Cancer Research

Interaction of Developmental Transcription Factor HOXC11 with Steroid Receptor Coactivator SRC-1 Mediates Resistance to Endocrine Therapy in Breast Cancer

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Abstract

Mechanisms of acquired resistance to endocrine therapy in breast cancer, a major clinical challenge, are poorly understood. We have used a mass spectrometry–based screen to identify proteins that are associated with the endocrine-resistant phenotype. In this study, we report the identification of a novel pathway of resistance to endocrine therapy involving interactions of the developmental transcription HOXC11 with the steroid receptor coactivator protein SRC-1, which is a strong predictor of reduced disease-free survival in breast cancer patients. HOXC11 and SRC-1 cooperate to regulate expression of the calcium-binding protein S100β in resistant breast cancer cells. Nuclear HOXC11 and S100β were found to strongly predict poor disease-free survival in breast cancer patients (n = 560; hazard ratios: 5.79 and 5.82, respectively; $P < 0.0001$). Elevated serum levels of S100β detected in patients also predicted reduced disease-free survival (n = 80; hazard ratio: 5.3; P = 0.004). Our findings define a biomolecular interaction network that drives an adaptive response to endocrine therapy with negative consequences for survival in breast cancer. *Cancer Res; 70(4); 1585–94. ©2010 AACR.*

Introduction

Classic pathologic parameters, such as tumor size and grade, have been invaluable in informing the clinical management of breast cancer; however, a significant number of patients with a good prognostic profile will have a tumor recurrence. Elucidation of the mechanisms of tumor adaptability and consequent markers of early recurrence is the basis of personalised medicine.

Aberrant expression of p160 steroid receptor coactivator (SRC) proteins has been associated with resistance to endocrine therapies and the development of tumor recurrence (1, 2) Unlike other oncogenes, recent studies provide evidence of a specific role for SRC-1 in the development of metastasis (3). Although previously thought to exclusively bind steroid receptors, a role for p160 coactivators in regulating nonsteroidal transcription factor activity has now been established (4–6). Indeed as distinct from other family members, SRC-1 is thought to mediate its tumorogenic effects primarily through interactions with these transcription factors. In particular SRC-1 has been shown to interact with the Ets family members Ets-2 and PEA3 to mediate the production of c-Myc and Twist (7, 8). Further investigation into SRC-1 binding partners is now timely to illuminate its role in steroid-independent breast tumor growth.

In this study we took an unbiased approach to identify new SRC-1 transcription factor hosts relevant to endocrine resistance. The homeodomain protein HOXC11 was identified as a potential transcription factor partner. Using a molecular and translational approach, we show that this is a functional partnership important in the development of endocrine resistance in breast cancer patients.

Materials and Methods

Cell lines, primary cell cultures, and treatments. Endocrine-sensitive MCF-7 [American Type Culture Collection (ATCC)] and endocrine-resistant LY2 (9) cells (kind gift from R. Clarke, Georgetown, DC) were grown as previously described (7). Primary cell cultures were derived from patient tumors and cultured for 72 h (10). Letrozole (aromatase inhibitor)–sensitive cells (MCF-7aro) were developed by stable transfection of the aromatase gene (CYP19; Invitrogen). Letrozole-resistant cells (MCF-7aro Let-R) were created by long-term treatment of aromatase-expressing MCF7 cells (MCF7aro) with letrozole (Novartis). MDA-MB231 (ATCC) were cultured as standard in DMEM, 10% fetal bovine serum, and 2 mmol/L L-glutamine. Cells were maintained in steroid

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depleted medium for 72 h before treatment with hormones [estadiol (E_2) 10^{-8} mol/L, 4-hydroxytamoxifen (4-OHT) 10^{-8} mol/L; Sigma Aldrich] over varying time periods. All cells were incubated at 37°C in 5% CO_2 in a humidified incubator. All in-house cells were authenticated and are routinely verified as endocrine resistant.

Mass spectrometry analysis. Protein bands were excised from Coomassie blue (Sigma-Aldrich)–stained gels. Analysis of the peptides produced from the tryptic digestions was performed on an Applied Biosystems 4700 matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometer using 10 mg/mL cyano-4-hydroxycinnamic acid, as the matrix and mass spectra were acquired in the reflector mode. The precursor ion masses were searched using Mascot Search software[3] incorporated in GPS Explorer v3.5 software. The UniProt SwissProt nonredundant database and human taxonomy were used for all searches. Methionine oxidation and cysteine carboxyamidomethylation were specified as variable modifications, and a maximum of one missed cleavage site was allowed.

Coimmunoprecipitation and Western blot. Protein (500 μg) was immunoprecipitated with rabbit anti-SRC-1 (SC-8995; Santa Cruz) and all of the resultant protein was subsequently blotted with either rabbit anti-SRC-1 (Santa Cruz) or chicken anti-HOXC11 (15-288-22000F; Genway Biotech). Immunoblotting for S100β was carried out using mouse anti-S100β (Ab-8330-100; Abcam). Protein from breast cancer cells was lysed, electorphoresed, and immunoblotted with either chicken anti-HOXC11 (1 μg/mL), anti-SRC-1 (1 μg/mL), or mouse anti-S100β (2 μg/mL).

PCR. Levels of HOXC11 and SRC-1 mRNA were assessed in LY2, MCF7 cells, and primary breast cancer tissue with quantitative real-time PCR (Lightcycler, Roche Laboratories) using primers for HOXC11, forward: AACACAAATCCCAGCTCGTC and reverse: AAAAACTCTCGCTCCAGTTCC, and for SRC-1 forward: TTGACAGCTTGAGTGTAAAACCA and reverse: CATCGTCATCAGTTGTTGATTTC.

Plasmids and siRNA. pDest47 HOXC11 was cloned using the Gateway System (Invitrogen). Predesigned and validated siRNA directed against HOXC11 (Qiagen) and SRC-1 (Ambion) were used in the knockdown studies.

Proliferation assay. LY2 and MCF-7 cells were plated at equal confluence. Cells were steroid depleted for 72 h; transfected with scrambled siRNA, HOXC11 siRNA, and/or SRC-1 siRNA; and then treated with either estrogen (10^{-8} mol/L) or 4-OHT (10^{-8} mol/L) for a further 72 h. Total cells were harvested and counted.

TFF1(pS2) enrichment assay. LY2 cells treated with 4-OHT or untreated were subjected to chromatin immunoprecipitation (ChIP) analysis using an SRC-1 antibody. The resultant DNA was amplified and queried at the TFF1 promoter (Genpathway).

ChIP sequencing. LY2 cells treated with 4-OHT or untreated were subjected to ChIP using an SRC-1 antibody. DNA libraries were generated from 10 ng of ChIP output DNA by adaptor ligation, gel purification, and cycles of PCR as described by Illumina. Sequencing was carried out using the Illumina/Solexa Genome Analyzer system (Illumina). The 35-nt sequence tags were mapped to the human genome using the Eland software. Tags were extended at their 3′ ends to a length of 110 bp, the average fragment length in the size selected library. For each basepair in the genome, the number of overlapping sequence reads was determined, averaged over a 10-bp window and visualized in the University of California, Santa Cruz genome browser.[4] ChIP peaks were identified using MACS software.

ChIP analysis. LY2 cells were treated with 4-OHT. ChIP was performed as previously described (7). Rabbit anti-SRC-1 (6 μg), chicken anti-HOXC11 (7 μg), mouse anti–estrogen receptor (ER) α (6 μg; Santa Cruz, F10), or H4 antibody (7 μg; Upstate; positive control) was added to the supernatant fraction and incubated overnight at 4°C with rotation. Proteins were uncross-linked, and primers were used to amplify the DNA −424 to +24 of the S100β transcriptional start site: S100β promoter specific primers, forward: TGGCAGAGGAGAGAAGCTC and reverse: TTCCTGAGCGTCCTCTTGG.

Dual-luciferase reporter assay. MCF7 and LY2 cells were transfected with pGL3 vector (1 μg; Invitrogen) or pGL3-S100β promoter (1 μg; ref. 11), pRL (100 ng), and siRNA-HOXC11 (30 nmol/L) using Lipofectamine 2000 reagent (Invitrogen). At 24 h posttransfection cells were harvested for detection of luciferase activity.

Immunolocalization and immunofluorescent colocalization. Breast cancer tissue and cells were immunostained with chicken anti-HOXC11 (3 μg/mL) or S100β (3 μg/mL) using the VectaStain Elite kit (Vector Labs) and counterstained with hematoxylin. HOXC11 was colocalized with either SRC-1 or S100β using chicken anti-HOXC11 (30 μg/mL), rabbit anti-SRC-1(10 μg/mL), or mouse anti-S100β (30 μg/mL), followed by the corresponding fluorescent-conjugated antibodies FITC anti-chicken, TRITC anti-rabbit, or TRITC anti-mouse (Molecular Probes). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole.

Patient information and construction of tissue microarray. Breast tumor samples were collected, and data were recorded as previously described (12). Data on the patients included pathologic characteristics (tumor size, grade, lymph node status, ER status, recurrence) as well as treatment with radiotherapy, chemotherapy, or tamoxifen. Detailed follow-up data (median, 6.5 y) were collected on the patients to determine disease-free and overall survival. Tissue microarray (TMA) construction was conducted as previously described (12). Immunostained TMAs were scored using the Allred scoring system (12). A nuclear score of 3 or higher was defined as positive staining. Independent observers, without knowledge of prognostic factors, scored slides.

ELISA in breast cancer patient serum. Preoperative serum levels of S100β were analysed in a cohort of breast cancer patients (n = 80) using a commercial ELISA kit (Diasorin)

[3] http://www.matrixscience.com

[4] http://genome.ucsc.edu

according to manufacturer's instructions. Cutoff was taken at 0.15 μg/L. S100β serum status was analysed in relation to patient pathologic characteristics and disease-free survival (median follow-up, 3.2 y).

Statistical analysis. STATA 10 data analysis statistical software (Stata Corp. LP) was used in the analysis. Univariate analysis was performed using Fisher's exact test for categorical variables and Wilcoxon's test for continuous variables. Multivariate analysis was carried out using Cox's proportional hazard model, using the Breslow method for ties. A P value of <0.05 was considered to be significant. Survival times between groups were compared using the Wilcoxon test adjusted for censored values.

Results

Identification of HOXC11 as a novel SRC-1 interacting protein in endocrine-sensitive and -resistant breast cancer cells. SRC-1 interacting proteins were isolated from LY2 and MCF-7 cells after treatment with 4-OHT. We applied gel separation and MALDI-TOF mass spectrometry analysis to identify transcription factors that differentially interact with SRC-1 in endocrine-resistant (LY2) compared with endocrine-sensitive (MCF7) breast cancer cells in the presence of 4-OHT (Fig. 1A). Successful pullout of SRC-1 was confirmed by immunoblot. Three bands containing potential SRC-1 interacting proteins specific to the tamoxifen-resistant cells were excised and analysed (Supplementary Table S1). The homeobox protein HOXC11 was identified as a potential interaction partner for SRC-1. The differential association of HOXC11 with SRC-1 in the resistant and sensitive cells was confirmed via coimmunoprecipitation with SRC-1 and subsequent immunoblotting for HOXC11 (Fig. 1A). SRC-1/HOXC11 interactions were observed in the endocrine-resistant cells. Interactions were enhanced in cells treated with 4-OHT and conversely reduced in the presence of estrogen. In contrast, estrogen drove minor increases in HOXC11/SRC-1 interactions in endocrine-sensitive MCF7 cells, which were due in part to increased SRC-1 expression (Fig. 1A). These interactions were inhibited by 4-OHT (Fig. 1A). We observed higher basal levels of HOXC11 mRNA and protein in LY2 cells than in MCF-7 cells (Fig. 1B). Nuclear protein levels of both SRC-1 and HOXC11 were also higher in endocrine-resistant (tamoxifen and aromatase inhibitor; LY2 and MCF-7aro Let-R) and endocrine-insensitive (MDA-MB231) breast cancer cell lines compared with endocrine-sensitive (MCF-7 and MCF-7aro) cells (Fig. 1B). Furthermore strong associations were observed between SRC-1 and HOXC11 mRNA levels in breast cancer patients ($P < 0.001$; Fig. 1C). In endocrine-resistant cells, treatment with 4-OHT increased nuclear localization as well as protein and mRNA expression of the transcription factor HOXC11 (Fig. 1D).

Associations between HOXC11 and SRC-1 in breast cancer. Relative levels of *ex vivo* HOXC11/SRC-1 coassociations were assessed in primary cultures derived from breast cancer patient tumors (Fig. 2A and B). In line with the coimmunoprecipitation studies, estrogen induced a modest increase in HOXC11/SRC-1 association, which was reversed by 4-OHT. A similar pattern was observed in endocrine-sensitive MCF7 cells (Supplementary Fig. S1A). In contrast, in LY2 cells, 4-OHT alone and in combination with estrogen increased nuclear expression and colocalization of HOXC11 and SRC-1, whereas treatment with estrogen alone depleted HOXC11 (Supplementary Fig. S1A). At a functional level knockdown of SRC-1 was found to resensitize endocrine-resistant cells to the inhibitory effects of 4-OHT (Supplementary Fig. S1B and C; ref. 2); moreover, combined knockdown of SRC-1 and HOXC11 had a synergistically negative effect on cell proliferation, suggesting that HOXC11 may require SRC-1 to induce endocrine resistance (Fig. 2C).

SRC-1 and HOXC11 cooperate to produce S100β in endocrine-resistant cells. After confirmation that 4-OHT can inappropriately activate ER target gene activity in LY2 cells (Supplementary Fig. 2A), we undertook bioinformatic analysis to uncover potential HOXC11 response elements in the human genome. Putative homeodomain binding sites, along with ER elements, were found in the promoter of the calcium-binding protein S100β (Fig. 3A). As SRC-1 does not bind directly to the DNA, we took a corresponding ChIP-seq approach to identify potential SRC-1–DNA binding sites relevant to the resistant phenotype. The total number of SRC-1–related peaks found between tamoxifen-treated and untreated samples was 3,785 (with a max false discovery rate of 5%), 44.6% of which contained an ERE binding site (both forward and reverse strand). No significant peaks were identified at the S100β gene; however, a greater number of reads mapped to the S100β promoter in LY2 cells after 4-OHT treatment compared with untreated controls (Fig. 3A). We therefore examined ERα, SRC-1, and HOXC11 binding at the S100β promoter. ChIP analysis in the endocrine-resistant cell line at 45 minutes after treatment with 4-OHT revealed SRC-1 and HOXC11 recruitment to the S100β promoter along with ERα (Fig. 3B). By 2 hours after treatment with 4-OHT SRC-1 was rolling off the promoter whereas HOXC11 continued to engage. No recruitment to S100β nonpromoter regions of either transcription factors or the coactivator protein was found (Supplementary Fig. S2B). Furthermore, in endocrine-resistant cells, knockdown of HOXC11 significantly reduced S100β promoter activity, whereas in the MCF-7 cells reduction of HOXC11 had no effect (Fig. 3C). Exogenous expression of HOXC11 in LY2 cells resulted in upregulation of S100β protein expression, which was abrogated by siRNA-mediated knockdown of SRC-1 (Fig. 3D). Furthermore, overexpression of SRC-1 alone in combination with HOXC11 increased S100β (Fig. 3D). In endocrine-resistant cells, HOXC11 colocalized with S100β and the calcium binding protein was upregulated in the presence of tamoxifen (Supplementary Fig. S2C).

HOXC11 predicts poor disease-free survival in breast cancer patients. To determine the significance of HOXC11 and S100β in mediating disease progression in breast cancer, we analysed 560 patients for expression of HOXC11 and S100β and compared this to classic clinicopathologic parameters. Significant associations were found between SRC-1 and HOXC11 ($P < 0.0001$) and between each of these proteins and their putative target S100β ($P < 0.0001$ and $P < 0.0001$,

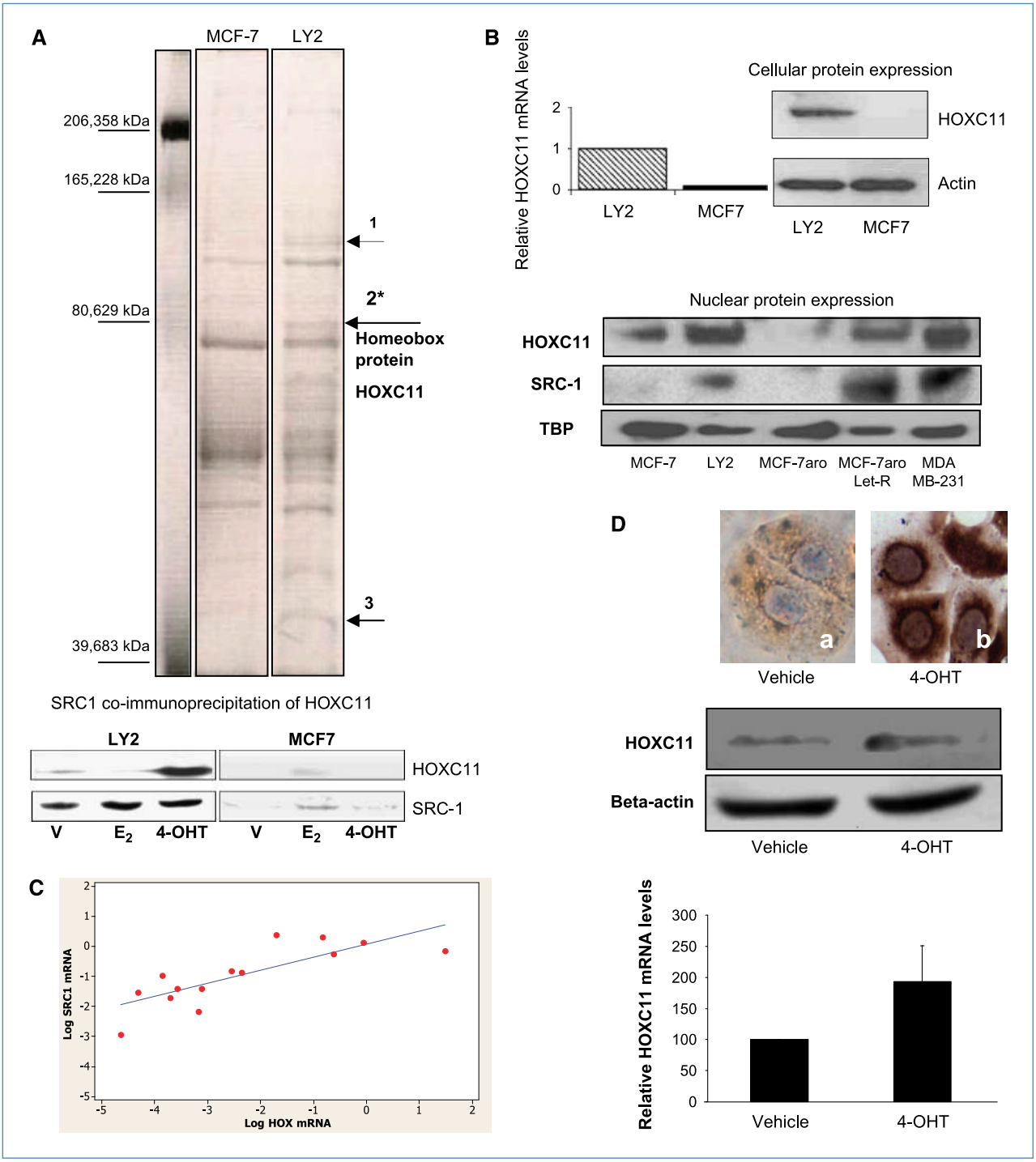

Figure 1. Identification of HOXC11 as a novel SRC-1 interacting protein in endocrine-sensitive and -resistant breast cancer cells. A, endocrine-sensitive MCF-7 and endocrine-resistant LY2 cells were treated with 4-OHT and were immunoprecipitated with anti-SRC-1. SRC-1 interacting proteins were separated on a one-dimensional gel, and resultant lanes were analyzed using MALDI-TOF mass spectrometry. The transcription factor HOXC11 was identified as a possible interacting partner for SRC-1. SRC-1/HOXC11 interactions were confirmed by coimmunoprecipitation. SRC-1 was immunoprecipitated from LY2 and MCF-7 cells treated with estrogen (E_2) and 4-OHT and subsequently immunoblotted for HOXC11. Blot shown is representative of three separate experiments. B, mRNA and cellular protein levels of HOXC11 in LY2 and MCF-7 cells ($n = 3$). Nuclear protein expression of HOXC11, SRC-1, and TBP (loading control) in tamoxifen-sensitive (MCF-7) and -resistant (LY2), letrozole-sensitive (MCF-7aro) and -resistant (MCF-7aro-LetR), and endocrine-insensitive (MDA-MB231) cancer cell lines. C, levels of HOXC11 and SRC-1 mRNA (expressed as log values) in individual patient tumors significantly associate with each other ($n = 14$, $R^2 = 0.63$, $P < 0.001$). D, immunolocalization, protein and mRNA expression of HOXC11 in LY2 cells after treatment with 4-OHT and estrogen (24 h; $n = 3$).

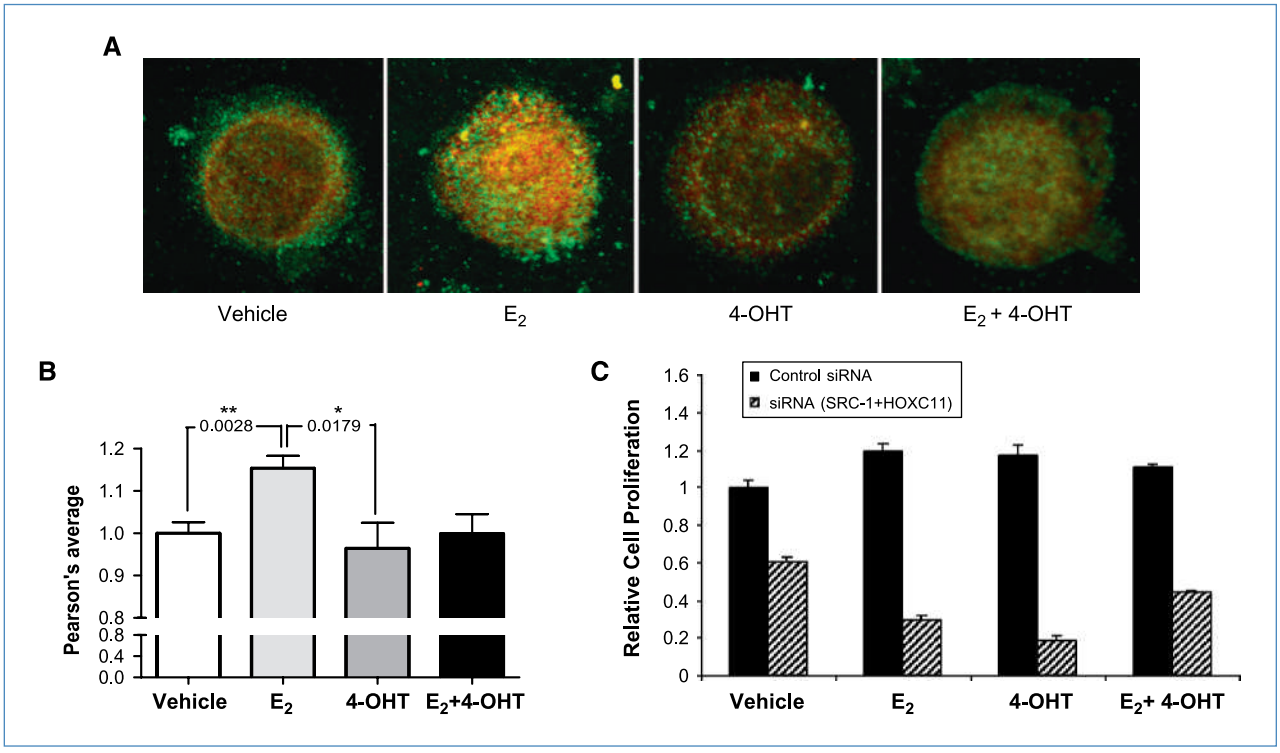

Figure 2. Associations between HOXC11 and SRC-1 in breast cancer. A, colocalization of SRC-1 (FITC) and HOXC11 (TRITC; X100) in primary culture derived from breast cancer patients after treatment with E_2 and 4-OHT for 45 min. Image is representative of five separate patient tumors. B, quantitative coassociation analysis of SRC-1 with HOXC11. Histogram represents normalized Pearson's correlation coefficient between SRC-1 and HOXC11 (mean ± SEM). Primary cell cultures (n = 5/treatment) were analyzed under a Zeiss LSM 510 META confocal fluorescent microscope. C, functional role of HOXC11 and SRC-1 in breast cancer cells. LY2 cells were used to examine the effect of HOXC11 and SRC-1 on cell proliferation in response to E2 and 4-OHT (72 h). 4-OHT induces cell proliferation in endocrine-resistant LY2 cells. Combined knockdown of HOXC11 and SRC-1 significantly reduces cell proliferation. Scrambled siRNA was used as a control; results are mean ± SEM (n = 3).

respectively). HOXC11 was found to be expressed in both the nucleus and cytoplasm of breast tumor epithelial cells. Kaplan-Meier estimates of disease-free survival indicated that nuclear expression of HOXC11 is a strong predictor of poor survival (hazard ratio: 5.79, $P < 0.0001$; Fig. 4A). To explore the effect of SRC-1 on HOXC11-mediated tumor progression, we examined the coexpression of these proteins in relation to tumor recurrence. Coexpression of HOXC11 and SRC-1 associated with reduced disease-free survival in patients treated with 4-OHT compared with patients who expressed HOXC11 alone (Fig. 4B). These associations were not significant in patients who received no endocrine therapy.

S100β tumor tissue expression and blood serum levels predict poor disease-free survival in breast cancer patients. S100β was localized predominantly to the cytoplasm of the tumor epithelial cells, with some nuclear and extracellular expression also observed. Tumor tissue expression of S100β was associated with reduced time to disease recurrence (hazard ratio:5.82, $P < 0.0001$; Fig. 5A). Both HOXC11 and S100β are stronger predictors of disease-free survival than any of the classic parameters of disease recurrence, including tumor size, nodal, and HER2 status (hazard ratios: 1.65, 1.82, and 1.50, respectively). HOXC11 and S100β were found to associate with local and distant metastasis but not the classic pathologic parameters (Table 1).

Based on strong associations between S100β tissue expression and reduced disease-free survival in breast cancer, we examined preoperative levels of serum S100β in a cohort of primary breast cancer patients (n = 80). Elevated levels of serum S100β were detected in a subset of breast cancer patients compared with the breast cancer patient population and with age matched controls (Supplementary Fig. S3). Moreover, Kaplan-Meier estimates of disease-free survival reveal that elevated serum levels of S100β strongly predict poor survival (hazard ratio: 5.3, P = 0.004; Fig. 5B). In line with observations made for tissue expression of HOXC11 and S100β, serum levels of S100β associated with disease recurrence (P = 0.017) but not tumor grade or nodal status (Table 1).

Discussion

Endocrine therapies are the treatment of choice for ER-positive tumors, and although most patients initially respond to treatment, many eventually relapse. Molecular and clinical studies have confirmed SRC-1 as a key mediator of disease recurrence. Although initially described as a nuclear receptor

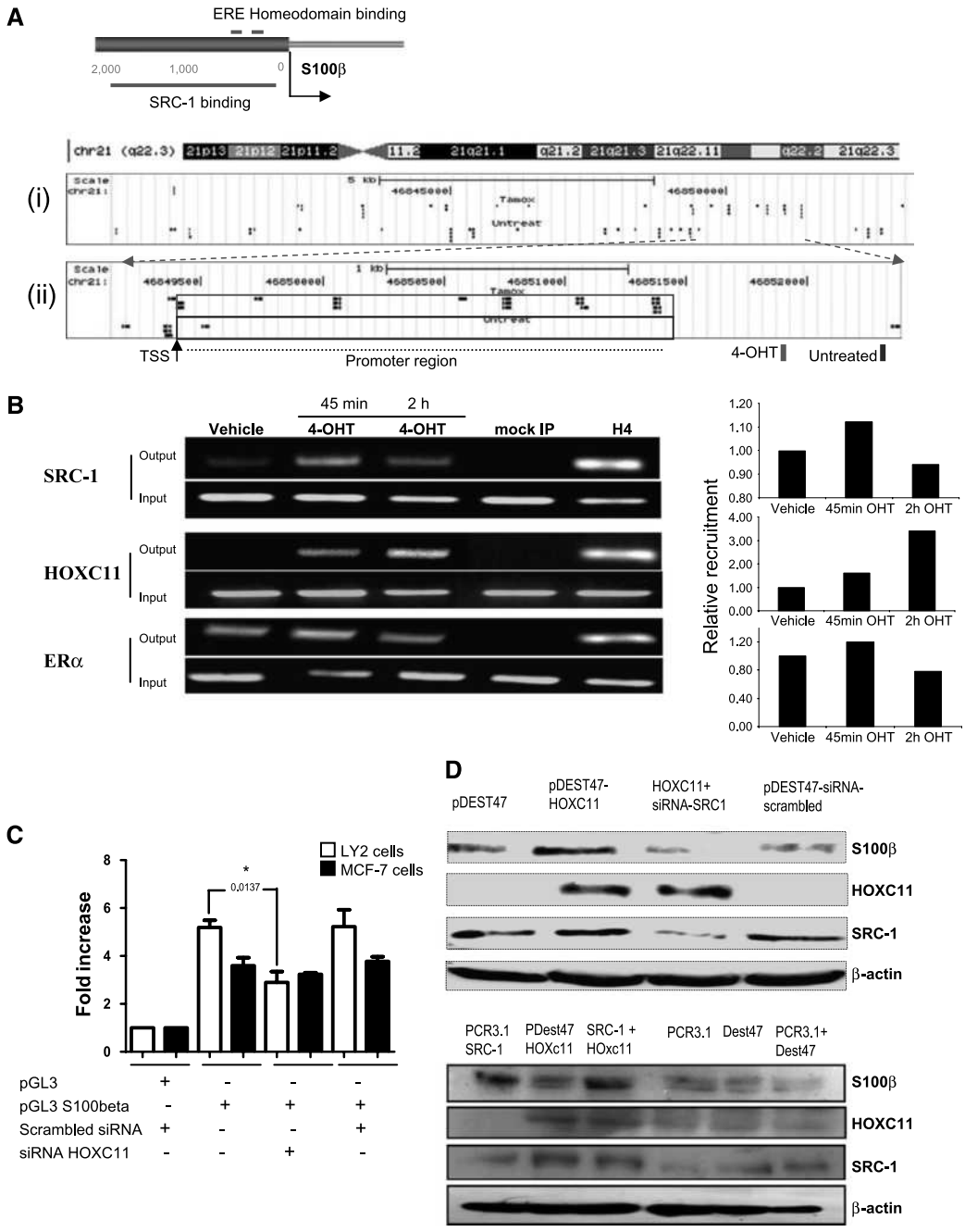

Figure 3. SRC-1 and HOXC11 cooperate to produce S100β in endocrine-resistant cells. A, cartoon representing putative HOXC11 and ER DNA interactions in the promoter region of S100β (geneID:6285) were located using Genomatix MatInspector (http://www.genomatix.de). Predicted SRC-1 binding regions on the S100β promoter were identified by ChIP-seq. ChIP-seq analysis of SRC-1 binding regions on the human genome revealed a greater number of reads in the promoter region of S100β with 4-OHT treatment compared with the untreated sample. Image of the S100β gene in UCSC genome browser (i), with an enhanced image of the promoter region (ii). Detected reads spanned between 257 and 1,936 upstream of the start-site on the reverse strand, highlighted with a red box (4-OHT sample) and a blue box (untreated sample). B, ChIP of SRC-1, HOXC11, and ERα recruitment to the S100β promoter region in LY2 cells after treatment with 4-OHT. Results are representative of those obtained from three separate experiments. Absorbance readings of recruited proteins after treatment relative to vehicle are expressed as mean (n = 3). C, LY2 and MCF-7 cells were transfected with pGL3 S100β promoter and the internal control vector pRL in combination with siRNA HOXC11 or scrambled siRNA. Luciferase was measured using the dual-reporter system and normalized to *Renilla*. Results are expressed as mean ± SEM (n = 3). D, overexpression of HOXC11 in endocrine-resistant LY2 cells (pDEST47 HOXC11) upregulated protein expression of the putative target gene S100β compared with control (pDEST47). Overexpression of SRC-1 (PCR3.1 SRC-1) and HOXC11 in LY2 cells upregulated S100β protein expression. Blots are representative of three separate experiments.

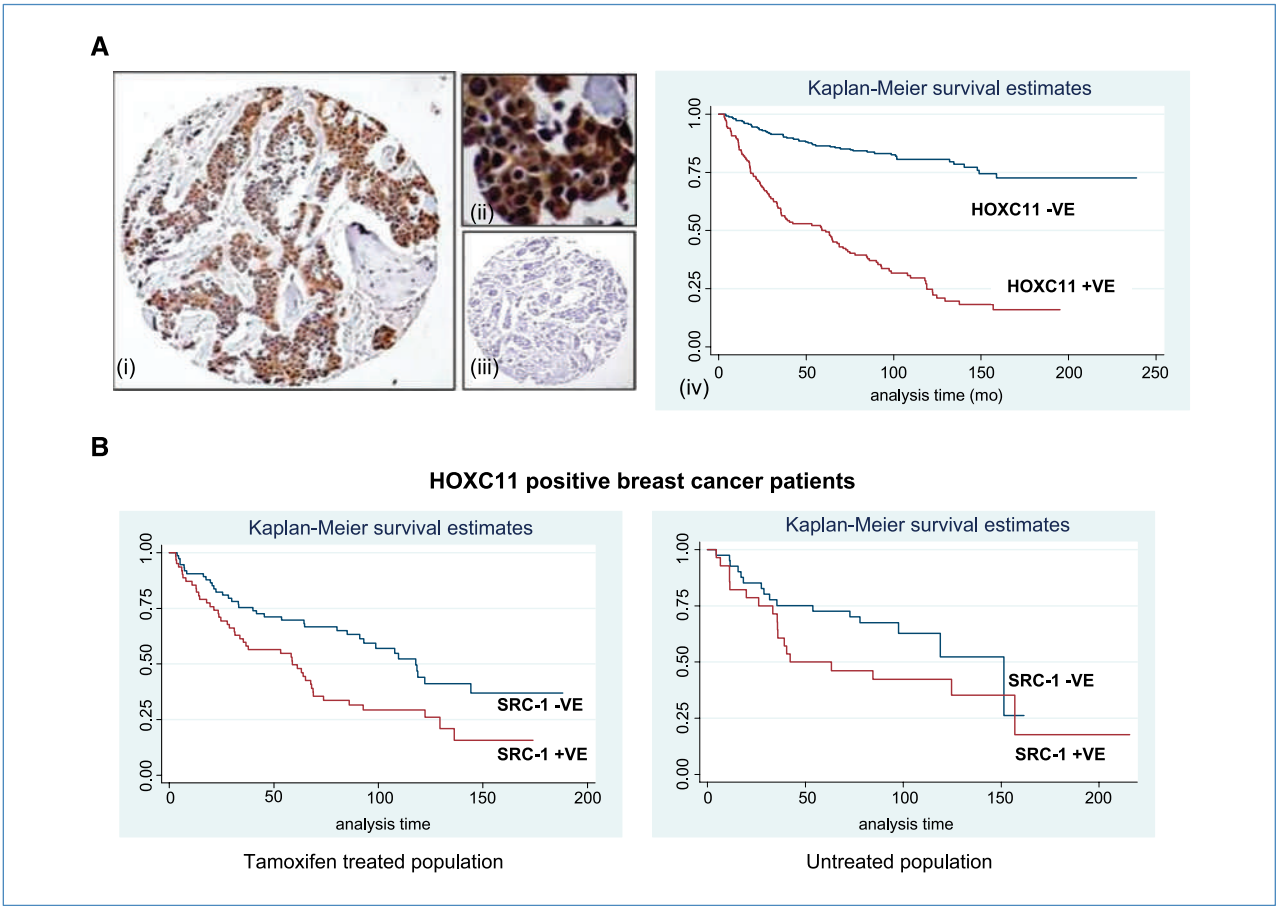

Figure 4. HOXC11 predicts poor disease-free survival in breast cancer patients. A, immunolocalization of HOXC11 in breast cancer patient TMA-positive tissue sections (100×, i and 200×, ii), TMA-negative tissue sections (iii), and Kaplan-Meier estimates of disease-free survival (iv) according to HOXC11 in primary breast cancer patients (n = 560). B, disease-free survival according to SRC-1 in HOXC11-positive patients. In tamoxifen-treated patients, coexpression of HOXC11 and SRC-1 significantly increased the rate of recurrence compared with patients who expressed HOXC11 but not SRC-1 (P = 0.0038). Significant associations were not observed in patients who received no endocrine therapy (P = 0.066).

coactivator protein, SRC-1 interactions with other transcription factors, in particular those running downstream of an activated mitogen-activated protein kinase pathway, have been implicated in poor response to treatment. These transcription factor interactions may represent one of the consequences of increased growth factor pathway cross talk described in endocrine resistance. Work from our group and others has reported functional interactions between SRC-1 and the Ets family of the transcription factors Ets-2 and PEA3 and that this relationship is important in tumor progression and the development of metastasis (7, 8). In this study we used proteomic discovery tools to uncover new SRC-1 signaling networks. We describe an interaction between SRC-1 and the homeobox protein HOXC11, which is central to the resistant phenotype in breast cancer. S100β was identified as a target of HOXC11/SRC-1 interaction, and serum levels of this protein were found to provide an accurate marker of early-disease recurrence.

The homeobox protein HOXC11 differentially interacts with SRC-1 in the endocrine-resistant versus the endocrine-sensitive phenotype. Homeobox genes encode transcriptional regulators implicated in normal organ development, but recent evidence indicates that the HOX family is upregulated in prostate cancer and suppresses androgen receptor signaling (13). Here we found higher basal levels of both SRC-1 and HOXC11 in endocrine-resistant compared with endocrine-sensitive breast cancer cells and observed strong correlations between transcript levels of the coactivator and transcription factor in breast cancer patients. Ma and colleagues found that HOXB13 in MCF10A cells potentiates epidermal growth factor–induced cell migration and invasion (14). These studies indicate that HOX proteins may participate in functional cross talk between the endocrine and growth factor pathways. Recently, using a combined computational and experimental approach, Dudek and colleagues showed that HOX proteins are involved in mediating agonist effects of 4-OHT seen in the presence of elevated cyclic AMP (15). In support of these observations, we found that treatment of resistant cells with 4-OHT increased nuclear localization and expression of HOXC11.

Ex vivo associations between coactivators and transcription factors provide evidence of disease progression in breast cancer patients (2). Steroid-dependent functional interactions between SRC-1 and HOXC11 were detected in primary cell cultures derived from breast cancer patient tumors. In endocrine-resistant cells treatment with 4-OHT alone and in combination with estrogen increased nuclear expression and colocalization of HOXC11 and SRC-1. Furthermore, at a functional level, combined knockdown of the coactivator and the transcription factor synergistically inhibited endocrine-resistant cell proliferation. These data support the concept that SRC-1 and HOXC11 cooperate to mediate resistance to therapy.

Direct HOX target genes remain elusive (16). Putative homeodomain binding sites, along with EREs, were identified in the promoter of the calcium-binding protein S100β. In line with this, forced expression of HOXC11 in neuronal cells was recently reported to induce expression of S100β protein (11). Complementary SRC-1 ChIP-seq analysis revealed that treatment with 4-OHT increased the number of reads mapping to the S100β promoter in endocrine-resistant cells. S100β is a member of the S100 family, it can act as a stimulator of proliferation and migration and is an inhibitor of apoptosis and differentiation (reviewed in ref. 17). As a dimer it binds to and activates STK38/NDR1, a protein kinase involved in cell proliferation and migration (17, 18) and can interact with p53 protein to inhibit tumor suppressor activity (19). ChIP analysis confirmed SRC-1 and HOXC11 recruitment to the S100β promoter. Luciferase activity assays and overexpression studies showed that the coactivator and transcription factor could cooperate to regulate S100β. These data provide evidence for a functional interaction between SRC-1 and the developmental protein HOXC11 and establish S100β as a target gene of HOXC11/SRC-1 interactions in endocrine-resistant breast cancer.

Molecular and clinical studies have confirmed SRC-1 as a significant biomarker of poor disease-free survival (2, 3). In a large, well-defined breast cancer cohort, strong associations were observed between SRC-1 and HOXC11 and between each of these proteins and their putative target gene

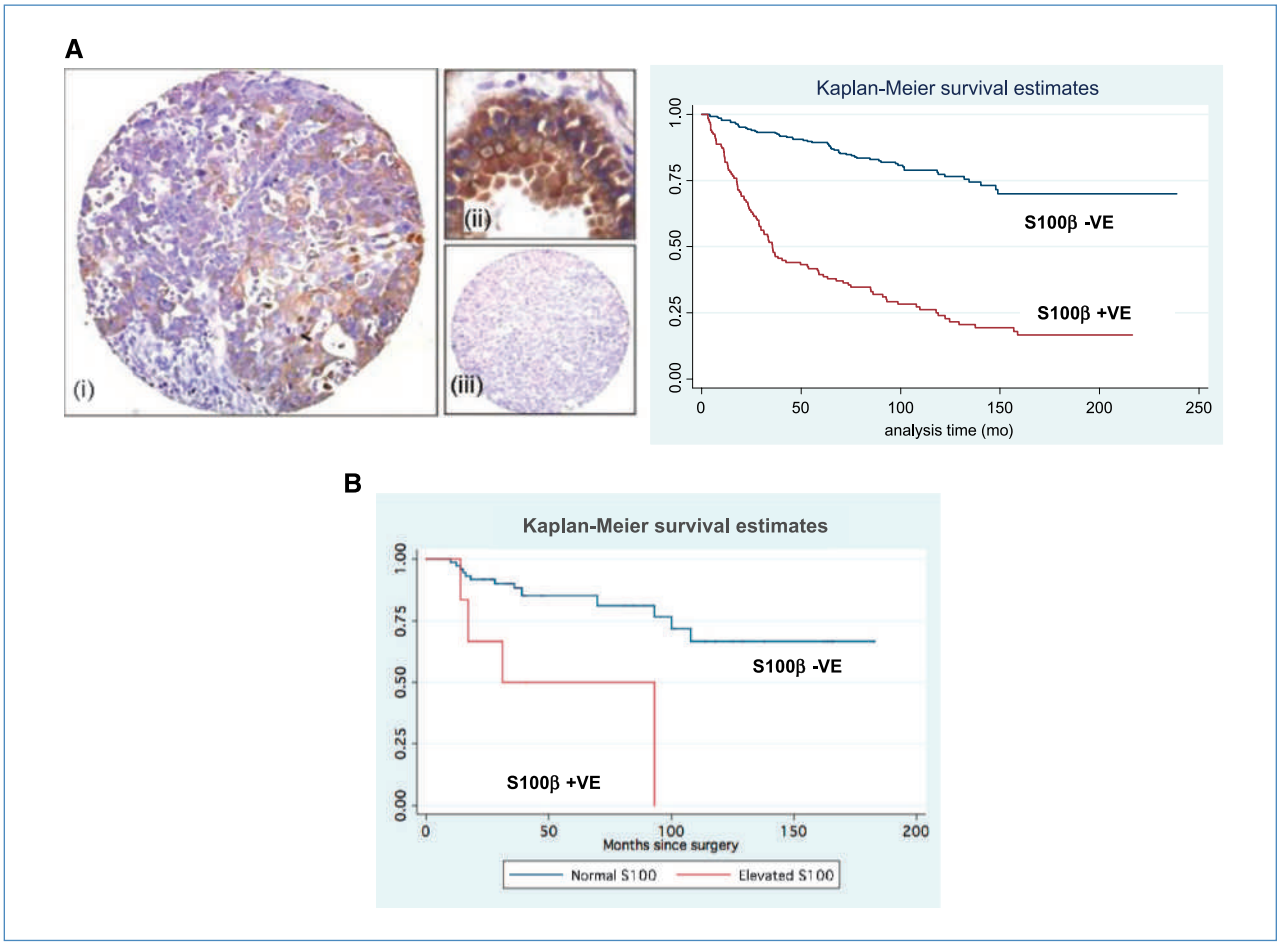

Figure 5. S100β tumor tissue expression and blood serum levels predict poor disease-free survival in breast cancer patients. A, immunolocalization of S100β in breast cancer patient TMA-positive tissue sections (100×, i and 200×, ii), TMA-negative tissue sections (iii), and Kaplan-Meier estimates of disease-free survival according to S100β in primary breast cancer patients ($n = 560$). B, serum preoperative S100β protein levels were measured in breast cancer patients with corresponding full clinical database including survival data (median follow up, 3.2 y). S100β serum levels significantly associate with reduced disease-free survival. Kaplan-Meier estimates of disease-free survival in breast cancer patients according to elevated S100β levels (>0.15 μg/L; $n = 80$).

Table 1. Associations of markers HOXC11 and S100β in breast cancer patient TMA and in patient blood with clinicopathologic variables and SRC-1 using Fisher's exact test

	Patient TMA					Patient blood samples		
Patient population		**HOXC11**		**S100β**			**S100β**	
***n* = 560**		***n* = 159**		***n* = 134**		***n* = 80**	***n* = 6**	
	%	%	*P*	%	*P*	%	%	*P*
Nodal status								
+ve	50	38	0.201	33	0.216	54	6	0.209
–ve	50	32		27		46	1	
HER2								
+ve	22	42	0.412	42	0.087	17.5	4	0.063
–ve	78	36		31		82.5	4	
Recurrence								
+ve	42	65	<0.001	59	<0.001	21	5	0.017
–ve	58	17		12		79	2.5	
Local metastasis								
+ve	18	64	<0.001	60	<0.001	4	1	0.211
–ve	82	31		26		96	6	
Distant metastasis								
+ve	30	70	<0.001	61	<0.001	17.5	4	0.063
–ve	70	22		19		82.5	4	
ER								
+ve	66	36	0.917	28	0.925	77.5	6	1.00
–ve	34	36		36		22.5	1	
Grades 1 and 2	52	32	0.314	24	0.058	56	1	0.083
Grade 3	48	23		34		44	6	
SRC-1								
+ve	36	54	<0.001	46	<0.001	-	-	-
–ve	64	27		24				

NOTE: Positive expression of variables (top row) is expressed as a percentage of patients in individual clinical classifications (1st column).

S100β. Although aberrant expression of HOX genes has previously been related to the development of breast cancer, no associations between HOXC11 and tumor progression in patients has been reported (20, 21). In this study HOXC11 was found to be a strong predictor of poor survival; moreover, coexpression of HOXC11 and SRC-1 associated with reduced disease-free survival in patients treated with 4-OHT compared with patients who expressed HOXC11 alone. These data provide robust evidence of a functional role for SRC-1 and HOXC11 in mediating clinical resistance to endocrine treatment. Tumor tissue expression of S100β also associated with reduced time to disease recurrence. Of significance, both HOXC11 and S100β were found to be stronger predictors of disease-free survival than any of the classic parameters of disease recurrence. These clinical *ex vivo* data firmly support our molecular observations that SRC-1 can cooperate with HOXC11 to regulate S100β and drive endocrine-resistant breast cancer.

S100β protein is readily detected in the serum of melanoma patients (22, 23). Although no consensus exists on its implementation in the routine clinical setting, recent meta analysis suggests that serum S100β detection has clinical value as an independent prognostic marker in patients with melanoma (24). In this study preoperative serum levels of S100β in breast cancer patients strongly predicted poor survival. Although a role for S100β as an effector of resistance has not been established, these clinical studies identify it as a powerful serum marker of early disease recurrence, which can have a profound effect on patient care.

The molecular interactions linking developmental biology and tumor progression present a dynamic mechanistic context for the ability of a tumor to adapt to its environment and evade targeted therapies. With the power to shape tissue morphogenesis, the HOX family of proteins is central to biological diversification. Establishing communications between steroid and developmental transcription factor pathways defines a network which may in part explain the phenomenon of endocrine tumor adaptability. Despite recent advances in targeted therapy, tumor resistance remains a significant problem. The identification of patients who require combined therapies and rigorous follow-up is the cornerstone of personalised medicine. In this translational study we have described a new transcriptional network linking the steroid and developmental pathways and, in doing so, have identified HOXC11 and S100β as

two new biomarkers of disease progression with great clinical potential.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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