.** Rat and human S100A4 were produced in *Escherichia coli* (38), and human S100A1 and S100A2 were gifts of Dr. G. Wang (University of Liverpool, United Kingdom). Soluble extracts of human breast tumor specimens were prepared by crushing in liquid nitrogen and homogenizing with 1 mM phenylmethyl sulfonyl fluoride. The extracts were added SDS, glycerol, bromophenol blue, and 2-mercaptoethanol prior to boiling, sonication, and electrophoresis on 15% (w/v) polyacrylamide gels (39). Molecular weight markers were run alongside the samples for molecular weight determination. Proteins were then transferred to Immobilon P membranes (Millipore Corporation, Watford, United Kingdom), which were blocked with “blocking buffer” containing 1% nonylphenylmethyl sulfonyl fluoride. The extract was centrifuged at 4°C for 1 min in a microfuge, and the supernatant was assayed for immunocytochemical staining for S100A4 as described in the Materials and Methods. Soluble extracts of carcinoma specimens were prepared by crushing the tissue in liquid nitrogen and homogenizing with 1 mM phenylmethyl sulfonyl fluoride. The extracts were added SDS, glycerol, bromophenol blue, and 2-mercaptoethanol prior to boiling, sonication, and electrophoresis on 15% (w/v) polyacrylamide gels (39). Molecular weight markers were run alongside the samples for molecular weight determination. Proteins were then transferred to Immobilon P membranes (Millipore Corporation, Watford, United Kingdom), which were blocked with “blocking buffer” containing 1% bovine serum albumin, and incubated with anti-S100A4 diluted as in the legend figure. In some experiments, 1 mg/ml rat S100A4 was present to provide a blocking antibody control. Filters were then incubated with 1 h with peroxidase-conjugated swine antirabbit IgG (Sigma, St. Louis, MO), and bound antibodies were detected with the Super Signal West Pico Chemiluminescence System (Pierce and Warriner, Rockford, Illinois) and exposing the filter against Fuji RX film.

**Statistical Methods.** Follow-up information was obtained from the Merseyside Cancer Registry for patients used in this study and was updated for patient survival to August 31, 1995. The accuracy of this data was subsequently checked by inspection of General Practitioner records to confirm whether patients were alive, dead of cancer, or dead of other causes. The association of immunocytochemical staining for S100A4 with other tumor variables was assessed using a Fisher’s exact test (40). These variables on the same group of patients included tumor size, histological grade, nodal status, menopausal status, patient age (12), and presence of c-erbB-2 (7), cathepsin D (18), ER, PgR, p53, and c-erbB-3 (37) in the primary tumor. The cutoff values between these groups of patients designated negatively or positively immunocytochemically stained for the marker proteins included the borderline staining group with the unstained group, unless otherwise specified (7, 18, 37).

The association of the staining for S100A4 in breast cancers with patient survival was evaluated using life tables constructed from survival data with Kaplan Meier plots and analyzed using generalized Wilcoxon (Gehan) statistics (7). Patients found to be dead from causes other than cancer were excluded from the analyses. To determine whether the association of patient survival with S100A4 was independent of other potential prognostic factors shown to approach significance in univariate analysis, a multivariate analysis was performed using the Cox proportional hazards model (41). Other potential prognostic factors measured on the same group of patients included tumor size, histology chromatography and pS2, p53, and c-erbB-3 (37). The degree of agreement between observers was assessed using the κ statistic; a value >0.61 was taken to be a satisfactory level of agreement (40). Data processing and statistical analyses were performed using Excel version 5.0 (Microsoft Corp., Washington, D.C.) and Statistical Package for the Social Sciences, version 6.1.2 (SPSS Inc., Chicago, IL).

**RESULTS**

Immunohistochemical Staining for S100A4. When histological sections from two normal breasts or 10 samples from uninvolved breast tissue from cancerous breasts were incubated with antiserum to S100A4, the majority of the parenchymal tissue was relatively unstained (Fig. 1A), although some areas showed staining of both ducts (Fig. 1B) and ductules. In contrast, individual invasive breast carcinomas presented a more uniform staining pattern. Of the 349 invasive breast carcinomas evaluated, 152 (44%) were unstained (Fig. 1C), 53 (15%) possessed borderline staining (Fig. 1D), and 144 (41%) were strongly stained (Fig. 1E) by antiserum to S100A4. The staining was confined mainly to the cytoplasm (Fig. 1F) and could be abolished by prior incubation of the antiserum with rat S100A4 (Fig. 1G). The assessment was made only on the malignant cells; however, positive staining was also present on normal blood vessels and certain reactive stromal cells (Fig. 1H) and lymphocytes (Fig. 1I) present in the carcinomas. The borderline group was defined as carcinomas possessing 1–5% of the malignant cells stained for S100A4 (see “Materials and Methods”). For the purposes of most analyses, the borderline staining carcinomas were combined with the unstained carcinomas into one group of negatively stained carcinomas, leaving the clearly positive staining carcinomas as the other group.

There was some variability in the assessment of staining by the two observers on the same histological section. However, there was agreement in 87% of the slides, corresponding to a κ value of 0.79, which represents a good degree of consistency between observers. In addition, intratumor heterogeneity was assessed by comparing the category of staining allocated when two well-separated sections from the same tumor were analyzed independently. In 7% of all histological sections studied, intratumor heterogeneity was sufficiently high to affect whether a section was regarded as negatively or positively stained, and then recourse was made to staining and analyzing two further sections to obtain a consensus result.

The antibody to rat S100A4 recognized both rat (not shown) and human S100A4 on Western blots, but did not cross-react with the closely related human S100A1 and S100A2 proteins at the same loading on the gel (Fig. 2A). Western blots of extracts of selected carcinoma specimens confirmed that the antibody detected a Mr 9000 protein in extracts of specimens that exhibited high levels of immunocytochemically detectable S100A4 (Fig. 2B) but not in extracts of specimens that displayed no staining (not shown). The binding of antibody to the Mr 9000 protein was blocked when human S100A4...
(at a concentration of 1 mg/ml) was present during incubation of the antibodies to S100A4 with the filter (Fig. 2C). In extracts of some, but not all tumor specimens tested, a high molecular weight band of immunoreactivity was also present at about $M_r$ 60,000 – 65,000. Although this band corresponded approximately to the molecular weight of serum albumin, there was no cross-reaction of the antibody onto purified serum albumin (Fig. 2A). It is possible that this immunoreactivity consisted of higher molecular weight aggregates of S100A4 because essentially similar results were obtained with a second commercially produced anti-S100A4 (not shown).

**Association of S100A4 with Other Tumor Variables.** The presence of definite immunocytochemical staining for p9Ka in the carcinoma cells was cross-tabulated with other tumor variables potentially predictive of patient outcome, including tumor size, histological grade, nodal status, menopausal status, and the presence of c-erbB-2, cathepsin D, ER, PgR, pS2, p53, and c-erbB-3 using the Fisher’s exact test (Table 1). Of the pathological factors, only the presence of carcinoma in the axillary lymph nodes showed a borderline association with immunocytochemical staining for S100A4 in the primary tumor; 54% of S100A4-positive tumors had tumor in the associated

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**Fig. 1. Immunocytochemical staining with anti-S100A4.** A and B, normal human breast showing either (A) an unstained duct (d) and ductules (du) or (B) a stained duct (d). C, invasive carcinoma (c) showing no immunocytochemical staining. D, invasive carcinoma showing borderline staining of the occasional malignant cell (arrows). E, invasive carcinoma showing strong staining of most malignant cells but not of the host ductule (du). F, higher magnification of E showing cytoplasmic staining of malignant cells (arrows). G, the same area of invasive carcinoma as in E but incubated with anti-S100A4 preincubated with rS100A4, showing no immunocytochemical staining. H and I, reactive stromal cells (arrows; H) and lymphocytes (I) in an invasive but unstained carcinoma (c), both showing strong staining. Magnification, A–E and G–I, ×230; F, ×580. Bars, A–E and G–I, 50 μm; F, 20 μm.
peroxidase-conjugated swine antirabbit IgG for 1 h. Bound antibody was detected by Western blotting, incubation with anti-S100A4 in the absence (B) or presence (C) of 1 mg/ml rat S100A4, secondary antibody, and detection of bound antibody by chemiluminescence. Molecular weights of markers are shown on the left-hand side of the panels, and the position of the S100A4 band is shown by the arrows. A high-molecular-weight signal observable in some specimens is shown by the arrowshead.

Fig. 2. Detection of S100A4 by Western blotting. A, samples (1 μg) of human S100A4 (Lanes 1 and 3), S100A1 (Lane 2), S100A2 (Lane 3), and BSA (Lane 4) were subjected to electrophoresis on 15% polyacrylamide SDS gels, and the proteins were transferred to the Immobilon P membrane (see “Materials and Methods”). The filter was incubated with a 1:5000 dilution of affinity-purified anti-S100A4 for 1 h and with a 1:5000 dilution of peroxidase-conjugated swine antirabbit IgG for 1 h. Bound antibody was detected by chemiluminescence and exposure to Fuji RX film. B and C, human S100A4 (Lane 1) and a SDS extract of a S100A4-positive carcinoma specimen (Lane 2) were subjected to electrophoresis. Western blotting, incubation with anti-S100A4 in the absence (B) or presence (C) of 1 mg/ml rat S100A4, secondary antibody, and detection of bound antibody by chemiluminescence.

Association of S100A4 with Patient Survival. The association of staining for S100A4 and the cumulative proportion of patients surviving at yearly intervals after the time of presentation is shown in Fig. 3. Of 205 patients who were classified as S100A4-negative, about 80% were alive at the census date, in comparison with about 11% of the 144 patients classified as S100A4-positive. The median survival of patients whose tumors were classified as S100A4-negative was >228 months in comparison with that of patients with a tumor classified as positive for 47 months. The data show that over the time period of 19 years, the survival of patients with S100A4-negative carcinomas was highly significantly worse than those patients with carcinomas classified as S100A4-negative (Wilcoxon test, P < 0.0001; Fig. 3). This difference in survival of patients between the two groups became statistically significant after 6 months of follow-up (Wilcoxon test, χ² = 4.39, 1 d.f., P = 0.036). Thereafter, increasing follow-up times increased the significance of the difference (e.g., for 1 year, χ² = 7.90, 1 d.f., P = 0.005; for 2 years, χ² = 22.14, 1 d.f., P < 0.0001; for 3 years, χ² = 47.63; for 4 years, χ² = 70.55; for 19 years, χ² = 131.5). When the results are expressed in terms of the RR of a patient surviving, women with S100A4-negative and S100A4-positive carcinomas had an unadjusted RR for survival of 8.7 (95% CI, 6.7–12.7) compared to the S100A4-positive group.

If the group of 53 patients with borderline staining was separated from the group of patients originally classified as negatively stained for S100A4 and analyzed separately for their outcome, then the three curves were highly significantly different (Wilcoxon test, P < 0.0001; Fig. 4). These overall differences became significant after 1 year (Wilcoxon test, χ² = 13.01, 2 d.f., P = 0.0015). The completely negatively stained group of 152 patients showed a very high level of cumulative survival of 98% and a median survival of >228 months. The group of 53 patients with borderline staining showed a level of survival of 29% and a median survival of 82 months, which was significantly different from either the completely negatively (P < 0.0001) or positively stained group of patients (P = 0.017). The former difference became statistically significant after 1 year (χ² = 11.73, 1 d.f., P = 0.0006) and the latter difference after 5 years (χ² = 4.22, 1 d.f., P = 0.04). The same positively stained group of 144 patients as above showed a level of survival of 11% and a median survival of 47 months as before (Fig. 4). Women with S100A4-negative carcinomas had an unadjusted RR for survival of 53.4 (95% CI, 16.4–174) compared to those with S100A4-borderline carcinomas and of 91.6 (95% CI, 29.0–289) compared to those with S100A4-positive carcinomas. The comparisons with the group of patients with unstained carcinomas, however, may be open to question because there were only three deaths and therefore 149 censored observations in this group (Fig. 4).

Association of S100A4 and Other Tumor Variables with Patient Survival. In addition to staining for S100A4, the other tumor variables that showed a significant association with survival time for this set of 349 patients at the same census date were: nodal status (Wilcoxon test, χ² = 18.00, 1 d.f., P < 0.0001), tumor size (χ² = 13.6, 3 d.f., P = 0.0035), histological grade (χ² = 8.66, 2 d.f., P = 0.013), staining for c-erbB-2 (χ² = 10.03, 1 d.f., P = 0.0015), ER (χ² = 4.83, 1 d.f., P = 0.028), and p53 (χ² = 4.61, 1 d.f., P = 0.032). The association of staining for cathepsin D, PgR, pS2, and c-erbB-3 with survival time failed to reach statistical significance (not shown). The association of patients with carcinomas staining for S100A4 and their survival in subgroups defined by the different tumor variables described above was analyzed. The borderline cases of carcinoma cell staining for S100A4 were included once again in the unstained carcinoma cell group of patients to ensure sufficient numbers in the subgroups for statistical validity. In all of these subgroups of patients, staining for S100A4 was associated with poorer survival, including that for patients with involved lymph nodes (Fig. 5). There were only three tumor variables which, when they occurred with S100A4, showed a statistically significant reduction in patients’ survival time over that obtained with S100A4 alone: involved lymph nodes (Fig. 5), fixed tumors (T₄), and staining for c-erbB-2 (Fig. 6). The other tumor variables, including tumor sizes T₁-T₃ and histological grades II and III, showed no such effect (not shown). Thus, the 51 patients who were positive for S100A4 and negative for involved lymph nodes...
exhibited longer survival times than the 60 patients who were positive for S100A4 and also positive for involved lymph nodes (P < 0.0001). The differences were 24% and 3% in cumulative proportion surviving and 85 months and 36 months in median survival times, respectively (Fig. 5). Similarly, 129 patients positive for S100A4 without fixed tumors (T1, T2, T3) had longer survival times than the modest 10 patients positive for S100A4 and possessing fixed tumors (T4) (χ² = 5.93, 1 d.f., P = 0.015; not shown). In this case, the differences were 81% and 48% in cumulative proportion surviving and >228 months and 106 months in median survival times, respectively. Although only at borderline significance, the 102 patients who were positive for S100A4 and negative for c-erbB-2 had longer survival times than the 39 patients who were positive for S100A4 and also positive for c-erbB-2 (P = 0.050; Fig. 6). In this case, the differences were 11% and 6% in cumulative proportion surviving and 49 months and 35 months in median survival times, respectively. Neither the presence of involved lymph nodes nor staining for c-erbB-2 in the S100A4-negative group of patients showed any correlation with poorer prognosis (Figs. 5 and 6). It is possible that the statistical validity may, however, have been influenced by the numbers of patients in the subgroups analyzed.

To determine whether the seven tumor variables that were significant in the univariate analyses are independent of one another, they were all included in a multivariate regression analysis for the 137 patients available with full data sets (see “Materials and Methods”). Following analysis using this model, the first variable to emerge and the most significant of all was staining for S100A4, followed by fixed tumors (T4), nodal status, and high histological grade (III) (Table 2). On controlling the data for T4, nodal status, grade III, and S100A4, there was no significant association between tumor sizes T1-T3, grades I-II, c-erbB-2, p53, ER, and patient survival (Cox analysis, residual χ² = 3.53, 6 d.f., P = 0.74). Consistent with this result, the univariate analysis for tumor size gave the most significant and shortest survival for patients when groups with fixed tumors (T4) were...
Fig. 5. Association of staining for S100A4 with survival of patients divided into groups by their nodal status. The cumulative proportion of surviving patients as a percentage of the total is shown for each year after presentation for the following: a, patients with S100A4-negative, node-negative carcinomas (———; 100% = 84 patients); b, patients with S100A4-positive, node-negative carcinomas (———; 100% = 51 patients); c, patients with S100A4-negative, node-positive carcinomas (———; 100% = 60 patients); and d, patients with S100A4-positive, node-positive carcinomas (———; 100% = 60 patients). There were 68 censored observations in a (19 dead of other causes); 15 in b (5 dead of other causes); 49 in c (21 dead of other causes); and 3 in d (2 dead of other causes). The cumulative proportions surviving ± 95% CIs were (a) 0.90 ± 0.06, (b) 0.59 ± 0.14, (c) 0.86 ± 0.09, and (d) 0.27 ± 0.11 at 5 years; (a) 0.85 ± 0.08, (b) 0.34 ± 0.14, (c) 0.82 ± 0.10, and (d) 0.06 ± 0.06 at 10 years; (a) 0.79 ± 0.09, (b) 0.27 ± 0.13, (c) 0.80 ± 0.11, and (d) 0.06 ± 0.06 at 15 years; and (a) 0.79 ± 0.09, (b) 0.24 ± 0.13, (c) 0.80 ± 0.11, and (d) 0.03 ± 0.05 at 20 years. In pairwise tests, a and b (Wilcoxon statistic $\chi^2 = 32.4$, 1 d.f., $P < 0.0001$), c and d ($\chi^2 = 50.5$, 1 d.f., $P < 0.0001$), and b and d ($\chi^2 = 18.61$, 1 d.f., $P < 0.0001$) were significantly different, whereas a and c were not ($\chi^2 = 0.209$, 1 d.f., $P = 0.65$). Data for nodal status were available for only 255 patients.

Fig. 6. Association of staining for S100A4 with survival of patients divided into groups by their c-erbB-2 staining status. The cumulative proportion of surviving patients as a percentage of the total is shown for each year after presentation for the following: a, patients with S100A4-negative, c-erbB-2-negative carcinomas (———; 100% = 164 patients); b, patients with S100A4-negative, c-erbB-2-positive carcinomas (———; 100% = 37 patients); c, patients with S100A4-positive, c-erbB-2-negative carcinomas (———; 100% = 102 patients); and d, patients with S100A4-positive, c-erbB-2-positive carcinomas (———; 100% = 39 patients). There were 136 censored observations in a (51 dead of other causes); 30 in b (11 dead of other causes); 16 in c (9 dead of other causes); and 3 in d (1 dead of other causes). The cumulative proportions surviving ± 95% CIs were (a) 0.92 ± 0.04, (b) 0.41 ± 0.10, (c) 0.80 ± 0.13, and (d) 0.25 ± 0.14 at 5 years; (a) 0.85 ± 0.06, (b) 0.20 ± 0.08, (c) 0.80 ± 0.13, and (d) 0.06 ± 0.08 at 10 years; (a) 0.80 ± 0.07, (b) 0.15 ± 0.08, (c) 0.80 ± 0.13, and (d) 0.06 ± 0.08 at 15 years; and (a) 0.80 ± 0.07, (b) 0.11 ± 0.07, (c) 0.80 ± 0.13, and (d) 0.06 ± 0.08 at 20 years. In pairwise tests, a and c (Wilcoxon statistic $\chi^2 = 106.5$, 1 d.f., $P < 0.0001$), and b and d ($\chi^2 = 26.41$, 1 d.f., $P < 0.0001$) were highly significantly different; a and b were not ($\chi^2 = 0.678$, 1 d.f., $P = 0.41$), and c and d were just significantly different ($\chi^2 = 3.81$, 1 d.f., $P = 0.05$). Data for c-erbB-2 staining status were available for only 342 patients.
patients presenting with primary breast cancer and its clinical significance. We have found that 41% of the carcinomas are stained strongly by the rabbit antisera to rat rS100A4 and that 15% possessed border-line staining of between 1–5% of the carcinoma cells stained for S100A4, in line with our earlier pilot experiments (25). In the present work, the borderline cases have been included with the negatively stained carcinomas, unless otherwise stated. The staining in specimens is not restricted to only carcinoma cells because variable, often reduced levels are detected in normal parenchymal breast tissue and in some reactive fibroblast-like cells, lymphocytes, and blood vessels in the invasive carcinomas. The present assessment, however, has been undertaken on only the carcinoma cells. This heterogeneous cellular staining pattern for S100A4 is not a simple artifact of tissue preservation due, for instance, to lack of accessibility of the antigen for its specific antibody for the following reasons. (a) The same results have been achieved in pilot experiments using frozen sections and with carcinomas preserved in Methacarn (32) or formalin as paraffin-embedded sections. (b) Increasing the concentration of the antibody 10-fold or incubating for periods >16 h fails to increase the assessment of staining, although incubating for short 1–3 h periods reduces the levels of staining and hence the assessment by 30%. (c) Attempts at antigen retrieval by prior microwaving (42) or pronase digestion (32) of the sections fail to increase the assessment. The immunocytochemical staining for S100A4 is also specific for this molecule for the following reasons: (a) Incubation of recombinant human S100A4 with the rabbit antibody to S100A4 before its use abolishes completely the immunocytochemical staining of all carcinoma cells as well as of normal breast parenchymal cells and reactive stromal cells in the invasive carcinomas. (b) The same staining patterns are achieved with our in-house rabbit polyclonal antisera raised in different rabbits to different preparations of rat rS100A4 and with a commercial polyclonal antisera raised to human rS100A4. (c) The anti-S100A4 serum detected authentic human S100A4 but not closely related calcium-binding proteins, as well as a single band of the same molecular weight as S100A4 in extracts of selected positively staining carcinomas when tested by Western blotting procedures. In addition, the observed interobserver and intratumor variability in immunocytochemical staining for S100A4 is sufficiently small (13% and 7%, respectively) not to affect the reported results. Pilot studies on another set of patients showed that S100A4 mRNA detected by in situ hybridization is present in the carcinoma cells (43), suggesting that in this present group of 349 patients, positive immunocytochemical staining for S100A4 may also reflect enhanced expression of its mRNA.

In this and previous studies with this group of patients, the tumor variables that show a significant association with survival time of the patients are nodal status (P < 0.0001), tumor size (P = 0.0035), histological grade (P = 0.013), staining for c-erbB-2 (P = 0.0015), ER (P = 0.028), and p53 (P = 0.032) for the full follow-up period of 19 years. Previous publications on this group of patients have reported that the presence of ER showed only a trend with improved prognosis for the patients after 14 years when measured biochemically (P = 0.09) (12). This difference is probably due to the method of detection used because after the full follow-up period of 19 years, no association of patient survival with ER measured biochemically is detected (χ² = 0.45, 1 d.f., P = 0.5), whereas a strong association is detected using immunocytochemical methods (P = 0.028) (37). Also, cathepsin D has been shown previously to be associated with a poorer prognosis for this group of patients (P = 0.025) after 14 years of follow-up (18), whereas after the full follow-up of 19 years, only a trend (P = 0.093) is observed (37). This difference may reflect the comparatively few patients dying of cancer with cathepsin D-positive carcinomas between the two census dates. Although previous reports have suggested associations between patient survival and the presence of PgR (14, 15), pS2 (13), and c-erbB-3 (8, 9) in breast carcinomas, these associations represent only trends in the present group of 349 patients and are not statistically significant (χ² = 3.6, 1 d.f., P = 0.058; χ² = 1.3, 1 d.f., P = 0.25; and χ² = 0.37, 1 d.f., P = 0.54, respectively [Ref. 37]).

Table 2 Regression coefficients for the Cox proportional hazards model for cancer-related deaths

<table>
<thead>
<tr>
<th>Tumor variable</th>
<th>Coefficient β</th>
<th>SE</th>
<th>d.f.</th>
<th>R value</th>
<th>P</th>
<th>RR</th>
<th>95% CI</th>
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</thead>
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<td>2.01</td>
<td>0.30</td>
<td>1</td>
<td>0.26</td>
<td>&lt;0.0001</td>
<td>7.5</td>
<td>4.2–13.5</td>
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<tr>
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<td>0.14</td>
<td>0.0002</td>
<td>11.6</td>
<td>3.1–42.8</td>
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<tr>
<td>Nodal status</td>
<td>0.88</td>
<td>0.27</td>
<td>1</td>
<td>0.12</td>
<td>0.001</td>
<td>2.4</td>
<td>1.4–4.1</td>
</tr>
<tr>
<td>Histological grade III</td>
<td>0.60</td>
<td>0.26</td>
<td>1</td>
<td>0.07</td>
<td>0.022</td>
<td>1.8</td>
<td>1.1–3.0</td>
</tr>
</tbody>
</table>

a Tumor variable that showed a statistically significant association with patient survival times in the univariate analysis. There were 137 patient cases available with full data sets. Only comparison between patients with involved lymph nodes, all tumor sizes (T1–T4), all histological grades (I–III), staining for S100A4, c-erbB-2, ER, and p53 were made.
b Value of β parameter in the Cox multiple regression analysis (see “Materials and Methods”).
c SE of β.
d Regression coefficient for the variable.

Probability from χ² test. Overall χ² = 107.5, 4 d.f., P < 0.0001.

f RR for survival and 95% CI from multivariate analysis.

† Tumor fixed to chest wall.

§ High histological grade.
lyzed using log-rank sums, a similar level of significance is achieved ($\chi^2 = 178, 1 \text{ d.f., } P < 0.0001$) and the median survival times of >231 and 46 (95% CI, 38–55) months (not shown) compare favorably with >228 and 47 months using Wilcoxon statistics (Fig. 3). This relationship for S100A4 achieved statistical significance after 6 months of follow-up and remained statistically significant for the full 19 years of follow-up of the patients, unlike some of the relationships between other tumor variables and patient survival [e.g., ER (12, 44) and cathepsin D (18, 37)]. Moreover, when the borderline cases (defined as 1–5% carcinoma cells stained) of immunocytochemical staining for S100A4 are analyzed separately, they are correlated with a level of patient deaths that is intermediate between that for the completely unstained group and that for the positively stained group for all follow-up times of 5 years and beyond (Fig. 4). Once again, Kaplan Meier plots followed by analysis of log-rank sums gave the same results [$\chi^2 = 224, 2 \text{ d.f., } P < 0.0001$, median survival times were >231, 78 (95% CI, 30–125), and 46 (95% CI, 38–55) months for patients with S100-negative carcinomas, with borderline carcinomas, and with positive carcinomas, respectively (not shown)]. This result suggests that not only the presence, but also the levels of immunoreactive S100A4 may be correlated with the time of demise of the patients. It should be noted, however, that only 3 deaths in 152 cases are observed in the group of patients with completely unstained carcinomas, and this very low percentage may cast some doubt on the validity of the overall statistical test for significance. Moreover, the fact that in our study a large number of patients is required to obtain a statistically significant result may mean that small fluctuations in data can alter considerably the significance of the results. Thus, when the positively stained group is separated into patients with 5–25%, 25–50%, and 50–75% of stained carcinoma cells in their tumors, there are too few highly stained carcinomas to verify this effect statistically using 5% confidence limits. Nevertheless, the fact that the presence of immunoreactive S100A4 in the carcinoma cells is so highly correlated with early demise of this group of patients may reflect that this change is more closely associated with their cause of death than some of the other tumor variables studied. Because S100A4 was first discovered as a metastasis-inducing protein in rodent models of breast cancer (20, 21), and metastasis is the major event responsible for death of patients from human breast cancer (3), it is possible that S100A4 is causing premature deaths by its ability to induce metastasis in humans as well.

When smaller subgroups of patients are analyzed for their survival times, small fluctuations in data may have an even more dramatic effect on the significance of the results than when analyzed as a whole and/or patient numbers may be too small to observe a significant effect. The magnitude of both interobserver error and intratumor heterogeneity in this study could conceivably result in such a situation. Nevertheless, when subgroups of patients with carcinomas classified as positive or negative for S100A4 and for another tumor variable were examined, those subdivided by lymph node status or by c-erbB-2 are of particular interest. Results of statistical analyses were virtually identical if Wilcoxon (Figs. 5 and 6) or log-rank tests (not shown) were used. There was virtually no difference in patient survival in the S100A4-negative group of patients with or without involved lymph nodes, but a significantly more rapid demise was observed for patients in the lymph node-negative group with rather than without S100A4 (Fig. 5). These results may suggest that the presence of S100A4 in the tumor is the more dominant factor at predicting patient outcome than that of involved lymph nodes. Moreover, once S100A4 is detected in the primary tumor, then patients with involved lymph nodes die more quickly than those without involved lymph nodes (Fig. 5). These results are consistent with those obtained in a Cox multivariate regression analysis model where the presence of S100A4 is found to be the most significant predictor of patient death, but nodal status is itself a significant independent predictive variable (Table 2). The other independent predictive variables in this proportional hazards model are small subsets of the remaining two pathological variables, tumor fixed to the chest wall ($T_4$), and high histological grade (III) (Table 2). When slightly larger data sets were analyzed for only lymph node status, histological grade, and S100A4 status, S100A4 status was retained as the most significant predictor of patient death, but high histological grade (III) was eliminated as an independent predictive variable. Similarly, there was virtually no difference in patient survival in the S100A4-negative group of patients with or without c-erbB-2, but a significantly more rapid demise was noted for patients in the c-erbB-2-negative group with, rather than without, S100A4 (Fig. 6). Moreover, once S100A4 was detected in the primary tumor, then patients with c-erbB-2-positive tumors died more quickly than those with c-erbB-2-negative tumors (Fig. 6). These results suggest once again that the presence of S100A4 in the tumor is the more dominant factor at predicting patient outcome than that of c-erbB-2, but that c-erbB-2 can synergize with S100A4 in accelerating the demise of patients. The fact that c-erbB-2 was rejected as an independent prognostic factor in the Cox multivariate regression analysis (Table 2) may suggest that c-erbB-2 was confounded with one or more of the independent pathological prognostic variables in the proportional hazards model. That c-erbB-2 can synergize with S100A4 in producing accelerated patient demise is consistent with one of the mouse models for breast cancer in which transgenic mice require both the expression of the mutated form of c-erbB-2, neu, and S100A4 to induce metastasis (27).

How S100A4 may be overexpressed and its role in human breast cancer are not clear. In rodent model systems, its expression is normally under the control of both positive and negative regulatory factors (45–47), and multiple copies of the rodent and human genes have been introduced into rodent and human cells to cause metastasis in rodents (20, 21, 26–28). In humans, the gene for S100A4 occurs in a cluster of 13 S100 genes on chromosome 1 (48), a region of the human genome, which is also often amplified in breast cancer and which contains jumping elements (49). In the rodent model systems, however, elevated levels of S100A4 can only synergize with growth-promoting oncogenic products like c-erbB-2 (27) or be expressed in already benign neoplasms before metastasis can be induced (20, 21, 28). By itself, it has no neoplastic or metastatic effect in normal rodent cells (50). Because the majority of invasive human breast carcinomas do not contain c-erbB-2 (7), interaction of S100A4 with other growth-promoting oncogenic products may also occur in those human breast carcinomas that fail to express c-erbB-2.

In the rodent model systems, S100A4 is thought to interact with components of the cytoskeleton (20, 51–53), thereby enhancing the motile properties of cells (54, 55). But motility per se is unlikely to be the sole property required to accomplish the metastatic cascade in rodent models; let alone in human breast cancer (56, 57). However, it is plausible that one step in the overall progression from an invasive breast carcinoma to a growing metastasis may be more or less rate-limiting and that, under appropriate conditions, S100A4 or similar molecules may accelerate that step. In conclusion, our results show that the presence of the calcium-binding protein S100A4, which can cause metastasis in rodent models, is now associated with a poor prognosis for one group of breast cancer patients. It remains to be determined how widespread this association will prove to be, not only in breast, but in other metastatic carcinomas.

ACKNOWLEDGMENTS

We thank Dr. Suzete de Silva Rudland for advice on immunocytochemical staining, the Cancer Tissue Bank Research Center, University of Liverpool, for supplying some of the samples used in this study, and Dr. E. M. I. Williams and the Merseyside Cancer Registry for providing patient outcome data.
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Prognostic Significance of the Metastasis-inducing Protein S100A4 (p9Ka) in Human Breast Cancer


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