

Identification of *NTN4*, *TRA1*, and *STC2* as Prognostic Markers in Breast Cancer in a Screen for Signal Sequence Encoding Proteins

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Abstract Purpose: In a previous screen using a signal-trap library, we identified a number of secreted proteins up-regulated in primary tumor cells isolated from invasive breast cancers. The purpose of this study was to assess the expression of these genes in human invasive breast tumors and to determine the significance of their expression for prognosis in breast cancer.

Experimental Design: A tissue microarray containing 245 invasive breast tumors from women treated with curative surgery followed by anthracycline-based chemotherapy and hormone therapy for the estrogen receptor (ER)–positive tumors was screened by *in situ* hybridization with probes against thrombospondin 3 (*TSP3*), insulin-like growth factor binding protein 7 (*IGFBP7*), tumor rejection antigen 1 (*TRA1*), stanniocalcin 2 (*STC2*), and netrin 4 (*NTN4*). Correlations between categorical variables were done using the χ^2 test and Fisher's exact test. Cumulative survival probabilities were calculated using the Kaplan-Meier method and multivariate survival analysis was done with Cox hazard model. A series of breast cancers were also stained with NTN4 antibodies.

Results: All five genes examined were expressed in invasive breast tumor cells. NTN4 protein expression was also confirmed by immunohistochemistry. Together, these data validate the design and screening of the signal-trap library. Univariate survival analysis revealed that expressions of *TRA1*, *STC2*, and *NTN4* are correlated with longer disease-free survival and that *TRA1* and *NTN4* are associated with longer overall survival. Multivariate analysis showed that *NTN4* is an independent prognostic factor of overall survival.

Conclusions: This article describes the identification of three secreted proteins, NTN4, TRA1, and STC2, as potential novel prognostic markers in breast cancer.

Paracrine signaling from tumor cell to stromal cells plays a key role in regulating leukocyte recruitment, angiogenesis, and activation of stromal fibroblasts. This, in turn, changes the tumor environment, thus regulating tumor cell proliferation, survival, migration, and invasion (1–4). As a consequence, identifying secreted proteins that are up-regulated in tumor cells is critical for understanding these complex signaling interactions and identifying key targets for therapeutic intervention. We have previously described the generation and

screening of a library to preferentially identify genes overexpressed in invasive breast tumor cells (5). The screen was designed to (a) specifically enrich for transcripts that are expressed by primary tumor cells *in vivo* rather than by cancer cell lines or total tumor tissue *in vivo*, and (b) to select for transcripts bearing a functional signal sequence and thereby specifically identify overexpressed transmembrane and secreted proteins. The purpose of the current study was to verify that the overexpressed genes that encode for secreted proteins are specifically expressed by breast tumor cells and to determine their prognostic significance in a large cohort of breast cancer patients. The advent of tissue microarrays allows a large number of tumors to be examined simultaneously, and, traditionally, this has involved immunohistochemical staining of the arrays. However, this approach is problematic when studying secreted genes as immunohistochemical staining only identifies the location of the protein and not the cell of origin. To overcome this, we have used *in situ* hybridization on a large cohort of breast cancer patients who had undergone uniform adjuvant anthracycline-based chemotherapy and for whom survival data were available.

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Materials and Methods

This project was approved by the Research Ethics Committee of the Royal Marsden NHS Trust.

Generation and screening of the signal-trap library. The generation of the subtracted library and its screening have been described elsewhere (5). Briefly, tumor cells were isolated from four invasive breast carcinomas; mRNA was isolated and double-strand cDNA was generated. The double-strand cDNA was subtracted against single-strand cDNA generated from normal breast tissue RNA. Subtracted cDNA was inserted into the pCMV- Δ ssPLAP vector and the library plated. In the primary library screen, plasmid DNA extracted from pools of 50 individual colonies was transfected into COS-1 cells. The pCMV- Δ ssPLAP vector contains a cytomegalovirus (CMV) promoter followed by a cloning site and placental alkaline phosphatase (PLAP) lacking an NH₂-terminal signal sequence. The presence of plasmids in which a cDNA with a functional signal sequence has been inserted upstream of the signalless PLAP was detected by cell-surface alkaline phosphatase staining. Positive pools were then transfected as subpools of 5 or 10 colonies (secondary screening) and colonies in positive subpools were then transfected individually (tertiary screening; ref. 5).

Breast tumor tissue microarray. The tissue microarray contained replicate 0.6-mm cores of 245 invasive breast carcinomas. Full details of the characterization of the tissue microarray and the cohort of patients are described elsewhere (6). All patients were primarily treated with surgery (69 mastectomy and 155 wide local excision) and anthracycline-based adjuvant chemotherapy, in combination with hormone therapy for estrogen receptor (ER)-positive tumors. Follow-up was available for 244 patients, ranging from 0.5 to 125 months (median, 67 months; mean, 67 months). Tumors were graded according to a modified Bloom-Richardson scoring system (7) and size was categorized according to the tumor-node-metastasis staging criteria (8). Although this series is representative of a large proportion of patients with early breast cancer, the findings of this series are particularly relevant to patients primarily treated with curative surgery and adjuvant anthracyclines.

I.M.A.G.E. clones (9) were purchased from Geneservice Ltd. For *in situ* hybridization, probes for netrin 4 (NTN4; IMAGE clone 3876557, nucleotides 1,501-2,186), stanniocalcin 2 (STC2; IMAGE clone 4120602, nucleotides 961-1,500), thrombospondin 3 (TSP3; IMAGE clone 3174496, nucleotides 1-457), tumor rejection antigen 1 (TRA1; IMAGE clone 6165138, nucleotides 2,161-2,659), and insulin-like growth factor binding protein 7 (IGFBP7; IMAGE clone 5296697, nucleotides 481-880) were generated by PCR amplification and cloned into the pGEM3Z vector (Promega). Generation and labeling of riboprobes and hybridization to the tissue microarray were as previously described (10). An *ACTB* (β -actin) probe was used as a positive control. mRNA levels in the tumor cells were concurrently scored by two observers (R.P. and J.S.R.F.) on a dark-field microscope coupled with a digital camera. Scoring was blind (i.e., without knowledge of the outcome of the patients) and classified semiquantitatively into four categories: 0, negative; 1, weak; 2, moderate expression; 3 strong expression. Tumors were scored positive if the staining was 2 or 3. For *TSP3*, tumors were scored only as negative or positive.

Immunohistochemistry to detect ER, progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), cytokeratin (Ck) 14, Ck 5/6, Ck 17, Ki-67, and p53 was done on the tissue microarray as previously described (6, 11). Tumors were classified according to the criteria of Nielsen et al. (12) into HER2 (HER2⁺, ER any, Ck 5/6, or EGFR any), luminal (HER2⁻, ER⁺, Ck 5/6, or EGFR any), or basal-like (HER2⁻, ER⁻, Ck 5/6, or EGFR⁺) groups.

Immunohistochemistry on breast tumor cryosections. Seven-micrometer cryosections were cut from normal adult human breast and from breast tumors stored at -80°C. Sections were thawed, fixed in cold methanol-acetone for 5 min, and then stained with anti-NTN4 antibody (AF1254, 1:50; R&D Systems) and Alexa 555 anti goat immunoglobulin (Molecular Probes, Invitrogen) followed by anti-collagen IV monoclonal antibody CIV 22 (1:50; DAKO) and Alexa 488 antimouse immunoglobulin. Nuclei were counterstained with TO-PRO-

3 (Molecular Probes) and images were collected sequentially in three channels on a Leica TCS SP2 (Leica Microsystems GmbH) confocal microscope. Control staining in which one or both primary antibodies were omitted showed that the secondary antibodies did not cross-react with the inappropriate primary antibody or show nonspecific tissue staining.

Statistical analysis. The Statview 5.0 (SAS Institute, Inc.) software package was used for all calculations. Correlations between categorical variables were done using the χ^2 test and Fisher's exact test. Correlations between continuous and categorical variables were done with ANOVA. Disease-free and overall survival were expressed as the number of months from diagnosis to the occurrence of an event (local recurrence/metastasis and disease-related death, respectively). Cumulative survival probabilities were calculated using the Kaplan-Meier method. Differences between survival rates were tested with the log-rank test. All tests were two tailed, with a confidence interval of 95%.

Multivariate analysis was done using the Cox multiple hazards model. A *P* value of 0.05 in the univariate survival analysis was adopted as the limit for inclusion in the multivariate model, and cases with missing values were excluded from this analysis.

Results

Identification of secreted proteins overexpressed in invasive breast tumor cells. The details of the signal-trap screen used to identify transmembrane and secreted proteins that were overexpressed in invasive breast tumor cells are described elsewhere (5). The signal-trap library selected effectively for transcripts encoding secreted and transmembrane proteins because these proteins had a functional signal sequence that directed a signalless form of PLAP into the secretory pathway. The presence of the PLAP transmembrane domain resulted in these proteins being anchored at the plasma membrane where they were detected using a colorimetric assay. From this screen, 12 secreted proteins were identified and then validated by dot-blot analysis as being up-regulated in the invasive breast tumor cells used to generate the library (5).

Expression analysis using a breast tumor tissue microarray. One problem in determining whether a secreted protein is indeed up-regulated in tumor cells is that the localization of the protein does not necessarily reflect the cell type in which the protein was synthesized. A further problem is the limited availability of antibodies that are suitable for immunohistochemical analysis on formalin-fixed, paraffin-embedded tissue sections. For these reasons, we assessed expression of a subset of these secreted genes by *in situ* hybridization on a tissue microarray of 245 invasive breast cancers of all grades for which outcome data were available (185 invasive ductal carcinomas, 25 invasive mixed carcinomas, and 8 invasive breast carcinomas of other special types). Figure 1 illustrates representative *in situ* hybridization results obtained. A summary of the results for the univariate analysis for each probe is shown in Table 1. Full details of the results obtained with each probe are shown in Supplementary Table S1.

TSP3 (*THBS3*) is a pentameric secreted glycoprotein and a member of the thrombospondin family of multifunctional cell-surface and extracellular matrix molecules. The prevalence of *TSP3* expression was low in breast cancer with only 11 (6%) tumors scoring positive. There was a trend for an association between expression of *TSP3* and presence of lymph node metastasis (*P* = 0.0536) but no correlation with patient survival (Table 1; Supplementary Table S1A). *TSP3* was only expressed in a small number of tumors, raising the question about

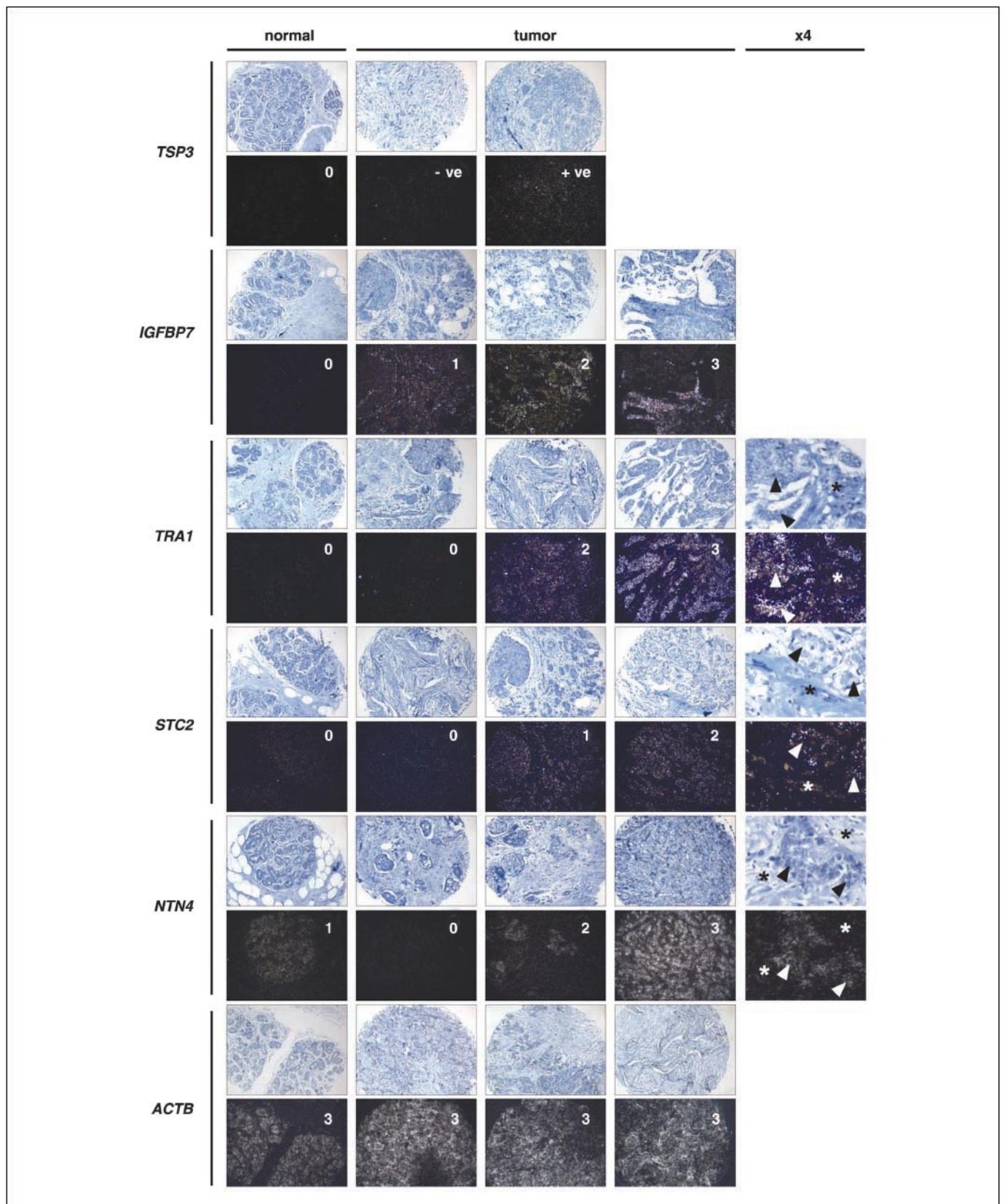


Fig. 1. *In situ* hybridization to detect mRNAs in a breast cancer tissue microarray. Riboprobes for *TSP3*, *IGFBP7*, *STC2*, *TRA1*, *NTN4*, and *ACTB* (β -actin) were hybridized to sections of a human breast cancer tissue microarray. Representative images of one normal breast section (*N*) and three tumor sections (where scores of 0 to 3 were given) or two tumor sections (where scores of positive or negative were given) are shown with the score given to each sample indicated (see Materials and Methods for further details of the scoring). Right, higher-magnification images ($\times 4$ of left images) to show expression of *STC2*, *TRA1*, and *NTN4* transcripts in tumor (arrowhead) but not stroma (asterisks). For each probe, top images show Giemsa counterstain and bottom images show silver staining. Cores were 0.6 mm in diameter.

Table 1. Univariate analysis of clinicopathologic and *in situ* hybridization data on a tissue microarray of 245 breast tumors

| Variable | N | Disease-free survival | | | Overall survival | | |
|-----------------------|-----|-----------------------|-------|---------------|------------------|------|---------------|
| | | Surv (mo) | SE | P | Surv (mo) | SE | P |
| Size | | | | 0.0008 | | | 0.4045 |
| T ₁ | 129 | 112.1 | 4.28 | | 115.7 | 4.05 | |
| T ₂ | 98 | 98.3 | 5.52 | | 114.4 | 4.44 | |
| T ₃ | 16 | 54.9 | 7.65 | | 76.4 | 7.47 | |
| Grade | | | | 0.0049 | | | 0.0650 |
| 1 | 23 | 116.8 | 3.94 | | 117 | 3.64 | |
| 2 | 69 | 116.4 | 4.98 | | 121 | 4.93 | |
| 3 | 148 | 95.9 | 4.52 | | 109 | 4.00 | |
| LVI | | | | 0.2623 | | | 0.1264 |
| Negative | 82 | 109.9 | 5.18 | | 121 | 4.15 | |
| Positive | 161 | 94.9 | 4.04 | | 104 | 3.73 | |
| LN met | | | | 0.0001 | | | 0.0004 |
| Negative | 83 | 122.2 | 3.96 | | 129 | 2.60 | |
| Positive | 154 | 93.5 | 4.61 | | 105 | 4.32 | |
| ER | | | | 0.0199 | | | 0.0001 |
| Negative | 48 | 81.2 | 6.94 | | 86.8 | 6.53 | |
| Positive | 191 | 107.9 | 3.73 | | 119.2 | 3.17 | |
| PgR | | | | 0.1316 | | | 0.0003 |
| Negative | 64 | 89.3 | 6.28 | | 92.8 | 5.84 | |
| Positive | 175 | 106.9 | 3.92 | | 119.7 | 3.33 | |
| HER2 | | | | 0.4755 | | | 0.2102 |
| Negative | 200 | 104.3 | 3.75 | | 115 | 3.43 | |
| Positive | 36 | 92.9 | 7.71 | | 102 | 7.28 | |
| EGFR | | | | 0.3527 | | | 0.0813 |
| Negative | 222 | 105 | 3.55 | | 115.5 | 3.18 | |
| Positive | 22 | 86 | 9.62 | | 92.3 | 8.79 | |
| Ck 14 | | | | 0.3251 | | | 0.0141 |
| Negative | 221 | 104.2 | 3.65 | | 116.0 | 3.13 | |
| Positive | 22 | 84.5 | 10.13 | | 86.6 | 9.47 | |
| Ck 5/6 | | | | 0.0896 | | | 0.0089 |
| Negative | 210 | 105.4 | 3.63 | | 116.2 | 3.19 | |
| Positive | 25 | 80.4 | 9.81 | | 86.8 | 8.95 | |
| Ck 17 | | | | 0.0171 | | | 0.0001 |
| Negative | 213 | 106.5 | 3.60 | | 118.5 | 3.06 | |
| Positive | 28 | 77.2 | 9.41 | | 80.3 | 8.72 | |
| P53 | | | | 0.0858 | | | 0.0009 |
| Negative | 158 | 107.8 | 3.99 | | 120 | 3.52 | |
| Positive | 67 | 94.9 | 6.93 | | 103 | 6.06 | |
| MIB1 | | | | 0.0133 | | | 0.0015 |
| <10% | 96 | 112.2 | 5.04 | | 122.4 | 3.66 | |
| 10-30% | 97 | 100.8 | 5.47 | | 111.4 | 5.48 | |
| >30% | 33 | 76.4 | 8.38 | | 88.8 | 8.44 | |
| Nielsen et al. groups | | | | 0.1134 | | | 0.0018 |
| Basal-like | 30 | 81.3 | 8.74 | | 87.2 | 8.01 | |
| HER2 | 36 | 92.9 | 7.71 | | 102.2 | 7.28 | |
| Luminal | 164 | 107.9 | 4.13 | | 119.1 | 3.68 | |
| TSP3 | | | | 0.9593 | | | 0.4868 |
| Negative | 175 | 103.5 | 4.02 | | 113.9 | 3.48 | |
| Positive | 11 | 76.3 | 7.36 | | 85.9 | 5.49 | |
| IGFBP7 | | | | 0.6604 | | | 0.4399 |
| Negative | 123 | 103.5 | 4.68 | | 115 | 4.03 | |
| Positive | 66 | 92.3 | 6.30 | | 103 | 5.73 | |
| TRA1 | | | | 0.7169 | | | 0.9494 |
| Negative | 98 | 98.1 | 4.96 | | 109 | 4.15 | |
| Positive | 97 | 106.1 | 5.17 | | 115 | 4.70 | |
| STC2 | | | | 0.0196 | | | 0.1022 |
| Negative | 159 | 98.6 | 4.37 | | 112 | 3.80 | |
| Positive | 37 | 114.6 | 5.35 | | 119 | 3.86 | |
| NTN4 | | | | 0.0373 | | | 0.0039 |
| Negative | 128 | 95.9 | 4.87 | | 106 | 4.52 | |
| Positive | 75 | 111.0 | 6.05 | | 127 | 3.35 | |

NOTE: *P* values were calculated by the log-rank test. Significant *P* values are in boldface. Nielsen et al. groups: HER2 (HER2 positive, ER any, Ck 5/6, or EGFR any), luminal (HER2 negative, ER positive, Ck 5/6, or EGFR any), and basal-like (HER2 negative, ER negative, Ck 5/6, or EGFR positive). Scoring of the tissue microarray is described in Materials and Methods.

Abbreviations: LVI, lymphovascular invasion; LN met, lymph node metastasis; *N*, number of cases; surv (mo), survival in months.

whether *TSP3* was misidentified in the screen for genes up-regulated in invasive breast cancers. Evidence that this is not the case comes from reverse Northern blot analysis which showed that *TSP3* transcripts were expressed at a higher level in a pool of four breast cancers used to generate the library compared with the normal breast tissue (5).

IGFBP7 belongs to the *IGFBP* family whose functions include binding to insulin-like growth factors (IGF) and regulating their bioavailability, as well as a range of IGF-independent biological activities. *IGFBP7* transcripts were detected in 35% of the tumors but there were no correlations with clinicopathologic features, immunohistochemical markers (Supplementary Table S1B), or patient survival (Table 1).

TRA1 (*GP96*, *GRP94*) is a member of the heat shock protein (HSP) family and, like other HSPs, functions as a molecular chaperone. *TRA1* expression was detected in 50% of the tumors and showed a statistically significant correlation with high grade ($P = 0.0364$), lack of lymph node metastasis at diagnosis ($P = 0.0246$), lack of ER and PgR ($P = 0.0037$ and $P = 0.0094$, respectively), high proliferation rate ($P = 0.0008$), and expression of basal markers (EGFR, $P = 0.0028$; Ck 14, $P = 0.0077$; Ck 5/6, $P = 0.0022$; Ck 17, $P = 0.0090$; Supplementary Table S1C). *TRA1* was also preferentially expressed in tumors with a basal-like phenotype, as defined by Nielsen et al. (ref. 12; see Materials and Methods) immunohistochemical criteria ($P = 0.0011$). Considering the patient cohort in its entirety, *TRA1* expression showed no prognostic significance; however, for the clinically relevant group of tumors expressing basal markers, *TRA1* expression correlated with disease-free survival ($P = 0.0140$) and overall survival ($P = 0.0075$; Fig. 2A and B).

STC2 is related to a secreted glycoprotein hormone of bony fish. *STC2* transcripts were detected in 19% of the tumors (Supplementary Table S1D) and its expression was preferentially found in tumors with a luminal phenotype ($P = 0.0415$) as defined by the criteria of Nielsen et al. (12). Owing to its association with luminal phenotype, it is not surprising that *STC2* expression significantly correlated with immunohistochemical features of luminal tumors, including expression of ER and PgR ($P = 0.0023$ and $P = 0.0402$, respectively), lack of EGFR ($P = 0.0463$) and p53 immunoreactivity ($P = 0.0461$), and a low proliferation index ($P = 0.0303$). In the overall cohort of patients, *STC2* expression correlated with improved disease-free survival ($P = 0.0196$) and showed a trend for a longer overall survival ($P = 0.1022$; Table 1; Fig. 2C and D).

NTN4 is a secreted growth factor of the evolutionally conserved netrin family. Of the tumors, 37.5% expressed *NTN4* (Supplementary Table S1E) and its expression was preferentially found in tumors of histologic grade 1 or 2 ($P = 0.0002$), positive for ER ($P = 0.0341$) and PgR ($P = 0.0158$), and with low proliferation rates ($P = 0.0007$). Expression of *NTN4* correlated with longer disease-free survival ($P = 0.0373$) and overall survival ($P = 0.0039$) in the whole cohort (Table 1; Fig. 2E and F).

Multivariate survival analysis. In this cohort, size, grade, lymph node metastasis, ER, Ck 17, proliferation index assessed by MIB1, and *STC2* and *NTN4* expression were statistically significant prognostic factors for disease-free survival on univariate analysis (Table 1). On multivariate analysis, only tumor grade and lymph node metastasis remained independent predictors of disease-free survival (data not shown).

Univariate survival analysis revealed lymph node metastasis, ER, PgR, Ck 14, Ck 5/6, Ck 17, p53, proliferation index as

defined by MIB1, and *NTN4* expression as statistically significant prognostic factors for overall survival (Fig. 2C; Table 1). On multivariate analysis, lymph node metastasis and *NTN4* emerged as the only independent predictors of overall survival (Table 2).

Expression of netrins and netrin receptors in breast cancer. It has previously been reported that, following secretion of *NTN4* by lung epithelial cells, the growth factor is deposited in the surrounding basal lamina (13). To investigate whether *NTN4* is similarly localized in the breast, cryosections of normal human breast and breast tumors were double labeled with antibodies against *NTN4* and a major basement membrane component, collagen IV. In normal breast, *NTN4* protein was predominantly localized to the basal lamina surrounding the epithelial structures and blood vessels (Fig. 3). As *NTN4* transcripts were detected in the epithelial cells of the normal breast (Fig. 1), this shows that, as in the lung, *NTN4* protein in the breast is secreted by the epithelial cells and sequestered by the surrounding basement membranes. Immunostaining of a series of breast tumors showed strong agreement with the *NTN4* *in situ* hybridization results (Fig. 1; Table 1; Supplementary Table S1E) in which 37.5% of invasive breast tumors scored positive for *NTN4* transcripts, and these predominantly represented ER⁺ tumors. For example, high levels of *NTN4* protein were observed associated with the epithelial cells of two ER⁺ tumors (tumors B21 and B22) whereas little *NTN4* protein was detected in an ER⁻ tumor (tumor B3). It was also noted that in the normal breast and all the breast tumors stained, *NTN4* protein was associated with the basal lamina of the vasculature. In the ER⁺ ductal carcinoma *in situ* lesion (tumor B22), *NTN4* protein strongly accumulated in the basal lamina, which remained associated with the tumor cells (Fig. 3).

Discussion

Our signal-trap screen identified 12 secreted proteins (*CCL3L1*, *COL3A1*, *COL8A1*, *CIQB*, *FN1*, *IL8*, *IGFBP7*, *SERPINH1*, *STC2*, *NTN4*, *TSP3*, and *TRA1*) that were validated by dot-blot analysis as being up-regulated within neoplastic cells of invasive breast cancers (5). In addition, our data on *CCL3L1*, *COL3A1*, *IL8*, and *FN1* have also been independently validated by other groups, which have found up-regulation of these genes in invasive breast cancers (14–18). The purpose of this study was to interrogate the distribution and prognostic significance of five of these genes (*TSP3*, *IGFBP7*, *TRA1*, *STC2*, and *NTN4*) by *in situ* hybridization in a large cohort of breast cancers from patients treated with anthracycline-based chemotherapy.

The thrombospondin (TSP) family of extracellular matrix glycoproteins are involved in cell-to-cell and cell-to-matrix interactions and have complex functions in the regulation of tissue genesis and remodeling. *TSP3*, like *TSP4* and *TSP5* (or cartilage oligomeric matrix protein), has a pentameric structure held together by disulfide bonds (19). The specific biological function of *TSP3* is not known but mice with a targeted deletion in the *TSP3* gene, although viable and fertile, show abnormalities in skeletal maturation (20). Similarly, little is known about the expression of *TSP3* in adult tissues, except that in a screen of 50 human tissues, the highest levels of transcripts were detected in the kidney, pituitary gland, trachea, uterus, and testis, whereas moderate expression was

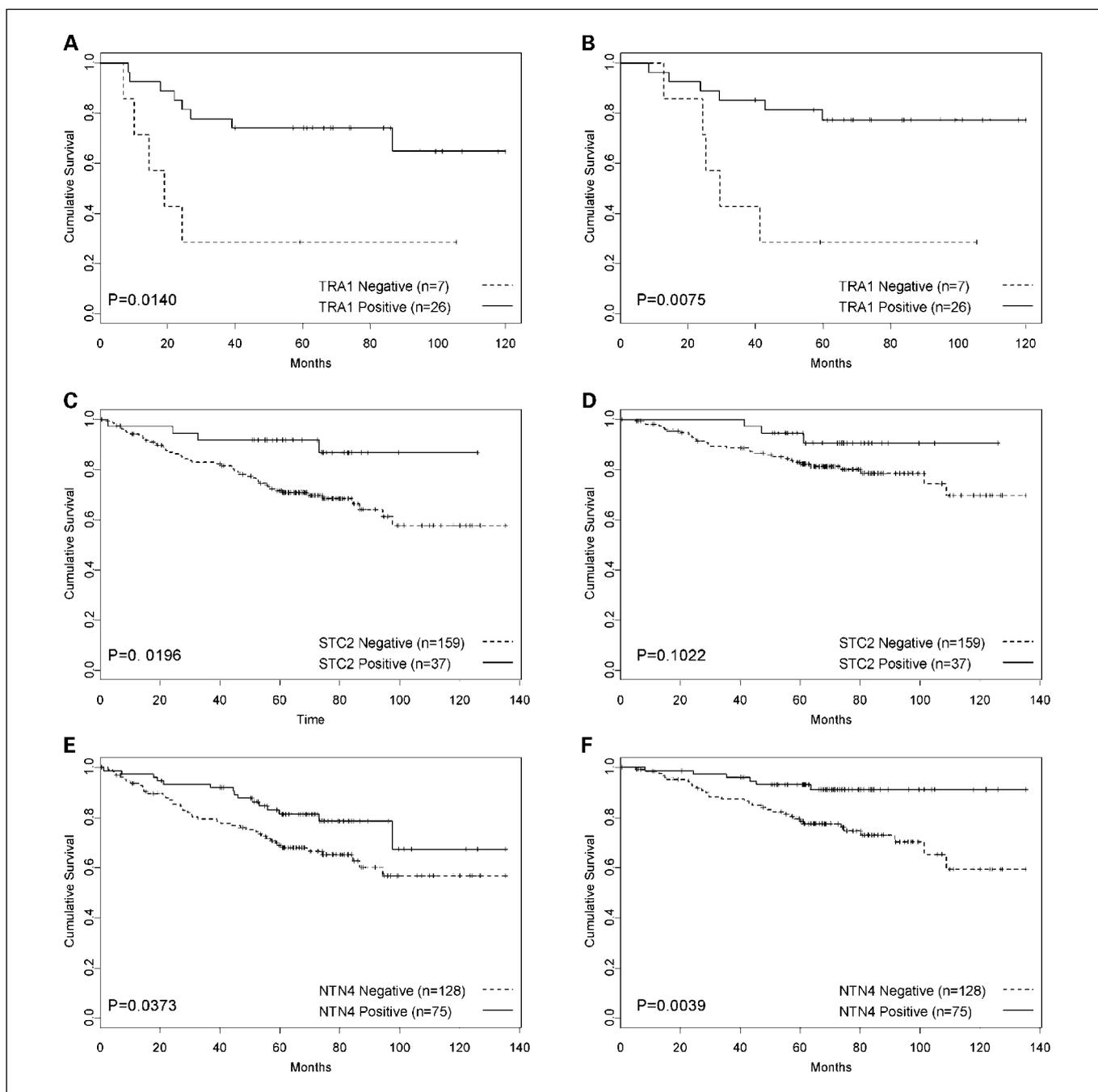


Fig. 2. Kaplan-Meier survival curves from tissue microarray analysis. *A*, *TRA1* expression in basal marker – positive cancers ($n = 33$); disease-free survival. *B*, *TRA1* expression in basal marker – positive cancers ($n = 33$); overall survival. *C*, *STC2* expression ($n = 196$); disease-free survival. *D*, *STC2* expression ($n = 196$); overall survival. *E*, *NTN4* expression ($n = 203$); disease-free survival. *F*, *NTN4* expression ($n = 203$); overall survival.

detected in the mammary gland (21). Importantly, a recent report on osteosarcoma has shown that expression of *TSP3* in primary tumors correlated with worse overall, event-free, and disease-free survival (22). Our data provide the first systematic analysis of *TSP3* mRNA expression in breast cancers. A small subset of tumors (6%) was indeed found to harbor *TSP3* transcripts but this expression did not correlate with clinical outcome.

IGFBP7 (also known as IGFBP-related protein 1, mac25, TAF, PSF, and angiomodulin) belongs to the multigene IGFBP

family but, compared with other family members, has a low binding affinity for IGF-I and IGF-II and a high binding affinity for insulin, activin, and collagen IV (23). An increasing body of evidence indicates an important role for IGFBP7 as a tumor suppressor in breast and other cancers (23). In particular, immunohistochemical studies have reported a loss of IGFBP7 protein during disease progression and a loss of heterozygosity at the *IGFBP7* locus (4q12) in 50% of invasive breast cancers (24). Importantly, reduction of *IGFBP7* expression correlates with decreased patient survival (25). In agreement with these

previous studies, we found that 65% of the invasive breast cancers in our cohort did not express *IGFBP7* transcripts. However, we could not find the same associations between lack of *IGFBP7* expression and high histologic grade, lack of hormone receptors, high proliferation rates, and a shorter overall survival as described by Landberg et al. (25). The reasons for these discrepancies may include different analysis methods [immunohistochemistry (25) versus *in situ* hybridization] and differences in the thresholds adopted and the composition of the cohorts.

TRA1 is also known as gp96 or GRP94 and is a member of the HSP family (26–28). *TRA1* is constitutively expressed in nearly all cell types and, during its synthesis, the presence of an NH₂-terminal signal sequence directs TRA1 to be translocated into the lumen of the endoplasmic reticulum. Like other HSPs, it functions as a molecular chaperone that ensures the correct folding of polypeptides and potentially the co-retention within the endoplasmic reticulum of misfolded or mutated proteins. However, unlike other HSPs, TRA1 has a limited range of client proteins and, in addition, it has been detected as being bound to specific receptors on the cell surface. There is limited information about the expression of *TRA1* expression in cancers, although in transgenic mice, LacZ expression driven by the *TRA1* promoter was strongly up-regulated in both spontaneous and induced tumors (29). Furthermore, immunohistochemical analysis of colorectal cancers showed an up-regulation of TRA1 staining in the majority of tumor samples compared with the surrounding stroma (30), whereas gene expression profiling has identified *TRA1* as an up-regulated gene in multiple myeloma (31). HSPs are up-regulated in response to cellular stress, and it has been suggested that *TRA1* would be up-regulated in tumor cells in response to glucose depletion, hypoxia, and decreased pH. However, there are two conflicting models for the function of up-regulated TRA1 in tumors (26–28). First, tumor cell-derived TRA1 can stimulate a strong antitumor immune response when injected into syngeneic mice. This has led to a model in which TRA1 bound to client proteins is released from tumor cells and bound to cell-surface receptors where it is recognized by antigen-presenting cells to promote tumor immunity. Second, it has been suggested that TRA1, like other HSPs, would function to protect the cell from apoptosis when exposed to a hostile microenvironment. In the present study, *TRA1* was significantly

more frequently expressed in basal-like cancers, which is consistent with the observation that the vast majority of basal-like cancers are characterized by the presence of central necrotic zones, which occur when tumor cells are subjected to hypoxic conditions (32). However, although *TRA1* expression did not correlate with patient outcome in the whole series, within the subset of basal breast cancers, patients with high levels of *TRA1* expression had a longer disease-free survival (Fig. 2A) and overall survival (Fig. 2B), arguing against a role for TRA1 in protecting these tumor cells from apoptosis. Further studies to determine whether up-regulation of *TRA1* results in increased sensitivity to chemotherapeutic agents and, in particular, to anthracycline-based chemotherapy are warranted.

Stanniocalcin was first identified as a glycoprotein hormone secreted from the endocrine gland of bony fish that is involved in calcium and phosphate homeostasis. Two related mammalian genes have been identified, *STC1* and *STC2*, both of which are predicted to be secreted glycosylated proteins (reviewed in refs. 33, 34). *STC1* and *STC2* are expressed in a variety of tissues including endocrine glands and hormone-responsive organs, and ectopic constitutive expression of either stanniocalcin in transgenic mice results in postnatal growth retardation and, in the case of *STC2*, neonatal morbidity. To date, there is a paucity of information on the biological activity of stanniocalcins in mammals and the identity of putative receptors. However, it is notable that *STC2* expression is up-regulated by exposure of cells to xenotoxic agents, such as tunicamycin, thapsigargin, Ca²⁺ ionophores, and reducing agents, and that down-regulation of *STC2* expression by small interfering RNA increases apoptotic cell death in response to these agents (35). *STC2* is reported to be up-regulated in estrogen-treated MCF7 breast cancer cells and selectively expressed in ER⁺ cell lines over ER⁻ cell lines (36, 37). In agreement with the data presented here (Fig. 2A; Supplementary Table S1D), *STC2* expression positively associated with ER status of breast carcinomas (36) and was associated with improved disease-free survival in ER⁺ patients (38). In the latter analysis that examined its expression in 110 invasive ductal carcinomas, an association between *STC2* expression and longer disease-free survival was found. Here, we confirmed that *STC2* expression showed an association with longer disease-free survival (Fig. 2B). However, patients with *STC2*-positive tumors showed

Table 2. Multivariate Cox hazard analysis of the prognostic significance of *NTN4* mRNA levels and clinicopathologic variables in ER⁺ tumors (*n* = 172)

| Variable | Coefficient (95% CI) | P | Risk ratio (95% CI) |
|----------|------------------------------|---------------|-------------------------|
| LN met | 2.1538 (1.0338-3.2739) | 0.0002 | 8.6179 (2.8117-26.4141) |
| ER | -0.5269 (-1.7031-0.6492) | 0.3799 | 0.5904 (0.1821-1.9141) |
| PgR | -0.3748 (-1.3238-0.5741) | 0.4388 | 0.6874 (0.2661-1.7756) |
| Ck 14 | 0.2206 (-1.4137-1.8549) | 0.7914 | 1.2468 (0.2432-6.3911) |
| Ck 5/6 | 0.6113 (-0.9412-2.1639) | 0.4403 | 1.8428 (0.3901-8.7046) |
| Ck 17 | 0.6143 (-0.4700-1.6987) | 0.2668 | 1.8484 (0.6250-5.4669) |
| P53 | 0.7413 (-0.0772-1.5598) | 0.0759 | 2.0986 (0.9257-4.7578) |
| MIB1 | -0.0286 (-0.6211-0.5640) | 0.9248 | 0.9719 (0.5374-1.7576) |
| NTN4 | -1.3687 (-2.4730 to -0.2644) | 0.0151 | 0.2544 (0.0843-0.7677) |

NOTE: The model includes all variables that were significantly associated with overall survival on univariate analysis. Significant *P* values are shown in boldface.

Abbreviation: 95% CI, 95% confidence interval.

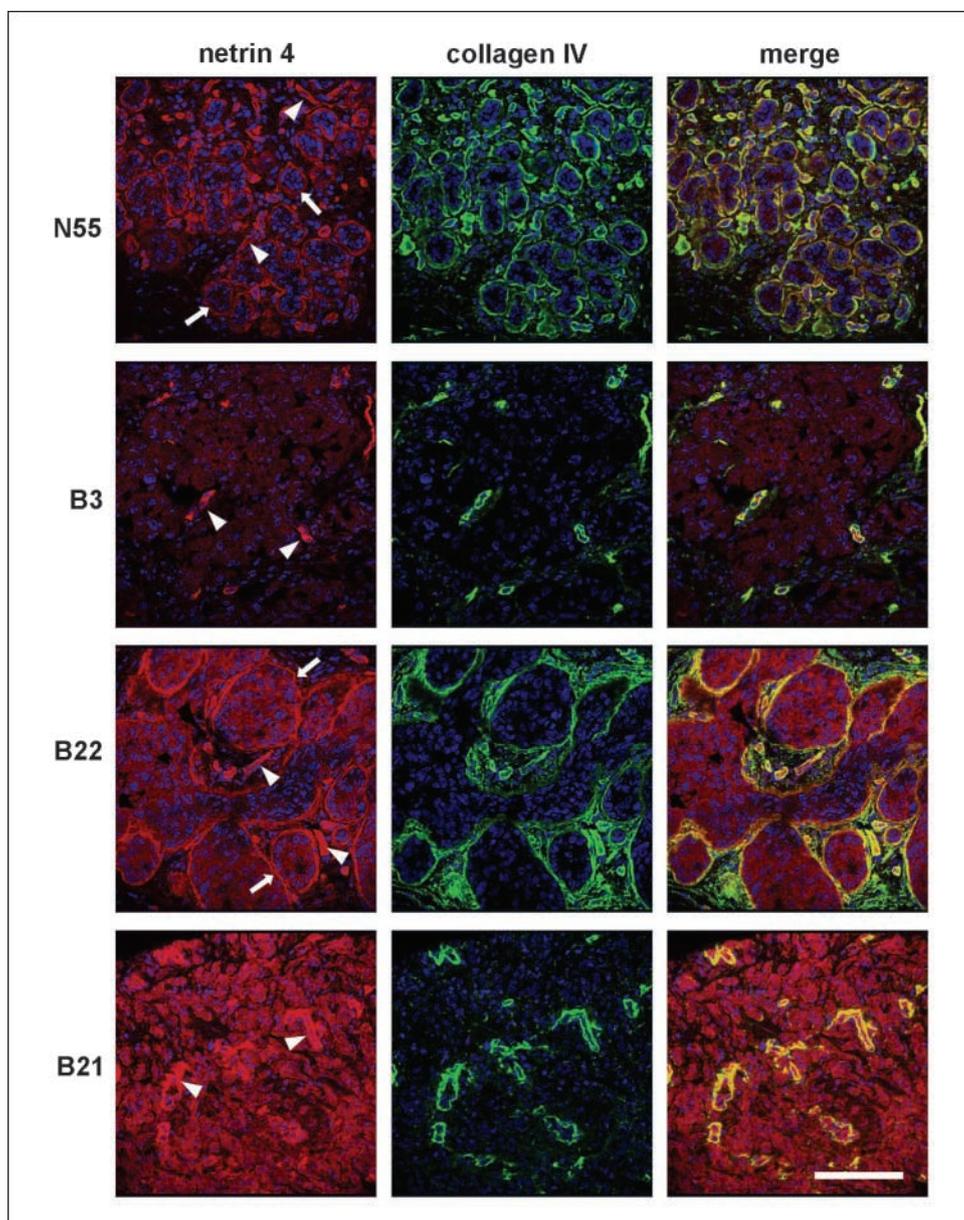


Fig. 3. Immunofluorescence staining of NTN4. Seven-micrometer cryosections of normal human breast (sample N55), ER⁻ invasive ductal carcinoma (sample B3), ER⁺ ductal carcinoma *in situ* (sample B22), and ER⁺ invasive ductal carcinoma (sample B21) were stained with NTN4 antibody and Alexa 555-conjugated anti-goat immunoglobulin followed by collagen IV antibody and Alexa 488 antimouse immunoglobulin. Nuclei were counterstained with TO-PRO-3. The accumulation of NTN4 in the basement membranes surrounding the vasculature (arrowheads) and epithelial structures (arrows) is indicated. Bar, 100 μ m.

only a trend for a longer overall survival and, consequently, further studies are required to determine the prognostic effect of *STC2* on the overall survival of breast cancer patients. Given the findings reported here, the biological activity of mammalian stanniocalcins warrants further investigation. In particular, it will be of interest to determine whether *STC2* secreted by ER⁺ tumor cells acts in a paracrine fashion to signal to the surrounding stromal cells and/or in an autocrine fashion to signal to the tumor cells themselves. Furthermore, it remains to be determined whether *STC2* expression confers a less aggressive phenotype to breast cancers or if it is merely a surrogate marker for tumors of the luminal group, which have a better outcome when compared with basal-like and HER2 cancers.

The netrins are a conserved family of secreted proteins that are highly conserved through evolution. Three netrins, netrin 1 (NTN1), netrin 3 (NTN3), and netrin 4/ β -netrin (NTN4), have been identified in mammals and six netrin receptors [deleted in

colon cancer (DCC), neogenin, and UNC5A, UNC5B, UNC5C, and UNC5D]. Both the netrins and their receptors are widely expressed and have been implicated in a range of developmental processes including axon guidance, lung branching morphogenesis, angiogenesis, and mammary gland development (39, 40). *NTN4* is expressed by a variety of cells including epithelial cells and vascular endothelial cells and, *in vivo*, *NTN4* protein is found predominantly localized to the basement membranes surrounding epithelial structures and the vasculature (13, 41). In agreement with these data, *NTN4* transcripts were preferentially detected in the normal breast epithelial cells (Fig. 1), whereas *NTN4* protein was localized predominantly to the basement membrane surrounding the lobular structures and the blood vessels (Fig. 3). The netrin receptor *DCC* was first identified as a tumor suppressor gene in colorectal cancer and subsequently shown to be deleted or inactivated in a number of other tumor types (42). More recently, *UNC5A*, *UNC5B*, and *UNC5C* have also been shown to be candidate tumor

suppressor genes (43) and, like DCC, to act as dependence receptors such that, in the absence of ligand, they promote a proapoptotic pathway, whereas in the presence of ligand, they promote cell survival. These findings have led to a model in which expression of these receptors in tumor cells that no longer have access to ligand would restrict tumor growth whereas their deletion or inactivation would allow the tumor to avoid apoptosis. To date, there are only two reports on the expression of netrins in human cancers, and it is notable that in both of these, *NTN1* expression was found to be down-regulated in tumors (44, 45). Further, in the reverse transcription-PCR analysis of 48 prostate tumors and 7 normal prostate specimens, a reduction in *NTN4*, *DCC*, and *NEO1*, as well as in *NTN1*, in the tumor samples compared with the normal controls was reported (44). In the present study, we showed that 37.5% of the breast cancers showed expression of *NTN4* and, surprisingly, that its expression was an independent predictor of improved outcome (Table 1; Fig. 2 and Table 2). These data contradict previous predictions that *NTN4* binding to its receptor would promote tumor cell growth and angiogenesis (40). Moreover, as a recent report provides evidence that the response of endothelial cells to *NTN4* is not mediated via any of the known netrin receptors (46), the mechanism by which tumor cell secretion of *NTN4* results in improved patient survival remains unclear. As a consequence, the study reported here has highlighted the need to reevaluate

the role of netrins in breast cancer. In particular, it will be important to resolve two important issues. First, it remains to be established whether the reported proangiogenic activity of *NTN4* in cultured endothelial cells *in vitro* and in an *in vivo* murine model of hind limb ischemia (46) also operates to promote breast cancer angiogenesis, and, if so, how this activity is counteracted to improve patient outcome. Second, to identify functional *NTN4* receptors in the breast and determine whether the main activities of *NTN4* are in paracrine or autocrine signaling.

In conclusion, we have surveyed the expression of a series of secreted genes identified using our signal-trap strategy (5) in a large cohort of primary invasive breast tumors from a well-defined patient series. This approach has validated the original screen by showing that these genes are indeed up-regulated in breast tumor cells. Furthermore, we have shown that *NTN4*, *TRAI1*, and *STC2* are novel prognostic makers in breast cancer and that, in particular, *NTN4* is an independent predictor of outcome in patients with early breast cancer treated with anthracycline-based chemotherapy.

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Identification of *NTN4*, *TRA1*, and *STC2* as Prognostic Markers in Breast Cancer in a Screen for Signal Sequence Encoding Proteins

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