Expression and Signaling Activity of Wnt-5a/Discoidin Domain Receptor-1 and Syk Plays Distinct but Decisive Roles in Breast Cancer Patient Survival

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ABSTRACT

Purpose: **The loss of Wnt-5a, a G-protein-coupled receptor ligand, or Syk, an intracellular kinase, has in separate studies been associated with poor prognosis of breast cancer patients. Both proteins are involved in cell adhesion, a key event in epithelial cancer metastasis. Here, we have investigated whether Syk is part of the Wnt-5a/discoidin domain receptor-1 (DDR1) signaling pathway and if a signaling interaction of these proteins is important for breast cancer–specific survival.**

Experimental Design: **The signaling interactions between Wnt-5a/DDR1 and Syk were addressed in mammary cell lines. Their mRNA and protein levels and the respective clinical correlates were investigated in 94 cases of primary breast cancer.**

Results: **The expression of Wnt-5a and Syk correlated in four of five tumor cell lines. However, despite a constitutive association between Syk and the Wnt-5a-dependent adhesion receptor DDR1, we found no evidence of a Wnt-5a/DDR1-mediated activation of Syk. Instead, β_1 integrins initiate the adhesion-induced activation of Syk. In tumors from breast cancer patients, the protein expression of Wnt-5a and Syk were differently regulated at the translational and transcriptional level, respectively. Analysis of breast cancer–specific survival revealed that the presence of Wnt-5a and Syk in primary tumors has good predictive value for a favorable outcome. Intriguingly, a simultaneous loss of both proteins did not reduce survival more than loss of either.**

Conclusions: **Despite the difference in regulation of Wnt-5a and Syk protein expression and their lack of signaling interaction, our clinical data indicate that a favorable prognosis in breast cancer requires the expression and signaling activity of both.**

INTRODUCTION

Wnt-5a belongs to the Wnt family of secreted, cysteine-rich, 38- to 45-kDa proteins that participate in development and tumorigenesis via autocrine or paracrine routes (1, 2). The Wnt proteins relate to cell surfaces and extracellular matrix and bind to members of the Frizzled family of G-protein-coupled receptors (3). Several Wnt proteins have a distinct function in carcinogenesis, and based on their ability to transform mouse mammary epithelial cells, they can be divided into three different classes. Wnt-1, Wnt-3a, and Wnt-7a have the greatest transforming capacity; Wnt-2, Wnt-5b, and Wnt-7b have an intermediate transforming capacity; and Wnt-4, Wnt-5a, and Wnt-6 are nontransforming (4).

We found recently that low-level protein expression of Wnt-5a in primary invasive breast carcinomas is associated with higher histologic grade (poor differentiation) and a shortened recurrence-free interval due to a more rapid development of distant metastases (5). These findings underline the clinical relevance of the expression of this Wnt protein. The only presently known explanation for how the presence of Wnt-5a can reduce the metastatic capacity of invasive breast cancer is its ability to enable collagen-induced activation of the discoidin domain receptor-1 (DDR1) receptor resulting in increased adhesion and consequently decreased invasive potential of breast cancer (6). In an investigation similar to ours but done on human melanoma cells, Wnt-5a was also shown to increase cell adhesion to the extracellular matrix (7). However, Wnt-5a expression has opposite effects in breast cancer and melanoma cells. In breast epithelial cells that are normally firmly adherent, a decreased expression of Wnt-5a and thus a decreased adhesion will increase their motility, whereas, in the more loosely adherent cells, an elevated expression of Wnt-5a and increased adhesion will increase their motility and thus the metastatic potential of these tumor cells.

The DDR1 tyrosine kinase receptor affects cell adhesion by binding native collagen, resulting in its activation. Recently, we found DDR1 to be an important target of noncanonical Wnt-5a signaling in human mammary cells (6). Additionally, we

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discovered that Src tyrosine kinase activity is an essential signal in enabling the effects of Wnt-5a on collagen-induced activation of DDR1 and adhesion of mammary cells to collagen (8).

Syk is a non–receptor tyrosine kinase, widely expressed in hematopoietic cells (9), that is potentially involved in the Wnt-5a and/or DDR1 signaling pathway as suggested by the fact that its activation is normally dependent on Src activity (10) and its role as a modifier of the metastatic behavior of breast cancer cells (11). We have observed previously that loss of Wnt-5a is related to the metastatic potential of breast carcinomas, which coincides with the finding by others, that loss of Syk expression is associated with increased metastatic potential of breast tumors in both experimental (11) and clinical (12) studies. The down-regulation of Syk seems to occur at the transcriptional level as shown by loss of Syk mRNA in malignant tissue (12–14). Furthermore, Coopman et al. (14) have observed that transfection of wild-type Syk into a Syk-negative breast cancer cell line, enabling adhesion-induced activation of this kinase, inhibited their capacity to form metastases in a mouse model.

In the present study, we examined the expression patterns and potential signaling interactions among Wnt-5a, Syk, and DDR1 in human mammary cell lines and their possible relationship as prognostic factors in invasive breast carcinomas.

MATERIALS AND METHODS

Cell Culture

We used the HB2 mammary epithelial cell line (8, 15) and the mammary carcinoma cells MCF-7, MDA-MB-468, MDA-MB-231, MDA-MB-435S, and T47D supplied by the American Tissue Type Collection (Manassas, VA).

Antibodies

Polyclonal rabbit IgG raised against Wnt-5a was developed against a Wnt-5a peptide (17 amino acids) that only exhibited 65% homology with the corresponding sequence in Wnt-5b. This makes it unlikely that the Wnt-5a antisera cross-react with Wnt-5b. Furthermore, the antiserum detects a protein band B with a slightly higher molecular weight than the endogenous Wnt-5a protein band in HB2 cells transfected and overexpressing hemagglutinin-tagged Wnt-5a and this protein band is also recognized by an anti-hemagglutinin antibody (6). Even more importantly, lysates from HB2 cells transfected with antisense Wnt-5a revealed no endogenous immunoreactivity against Wnt-5a when analyzed with our Wnt-5a antisera (6). The polyclonal rabbit anti-DDR1 and anti-Syk antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Adhesion

Plates were coated with collagen I (10 μg/mL, Sigma-Aldrich, St. Louis, MO). The indicated cells were allowed to adhere to the uncoated or collagen-coated plates for 90 minutes at 37°C as described previously (8). When appropriate, cells were kept in suspension and pretreated with pertussis toxin (Centre for Applied Microbiology and Research, Salisbury, United Kingdom; 500 nmol/L, 2 hours) or an anti-β_1 integrin monoclonal antibody P5D2 (Chemicon, Temecula, CA; used at a final concentration of 3.6 μL/mL for 45 minutes). Controls were incubated with vehicle alone. In Fig. 1, cells were lysed directly after detachment.

Immunoprecipitation and Western Blotting

The cells were lysed and samples prepared as described previously (8). The precleared lysates were subsequently incubated at 4°C for 1 hour with 3 μg of anti-DDR1 or anti-Syk antibody. The antibody-antigen complexes formed were collected and subjected to electrophoresis on a 10% or 12% SDS-PAGE, after which the separated proteins were electrotransferred to polyvinylidene difluoride membranes. The membranes were blocked and incubated with the indicated antibodies, and the protein bands were visualized by enhanced chemiluminescence (8). All reagents used for immunoprecipitation and Western blotting were from Sigma-Aldrich or Amersham Pharmacia Biotech (Little Chalfont, United Kingdom).

Tumor Samples

Samples were collected from primary tumors that had been removed from 94 consecutive patients with invasive breast carcinoma. The local ethical committee at Umeå University has approved the part of this study that relates to the analysis of the collected clinical material from breast cancer patients. Of the 94 tumors, 85 were ductal, 4 were mucinous, 2 were medullar, and 3 were lobular. According to the classification system of the International Union against Cancer, 23 patients had stage I, 56 had patients stage II, 1 patient had stage III, and 6 patients had stage IV breast carcinomas. In 8 patients, the stage of the disease was not known due to a lack of data on tumor size or axillary node status. None of the patients had received radiation treatment or chemotherapy before surgery. All tumors were attended to immediately after surgery, and at least two pieces of each tumor were snap frozen in liquid nitrogen and stored at −80°C. Protein extracts were prepared as described elsewhere (16). In parallel, adjacent tissue samples were fixed in formalin, embedded in paraffin, and used for routine morphologic examination (grading and immunostaining) and construction of

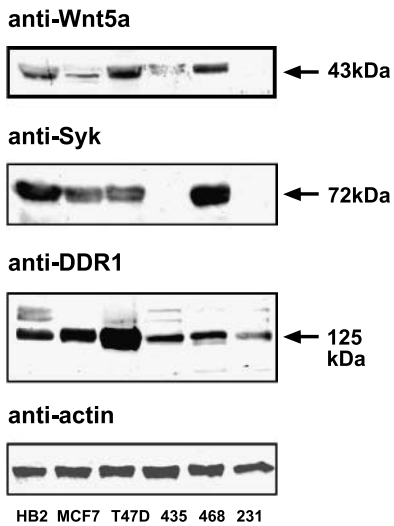

Fig. 1 Western blot analysis of expression of Wnt-5a, Syk, and DDR1 in one noncancer human breast epithelial cell line (HB2) and five human breast cancer cell lines (MCF-7, T47D, MDA-MB-435, MDA-MB-468, and MDA-MB-231). The blot was first developed for the presence of Wnt-5a and then consecutively reprobed for Syk, DDR1, and actin. Representative of five separate experiments.

tumor tissue arrays. To ensure that clearly defined areas of malignant tissue were used in the arrays, for each tumor sample, a slide with a fresh tissue section was prepared from the paraffin block and was stained with hematoxylin. Areas, including representative tumor cells, were identified and marked, and two biopsies (∅ 0.6 mm) corresponding to the marked areas on the slide were taken from each paraffin block. These biopsies were remounted in a new paraffin block in a tissue array machine according to the manufacturer's instructions (Beecher Instruments, Silver Spring, MD).

Immunohistochemistry

Immunohistochemistry was done using a DAKO ChemMate detection kit and a DAKO TechMate 500/1,000 staining instrument (Bio-Tek Solutions, Winooski, VT). Briefly, 6 μm sections were processed and incubated with the indicated primary antibody at room temperature and then washed and incubated with the secondary biotinylated anti-rabbit IgG antibody. Following visualization of immunoreactivity, the sections were counterstained and mounted. Two independent observers (J.D. and G.L.), with no knowledge of the clinical outcome, evaluated the stained slides. The tumors were graded based on the intensity of the staining: +++, immunoreactivity equivalent to that seen in normal epithelial cells; ++, immunoreactivity moderately decreased; +, weak immunoreactivity; and −, no immunoreactivity.

In situ Hybridization

Total RNA was purified with Trizol reagent (Invitrogen, Groningen, the Netherlands) from mammary HB2 cells and subjected to reverse transcriptase reaction using SuperScript II (Invitrogen). The cDNA produced was then used for the production of *in situ* hybridization probes as described below. The probes were prepared from amplified cDNA material of fragments corresponding to bp 486 to 738 (Wnt-5a) and bp 675 to 1,074 (Syk) using the following primers: Wnt-5a 5′-GGCTGGAAGTGCAATGTCTTCC, Wnt-5a 3′-GCCTGTCTTCGCGCCTTCTCC, Syk 5′-CTCTCGGGAAGAATCTGAGC, and Syk 3′-GGACTTTCTGTGGCCAGGCTTTGG. Primers for the cRNA probes were designed with T3 and T7 sites as follows: *T3*-Wnt-5a-sense, 5′-(GCAAT)*AATTAACCCTCACTAAAGGG*GGCTGGAAGTGCAATGTCTTCC, antisense cRNA; *T7*-Wnt-5a-antisense, 3′-(GCAAT)*TAATACGACTCACTATAGGG*CCTGCCAGTTGGCTGCAGAG, sense cRNA; *T3*-Syk-sense, 5′-(GCAAT)*AATTAACCCTCACTAAAGGG*CCGAGCCAGAGACAACAACGG, antisense cRNA; and *T7*-Syk-antisense, 3′-(GCAAT)*TAATACGACTCACTATAGGG*CCTGGAAGTTGTGGACGGCC, sense cRNA. The first reaction was carried out using TAQ polymerase (Invitrogen) to produce cDNA with flanking T3 and T7 sites. That yielded fragments corresponding to bp 486 to 659 (Wnt-5a) and bp 738 to 989 (Syk), which were sequenced and quantified. Digoxigenin-labeled rNTPs (Roche Applied Science, Mannheim, Germany) and T3 or T7 RNA polymerase (Promega, Madison, WI) were used according to the manufacturer's protocol to produce cRNA probes [Wnt-5a antisense; Wnt-5a sense (control); Syk antisense; and Syk sense (control)]. The Wnt-5a probe was carefully chosen to exclude the Wnt-5b homologue (5′ end). The probes were purified, quantified, and diluted in a hybridization buffer.

The actual *in situ* hybridizations with digoxigenin-labeled oligonucleotide probes were done as described previously (17, 18). The hybridization signals were classified as normal (retained) or reduced by three independent observers (A.B., K.L. and J.D.) with no knowledge of the clinical outcome.

Analysis of Ki-67, Cyclins, Hormone Receptors, and Retinoblastoma Phosphorylation

The percentage of Ki-67-positive cells in the different tumor biopsies was determined by immunostaining and counting as described previously (19). The levels of cyclin and retinoblastoma (Rb) expression in the tumor sample lysates were evaluated by Western blotting. After densitometric quantification, the tumor protein concentrations were normalized with respect to protein extract from either MG-63 (cyclin D1) or BL-42 (cyclin E) cells, which was run in parallel with tumor protein extracts on every gel (16). Calculating the absorbance ratio between the specific band of hyperphosphorylated Rb and total Rb in each sample enabled comparison of the degree of Rb phosphorylation in the different tumor samples. A commercial enzyme immunoassay (Abbott Laboratories, Abbott Park, IL) was used to analyze expression of estrogen receptors and progesterone receptors, and the resulting values were considered to be negative if the amount of protein detected for the two receptors was <0.1 or 0.05 nanomoles of ligand bound per milligram of protein, respectively (19).

Statistical Analysis

SPSS 10.1 statistical software was used for all calculations. All stained samples were subclassified into two groups with regard to Wnt-5a (see RESULTS), which were compared regarding established and potential predictors of outcome (e.g., age, tumor grade and stage, and hormone receptor status) as well as a range of tumor markers (e.g., Ki-67, Rb phosphorylation, and cyclins). χ^2 test and Student's t test were used to evaluate statistical differences between the various groups. All tests were two tailed, and $P < 0.05$ was regarded statistically significant. For individual patients, survival was expressed in years from the date of the primary surgery to the occurrence of an event and was analyzed as disease-specific survival based on deaths associated with or due to clinically advanced breast cancer. Cumulative survival was computed using the Kaplan-Meier method, and log rank testing was applied to assess differences in survival. A Cox proportional hazards analysis was used to obtain the relative risk with a 95% confidence interval of breast cancer death in the Wnt-5a ++/+++ group compared with the Wnt-5a −/+ group. A second model included potential confounders (i.e., tumor-related factors unevenly distributed between the two Wnt-5a groups).

RESULTS

Wnt-5a/DDR1 Signals Independently of Syk

Analysis of whole-cell lysates revealed Wnt-5a expression in HB2, T47D, and MDA-MB-468 cells but much lower levels in MDA-MB-231, MDA-MB-435S, and MCF-7 cells (Fig. 1). By comparison, Syk protein was expressed in MCF-7, T47D, and MDA-MB-468 cells but not in MDA-MB-231 or MDA-MB-435S cells (Fig. 1). Thus, with one exception (MCF-7), a

correlation between the expressions of Wnt-5a and Syk exists in the cell lines tested. In the noncancer breast epithelial cell line HB2, we detected significant expression of both Wnt-5a and Syk (Fig. 1). DDR1 was expressed in all the mentioned cell lines, albeit in different amounts, with the most pronounced expression in T47D cells (Fig. 1). The higher molecular weight bands in the anti-DDR1 immunoprecipitates from the different cell lines most likely reflect unspecific antibody binding to SH2-containing proteins that associate with active/phosphorylated DDR1 in the different cell lines (8).

To analyze a possible signaling interaction between Syk and DDR1, we next coimmunoprecipitated these proteins in noncancerous Wnt-5a-expressing HB2 cells as well as in MCF-7 tumor cells (Fig. 2*A*). The association between Syk and DDR1 was apparently constitutive, because it occurred in both suspended cells and cells allowed to adhere for 90 minutes to collagen or to tissue culture plastic (Fig. 2*A* and *B*). The time of adhesion was chosen to obtain maximal activation/phosphorylation of DDR1 (8). We confirmed that the bound protein was indeed Syk by using the MDA-MB-435 breast cancer cell line as a negative control (data not shown), because these cells do not express Syk protein due to hypermethylation of the *Syk* gene. In accordance with our previous results, tyrosine phosphorylation of DDR1 was confined to HB2 cells adhering to collagen (Fig. 2*B*; ref. 6). However, we found no signs of phosphorylation of DDR1-bound Syk in any of the experimental conditions (Fig. 2*B*). To determine whether adhesion induces phosphorylation of Syk, we immunoprecipitated Syk and blotted with an anti-phosphotyrosine antibody. We observed weak basal phosphorylation of Syk in HB2 and MCF-7 cell lysates prepared directly after detachment or after being kept in suspension for 30 minutes (Fig. 3*A*). Interestingly, both HB2 and MCF-7 cells displayed a prominent increase in phosphorylation of Syk after 90 minutes of plating on collagen (Fig. 3*A*). The fact that MCF-7 cells have a very low Wnt-5a protein expression implies that adhesion-induced Syk activation occurs through a Wnt-5a/DDR1-independent mechanism in breast epithelial cells. We have found previously that β_1 integrins are also involved in collagen adhesion of both HB2 and MCF-7 cells (8). That finding may indicate that the activation of Syk observed in our current investigation was triggered by β_1 integrins, because expression of those adhesion receptors and their ability to activate focal adhesion kinase signaling are not influenced by Wnt-5a expression (6). To test this hypothesis, we pretreated HB2 cells with either pertussis toxin, shown previously to inhibit collagen-induced phosphorylation of DDR1 (8), or a β_1 integrin blocking antibody. As shown in Fig. 3*B*, collagen-induced phosphorylation of Syk was not affected by pertussis toxin but significantly reduced by the blocking β_1 integrin antibody. Due to the preincubations and because the analysis were not dependent on maximal DDR1 activation, we reduced the time of adhesion to 60 minutes. This clearly shows that adhesion-induced Syk activation is unrelated to the Wnt-5a/DDR1 signaling pathway and apparently mediated by β_1 integrins. This conclusion is also compatible with the observations that MCF-7 cells exhibit a normal expression of Syk but only a vague expression of Wnt-5a (Fig. 1) and that adhesion of these cells to collagen is predominantly mediated via β_1 integrins and not via DDR1 (8).

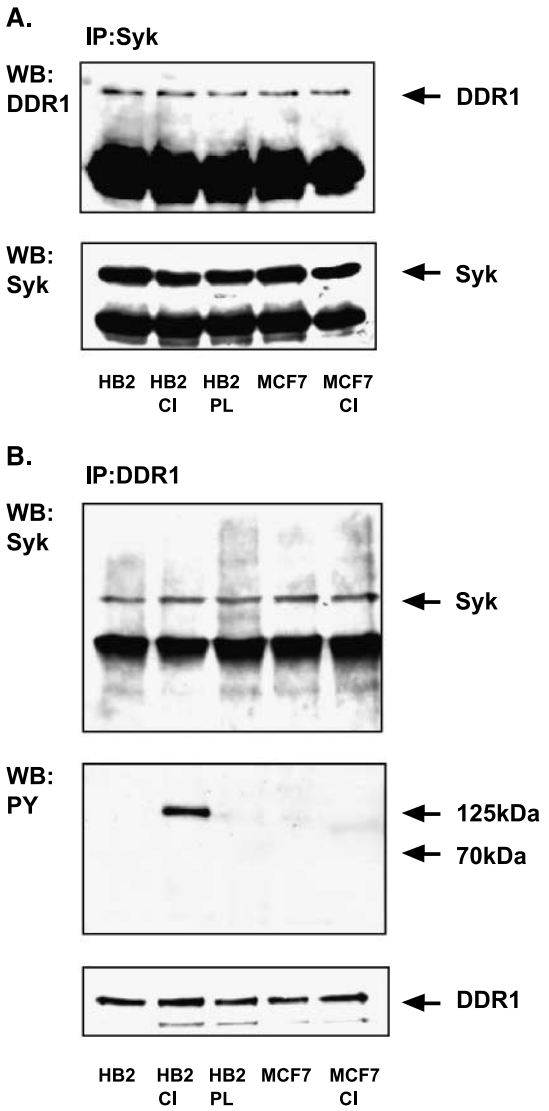

Fig. 2 Association between DDR1 and Syk in noncancer HB2 cells and MCF-7 breast carcinoma cells. Cells were allowed to adhere to uncoated tissue culture plates or to plates coated with collagen I (*CI*) for 90 minutes or were kept in suspension for the same period. Thereafter, the cells were lysed, and Syk (*A*) or DDR1 (*B*) was immunoprecipitated using a monoclonal anti-Syk or a polyclonal anti-DDR1 antibody, respectively (*A*). Immunoprecipitates were subjected to Western blotting with antibodies against DDR1 (*A*), Syk (*B*), or phosphotyrosine (monoclonal antibody 4G10; *B*). Membranes were subsequently reprobed for Syk (*A*) or DDR1 (*B*) to confirm equal amounts of immunoprecipitated protein in all lanes. Representative of 10 separate experiments.

Expression of and Correlation among Wnt-5a, DDR1, and Syk in Invasive Breast Carcinomas

The metastatic process requires not only an increased tumor cell motility and invasion but also an increased tumor cell survival and proliferation. Studies regarding the role for Wnt-5a in proliferation have been inconclusive, with some authors claiming Wnt-5a expression to decrease proliferation in a mammary myoepitelial cell line and in a renal tumor cell line (20, 21),

whereas other investigators find no such correlation in myocytes (22). In the support of the latter, we found no effect of varying the Wnt-5a expression on the proliferative rate of the human mammary epithelial cell line HB2 (6); furthermore, we found no expression of Wnt-5a in the mammary myoepithelial cells of human tissue (5). In the present tumor material, the Wnt-5a levels did not correlate with levels of the proliferation marker Ki-67, the protein cyclin D, or Rb phosphorylation (Table 1). Somewhat surprisingly, we found a negative correlation between Wnt-5a and cyclin E expression. Considering the recent finding that expression of cyclin E is an important predictor of breast cancer outcome (23), our data may indicate that the prognostic value of cyclin E is partly related to an associated reduction in Wnt-5a expression.

Next, we tested the expression of Wnt-5a, DDR1, and Syk proteins in this tumor material and compared them with the expression level in normal epithelial cells. In accordance with previous results obtained from a different tumor material (5), the data in Table 2 reveal that approximately half (n = 41) of all the investigated carcinomas exhibited normal or slightly reduced immunoreactivity to Wnt-5a protein, whereas the other half (n = 53) exhibited a significant reduced Wnt-5a protein expression. The level of expression of DDR1 protein was relatively stable, although a few tumors exhibited either decreased or increased immunostaining for DDR1 (Table 2; Fig. 4*B*). The expression of Syk protein was decreased in more than two thirds of all invasive tumors, and in 35% of those neoplasms, the Syk levels were reduced to <30% of the levels seen in normal breast epithelium (Table 2; Fig. 4*C*). Interestingly, we noted a covariation between decreased expression of Wnt-5a and Syk (Table 2). In contrast to the expression of Wnt-5a, none of the tumors displayed complete loss of either Syk or DDR1.

Table 1 Wnt-5a expression in the described clinical material in relation to markers of proliferation and other clinicopathologic variables in the same material

Factor	n	Wnt-5a −/+	Wnt-5a ++/+++	P
Ki-67	92	27.1	21.0	0.139
Cyclin E	94	1.01	0.43	0.040
Cyclin D	94	0.70	0.88	0.395
Rb phosphorylation	94	13.6	15.6	0.460
Elston-Ellis				
I	17	5	12	0.031
II	37	25	12	
III	40	23	17	
Menopausal status				
Pre	25	14	11	0.992
Post	68	38	30	
Stadium				
I	23	11	12	0.500
II	56	33	23	
III	1	0	1	
IV	6	4	2	
Estrogen receptor				
Positive	66	35	31	0.314
Negative	28	18	10	
Progesterone receptor				
Positive	57	27	30	0.037
Negative	36	25	11	

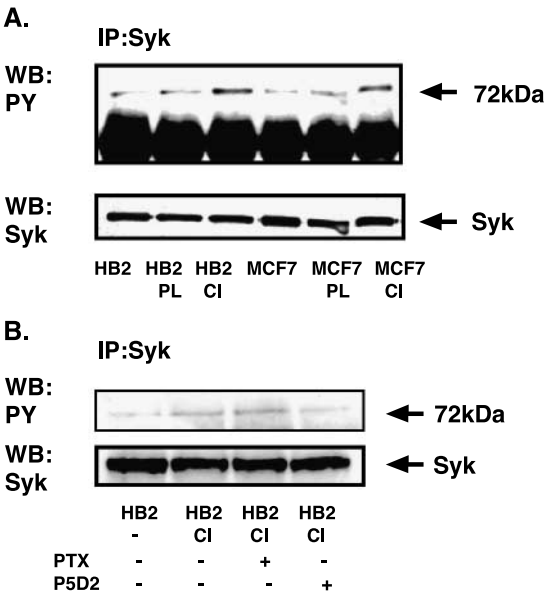

Fig. 3 Collagen-induced activation of Syk in noncancer HB2 cells and MCF-7 breast carcinoma cells. *A,* we analyzed cells in suspension or cells that had adhered for 90 minutes to an uncoated plastic surface (*PL*) or a surface coated with collagen I. *B,* cells were preincubated with 500 nmol/L pertussis toxin for 2 hours or 3.6 μL/mL β_1 integrin blocking antibody (P5D2) or vehicle alone and subsequently allowed to adhere to an uncoated plastic or collagen I–coated plastic culture plates for 60 minutes or were kept in suspension for the same amount of time. Thereafter, the cells were lysed, and Syk was immunoprecipitated with a monoclonal antibody. Immunoprecipitates were subsequently analyzed by Western blotting with antibodies against phosphotyrosine (monoclonal antibody 4G10) and Syk. Representative of 10 separate experiments.

In situ Analysis of Wnt-5a and Syk Transcripts

Interestingly, despite our previous and present data on the expression of Wnt-5a protein, two older studies have claimed that the level of Wnt-5a mRNA is up-regulated in invasive breast cancer tissue (24, 25). Therefore, we designed a probe that was specific for Wnt-5a and did not react with Wnt-5b (see MATERIALS AND METHODS). To the best of our knowledge, we are the first to use such a probe to detect Wnt-5a mRNA in breast tissue. Our probing for Wnt-5a and Syk mRNA clearly showed that specific transcripts of both proteins were well detectable in normal mammary epithelial cells but not in the stroma (Fig. 5*A*, *B*, *E*, and *F*), which

Table 2 Wnt-5a levels in relation to expression of Syk and DDR1 in tumor samples

	Wnt5a +/−	Wnt5a ++/+++	
Syk			
+	19	7	26
++	18	10	28
+++	2	19	21
	39	36	$P < 0.001$
DDR1			
+	9	6	15
++	23	14	37
+++	11	12	23
	43	32	$P = 0.516$

agrees with our immunohistochemistry data (5) on these proteins (data not shown). Analysis of Wnt-5a mRNA in 20 tumor samples revealed expression in all of the tumors, although the Wnt-5a protein level was reduced or lost in more than half of these tumor samples. A representative example of a tumor expressing Wnt-5a mRNA but not expressing Wnt-5a protein is shown in Fig. 5*C* and *D*. These observations suggest that Wnt-5a protein expression is regulated at the translational level as exemplified by cyclooxygenase-2 and tumor necrosis factor-α (26, 27), and because for the first time the Wnt-5a mRNA levels and protein expression were analyzed in parallel in the same tumor samples, it offers an explanation for the findings in previous studies (5, 24, 25). In contrast, analysis of Syk mRNA revealed reduced transcript levels in half (n = 7) of these samples with a corresponding reduction of Syk protein expression. Representative tumors with retained or reduced expression of Syk mRNA and protein are shown in Fig. 5*G* to *J*. These findings indicate that the absence of Syk protein expression in Syk-lacking breast tumors is affected at the transcriptional level, which concurs with previously reported results (13, 14).

Analysis of Disease-Specific Mortality

The presence of Wnt-5a protein in the studied primary tumors was associated with a longer disease-specific survival (P < 0.01; Fig. 6*A*). The results of the univariate regression analysis indicate an increased risk of death from breast cancer in the Wnt-5a-negative group (relative risk, 2.5; 95% confidence interval, 1.23-5.08). Loss of Wnt-5a expression remained an independent predictor of poor prognosis in a multivariate Cox regression analysis that included the covariates tumor stage, grade, hormone receptor status, and cyclin E levels (relative risk, 2.2; 95% confidence interval, 1.01-4.60). Preserved Syk expression was associated with a trend toward improved survival (P = 0.13; Fig. 6*B*), whereas no such correlation was seen for DDR1. Moreover, in tumors coexpressing Wnt-5a and Syk, survival was significantly better compared with the rest of the tumors (P = 0.018). Interestingly, the presence of either Wnt-5a or Syk alone did not yield a better prognosis (Fig. 6*C*).

DISCUSSION

The Wnt-5a protein has been implicated in breast cancer metastasis by affecting DDR1-dependent adhesion and motility of breast tumor cells (6, 8). The activation of DDR1 requires Wnt-5a-mediated stimulation of Src non–receptor tyrosine kinases (8), and a well-known downstream signaling target of Src kinases is the tyrosine kinase Syk (9). This is intriguing, because Syk expression is associated with an increased risk of

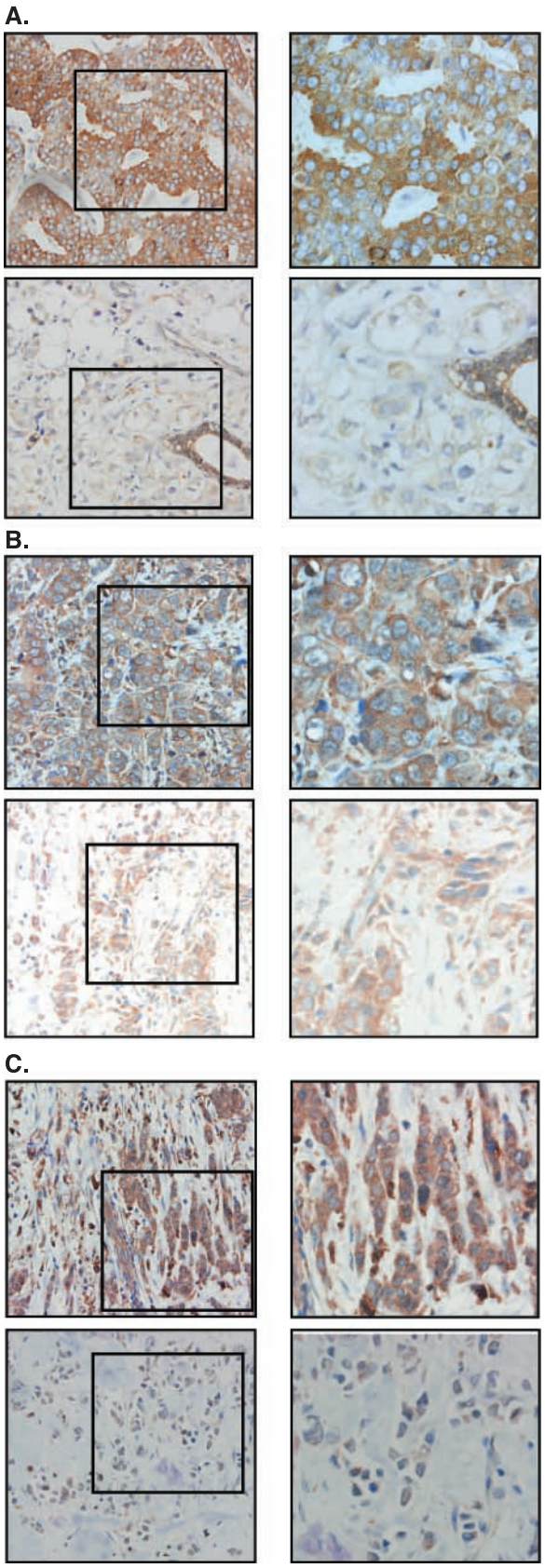

Fig. 4 Immunostaining of Wnt-5a, Syk, and DDR1 proteins in representative sections of invasive breast carcinomas. Staining was done with a polyclonal rabbit antibody against human Wnt-5a diluted 1:200 (*A*), a rabbit polyclonal anti-DDR1 antibody diluted 1:200 (*B*), and a polyclonal rabbit anti-Syk antibody diluted 1:400 (*C*). *Top micrographs,* tumor with retained expression (+++) of Wnt-5a, Syk, and DDR1; *lower micrographs,* tumor with reduced expression of Wnt-5a (−), Syk (+), and DDR1 (+). *Left,* magnification, 100×; *insets,* magnification, 400× images in *right*.

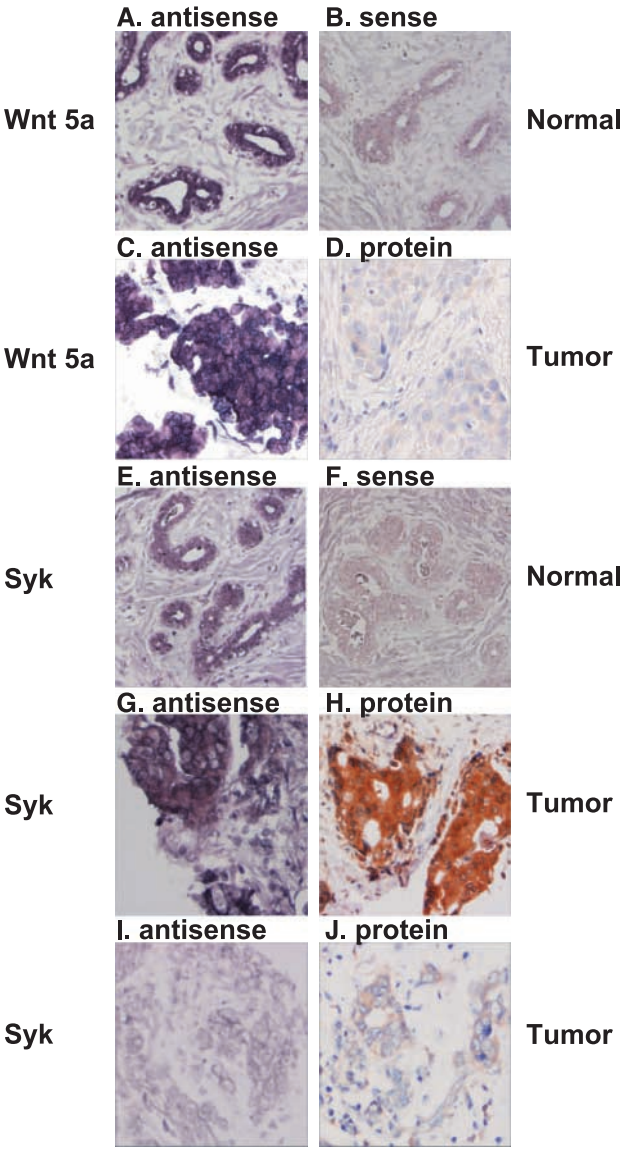

Fig. 5 Detection of Wnt-5a and Syk transcripts and proteins in human breast tissues. Sections of normal breast tissue (*A*, *B*, *E*, and *F*) and tumor arrays (*C*, *D*, and *G*-*J*) were hybridized with digoxigenin-labeled antisense-Wnt-5a (*A* and *C*), sense-Wnt-5a (*B*), antisense-Syk (*E*, *G*, and *I*), or sense-Syk (*F*) oligodeoxynucleotide probes or stained with anti-Wnt-5a (*D*) or anti-Syk (*H* and *J*) antibodies. Strong hybridization signals for Wnt-5a (*A*) and Syk (*E*) can be seen in the normal epithelial cells but not in the surrounding stroma. No hybridization occurred with the sense probes for Wnt-5a and Syk (*B* and *F*). Representative tumor samples show expression of Wnt-5a mRNA (*C*) but not Wnt-5a protein (*D*). For Syk, mRNA and protein levels were concordant, either retained (*G* and *H*) or reduced (*I* and *J*). Magnification, 200×.

metastatic spread in human breast carcinomas (11, 12). In the present study, we found a covariation between Syk and Wnt-5a protein expressions in mammary cell lines. It is hard to directly correlate the presence of Wnt-5a and Syk with the reported tumorigenic and metastatic potential of the presently used cell lines due to variations in the properties of a distinct cell lines between different laboratories and the use of different animals models. However, it is interesting to note that the two cell lines that exhibit no or a low expression of Wnt-5a and lack of Syk expression (MDA-MB-231 and MDA-MB-435) are those that in the literature are most often

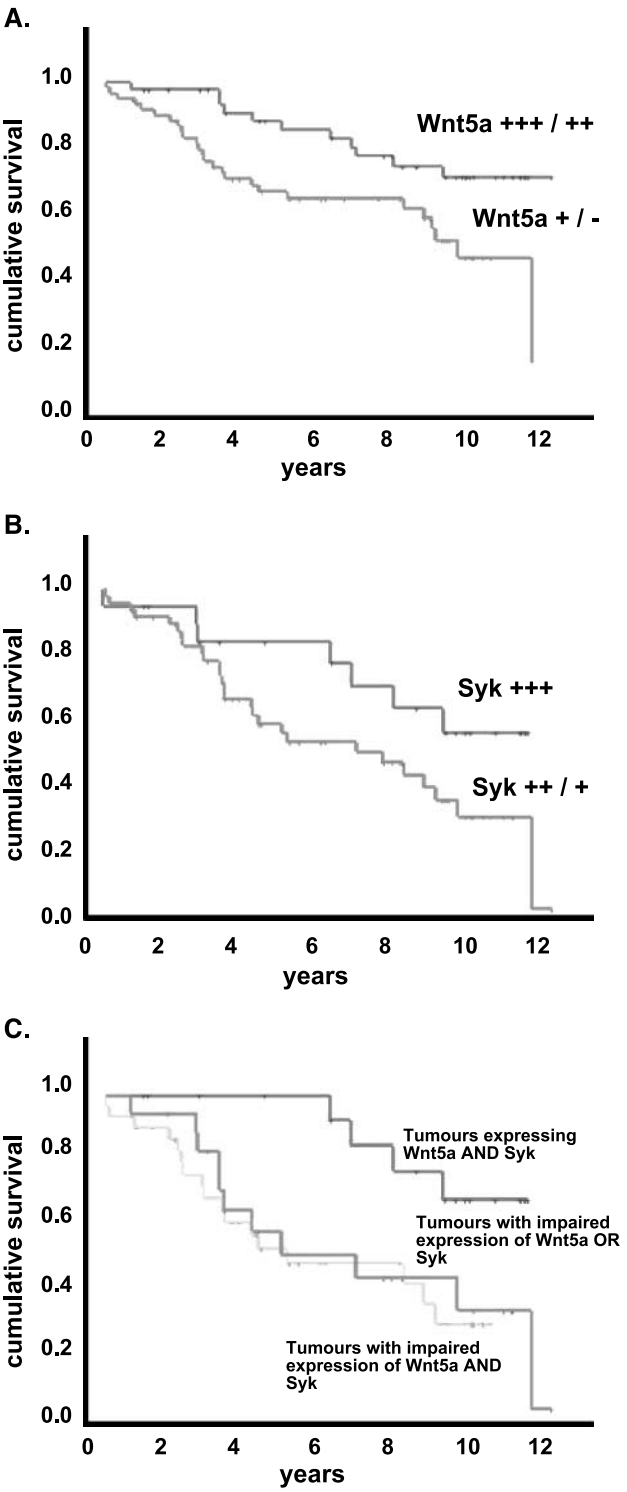

Fig. 6 Kaplan-Meier analysis showing breast cancer–specific survival stratified by expression of the proteins Wnt-5a (*A*), Syk (*B*), and both (*C*).

reported to be metastatic and therefore used in studies of this process (28, 29).

In search of a signaling interaction between Wnt-5a/DDR1 and Syk, we found a constitutive but Wnt-5a-independent association between DDR1 and Syk in both normal and tumor cell lines. The DDR1-associated Syk was not phosphorylated/activated regardless of the phosphorylation status of DDR1. However, we did notice that a significant amount of Syk that was not associated with DDR1 was readily phosphorylated/activated on cell adhesion to collagen. Hypothetically, two different pools of Syk could exist in the cell. One is constitutively bound to DDR1 but does not function as a substrate or downstream mediator of Syk, whereas the other portion is transiently activated by collagen through a β_1 integrin–mediated pathway. Such a cellular compartmentalization has been detected for Syk in natural killer cells, where activation by tumor cells leads to a polarization of Syk into lipid rafts (30). Alternatively, it is plausible that the large and small splice variants of Syk (SykL and SykS) are differentially activated by collagen receptors (31) and that only one of the two variants is capable of binding to the low constitutively tyrosine-phosphorylated DDR1 (32) by a SH2 domain-mediated mechanism. In this context, it should be noted that only SykL can translocate to the nucleus and suppress breast cancer invasiveness (31). The results with pertussis toxin, however, exclude a Wnt-5a/DDR1-dependent activation of Syk and most probably also a direct Wnt-5a-induced activation of this kinase, because pertussis toxin in all systems tested blocks the effects of Wnt-5a (8, 33). Instead, our data clearly show that the adhesion-induced activation of Syk is mediated through β_1 integrins, an observation in good accordance with the finding that β_1 integrin–induced activation of p125 focal adhesion kinase was independent on the expression level of Wnt-5a (6).

Interestingly, we found a covariation between Syk and Wnt-5a protein expressions also in invasive breast carcinomas. The loss of Syk protein and mRNA in breast carcinomas has been shown previously to be caused by hypermethylation of the *Syk* gene (13). As regards Wnt-5a, its regulation is clearly different and seems to occur at the translational level because no reduction in mRNA levels was observed in tumors lacking Wnt-5a protein. These results, obtained from the same tumor samples, are compatible with the results from our previous study (5) and that of Lejeune et al. (25) and clearly show that the Syk and Wnt-5a protein expression is differently regulated in human breast carcinomas.

Previous and present data reveal that adhesion-induced activation of Syk and DDR1 are mediated via β_1 integrins and a Wnt-5a-dependent mechanism, respectively (8). Furthermore, inhibition of β_1 integrins and loss of Wnt-5a activation in breast epithelial cells are additive in terms of their downstream effect on cell adhesion (8). Here, we show that loss of either or both Syk and Wnt-5a protein expression in invasive breast carcinomas have very similar prognostic values. Based on the presently available experimental data, we suggest that a reduction of breast tumor cell adhesion can be directly related to the metastatic potential and unfavorable prognosis of a tumor. However, once a certain reduction of adhesion has been obtained (as with loss of either Syk or Wnt-5a), a further reduction of adhesion (as with loss of both Syk and Wnt-5a) has no effect on the metastatic potential and thus the prognosis of a tumor.

We have shown that Wnt-5a protein expression is a good predictor of outcome in invasive breast carcinomas, as judged by recurrence-free interval (5) and disease-specific mortality (present study), presumably via its effect on tumor cell adhesion. It is well known that an increased rate of proliferation of tumor cells could be related to a higher metastatic frequency and consequently an increased mortality (34). We have concluded previously that Wnt-5a does not function by inhibiting proliferation, because our experimental data did not reveal any obvious differences in proliferation between Wnt-5a-expressing and nonexpressing human mammary epithelial cell lines (6). That assumption is confirmed and strengthened by our present investigation of clinical breast cancer material in which we found that Wnt-5a protein expression was not related to expression of the proliferation marker Ki-67 or cyclin D or to Rb phosphorylation.

In conclusion, our data clearly show that Wnt-5a and Syk affect breast cancer metastasis independently. Although the presently available experimental data suggest that both these signaling molecules affect tumor cell adhesion and motility, further studies of their potential role in breast cancer cell apoptosis is needed to obtain a more complete understanding of how their presence hinders breast cancer metastasis.

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REFERENCES

1. Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. Genes Dev 1997;11:3286–05.

2. Wodarz A, Nusse R. Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol 1998;14:59–88.

3. Bejsovec A. Wnt signaling: an embarrassment of receptors. Curr Biol 2000;10:919–22.

4. Shimizu H, Julius MA, Giarre M, Zheng Z, Brown AM, Kitajewski J. Transformation by Wnt family proteins correlates with regulation of β-catenin. Cell Growth Differ 1997;8:1349–58.

5. Jönsson M, Dejmek J, Bendahl PO, Andersson T. Loss of Wnt-5a protein is associated with early relapse in invasive ductal breast carcinomas. Cancer Res 2002;62:409–16.

6. Jönsson M, Andersson T. Repression of Wnt-5a impairs DDR1 phosphorylation and modifies adhesion and migration of mammary epithelial cells. J Cell Sci 2001;114:2043–53.

7. Weeraratna AT, Jiang Y, Hostetter G, et al. Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell 2002;1:279–88.

8. Dejmek J, Dib K, Jönsson M, Andersson T. Wnt-5a and G-protein signalling are required for collagen-induced DDR1 receptor activation and normal mammary cell adhesion. Int J Cancer 2003;103:344–51.

9. Yanagi S, Kurosaki T, Yamamura H. The structure and function of nonreceptor tyrosine kinase p72syk expressed in hematopoietic cells. Cell Signal 1995;3:185–93.

10. Sada K, Takano T, Yamamura H. Structure and function of Syk protein-tyrosine kinase. J Biochem 2001;130:177–86.

11. Stewart ZA, Pietenpol JA. Syk: a new player in the field of breast cancer. Breast Cancer Res 2001;1:5–7.

12. Toyama T, Iwase H, Yamashita H, et al. Reduced expression of the Syk gene is correlated with poor prognosis in human breast cancer. Cancer Lett 2003;189:97–102.

13. Yuan Y, Mendez R, Sahin A, Dai JL. Hypermethylation leads to silencing of the SYK gene in human breast cancer. Cancer Res 2001;61:5558–61.

14. Coopman PJ, Do MT, Barth M, et al. The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. Nature 2000;406:742–7.

15. Berdichevsky F, Alford D, D'Souza B, Taylor-Papadimitriou J. Branching morphogenesis of human mammary epithelial cells in collagen gels. J Cell Sci 1994;107:3557–68.

16. Nielsen NH, Arnerlov C, Emdin SO, Landberg G. Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to oestrogen receptor status. Br J Cancer 1996;74:874–80.

17. Bjartell A, Malm J, Möller C, Gunnarsson M, Lundwall A, Lilja H. Distribution and tissue expression of Semenogelin I and II in man as demonstrated by *in situ* hybridization and immunocytochemistry. J Androl 1996;17:17–26.

18. Paju A, Bjartell A, Zhang WM, et al. Expression and characterization of trypsinogen produced in the human male genital tract. Am J Pathol 2000;157:2011–21.

19. Nielsen NH, Emdin SO, Cajander J, Landberg G. Deregulation of cyclin E and D1 in breast cancer is associated with activation of the retinoblastoma protein. Oncogene 1997;14:296–304.

20. Olson DJ, Papkoff J. Regulated expression of Wnt family members during proliferation of C57MG mammary cells. Cell Growth Differ 1994;5:197–206.

21. Olson DJ, Oshimura M, Otte AP, Kumar R. Ectopic expression of Wnt-5a in human renal cell carcinoma cells suppresses *in vitro* growth and telomerase activity. Tumor Biol 1998;19:244–52.

22. Toyofuku T, Hong Z, Kuzuya T, Tada M, Hori M. Wnt/frizzled 2 signaling induces aggregation and adhesion among cardiac myocytes by increased cadherin-β-catenin complex. J Cell Biol 2000;150:225–41.

23. Keyomarsi K, Tucker SL, Buchholz TA, et al. Cyclin E and survival in patients with breast cancer. N Engl J Med 2002;347:1566–75.

24. Iozzo RV, Eichstetter I, Danielson KG. Aberrant expression of the growth factor Wnt-5a in human malignancy. Cancer Res 1995;55: 3495–9.

25. Lejeune S, Huguet EL, Hamby A, Paulsom R, Harris AL. Wnt5a cloning, expression, and up-regulation in human primary breast cancers. Clin Cancer Res 1995;1:215–22.

26. Dixon AD, Balch GC, Kedersha N, et al. Regulation of Cyclo-oxygenase-2 expression by the translational silencer TIA-1. J Exp Med 2003;3:475–81.

27. Zhang T, Kruys G, Huez G, Gueydan C. AU-rich element-mediated translational control: complexity and multiple activities of trans-activating factors. Biochem Soc Trans 2002;30:952–8.

28. Zhang RD, Fidler IJ, Price JE. Relative malignant potential of human breast carcinoma cell lines established from pleural effusion and a brain metastasis. Invasion Metastasis 1991;4:204–15.

29. Guise TA, Yin JJ, Taylor SD, et al. Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest 1996;98:1544–9.

30. Lou Z, Jevremovic D, Billadeau DD, Leibson PJ. A balance between positive and negative signals in cytotoxic lymphocytes regulates the polarization of lipid rafts during the development of cell-mediated killing. J Exp Med 2000;191:347–54.

31. Wang L, Duke L, Zhang PS, et al. Alternative splicing disrupts a nuclear localization signal in spleen tyrosine kinase that is required for invasion suppression in breast cancer. Cancer Res 2003;63:4724–30.

32. Vogel WF. Ligand-induced shedding of discoidin domain receptor 1. FEBS Lett 2002;514:175–80.

33. Liu X, Liu T, Slusarski DC, et al. Activation of frizzled-2/β-adrenergic receptor chimera promotes Wnt signaling and differentiation of mouse teratocarcinoma cells via Gαo and Gαt. Proc Natl Acad Sci 1999;96:14383–8.

34. Landberg G, Roos G. The cell cycle in breast cancer. APMIS 1997;105:575–89.

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