

Loss of Wnt-5a Protein Is Associated with Early Relapse in Invasive Ductal Breast Carcinomas¹

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ABSTRACT

Previous *in vitro* studies have implied that the *Wnt-5a* gene plays a role as a tumor suppressor. To explore the clinical relevance of this concept, 96 primary invasive breast carcinomas were stained with a novel anti-Wnt-5a antibody. Loss of Wnt-5a protein expression, evident in 44% of the invasive ductal carcinomas ($n = 59$), was significantly associated with higher histological grade ($P = 0.01$) and absence of estrogen ($P = 0.003$) and progesterone ($P = 0.02$) receptors. By contrast, loss of Wnt-5a protein in 24% of the invasive lobular carcinomas ($n = 37$) was not significantly related to any of the variables we investigated. The prognostic value of Wnt-5a for metastatic potential was evaluated by analyzing 83 additional invasive primary ductal carcinomas from patients with a longer follow-up time. We found that Wnt-5a expression was lost in tumors from 78% of the patients with recurrent disease ($n = 32$) compared with 35% of the recurrence-free patients ($n = 51$; $P < 0.001$), and that recurrence-free survival was significantly shorter in the Wnt-5a-negative group ($P < 0.001$). In multivariate analyses, loss of Wnt-5a expression proved to be an independent and powerful predictor of recurrence after adjustment for lymph node status and tumor size (hazard ratio = 4.8; $P = 0.002$). Our results show that loss of Wnt-5a increases the risk of early relapse and death because of recurrent ductal breast cancer, findings that support the notion that this protein retains tumor suppressor function by virtue of its effects on cell adhesion and motility.

INTRODUCTION

Members of the Wnt family encode secreted signaling molecules that play diverse biological roles in the regulation of several normal and pathological processes, such as cell growth, differentiation, and oncogenesis (reviewed in Refs. 1–3).

Experiments performed *in vitro* have shown that the *Wnt-5a* gene, which belongs to the nontransforming class of the Wnt gene family (4), can inhibit effects that are promoted during cell transformation. More specifically, direct inhibition of Wnt-5a (achieved using an antisense approach) was found to cause cuboidal mouse mammary cells to transform into elongated highly refractile cells that continued to replicate in a dense culture (5). Furthermore, loss of Wnt-5a endogenous expression in response to ectopically expressed *Wnt-1* or *neul/erbB-2* oncogene was correlated with cell transformation of C57 MG mouse mammary cells (6). In reciprocal experiments, ectopic expression of human Wnt-5a in mice reversed the phenotype of an uroepithelial cell line from tumorigenic to nontumorigenic (7). In good agreement with these cited results, we have found previously that overexpression of Wnt-5a did not affect the morphology and growth of normal breast cells, whereas inhibition of such expression was associated with elongated cell morphology, reduced affinity for collagen, and an enhanced capacity to migrate. Transfection of Wnt-5a into non-Wnt-5a-expressing MCF-7 breast cancer cells im-

proved many phenotypic aspects, including cell morphology, association between the cells, and contact inhibition at cell confluence (8). Thus, these findings support the notion that the *Wnt-5a* gene is related to tumor suppression (1).

On the other hand, evaluations of the Wnt-5a mRNA level in human cancers contradict the notion that Wnt-5a is involved in tumor suppression and instead suggest that the human *Wnt-5a* gene displays oncogenic activity. For instance, Lejeune *et al.* (9) have found that, compared with normal breast tissue, benign and invasive breast tumors respectively show 10-fold and 4-fold higher levels of Wnt-5a mRNA. Up-regulation of the Wnt-5a mRNA has also been reported in several other types of cancer, such as malignant melanoma and lung, colon, and prostate cancers (10, 11).

In view of the contradictory data obtained *in vitro* and *in vivo*, we explored the relationship between Wnt-5a protein expression and formation/progression of invasive primary breast carcinomas. Because other investigators have shown that several signaling components, including E-cadherin receptors, are differentially inactivated in various histological types of breast tumors (12, 13), we evaluated Wnt-5a protein expressions in both invasive DCAs³ and LCAs.

We found that loss of Wnt-5a expression was significantly associated with features indicative of an aggressive tumor phenotype in invasive ductal, but not lobular, tumors. To determine the relationship between loss of Wnt-5a protein and breast cancer recurrence, we extended our study to include an additional group of patients with longer follow-up time. We noted that loss of Wnt-5a was associated with an increased risk of recurrent ductal breast cancer and short RFS.

MATERIALS AND METHODS

Patients and Tissue Specimens. In the first part of the study (Table 1; Figs. 2–5), we consecutively collected tumors from 130 women (mean age, 62; range, 30–88 years) who had undergone surgery for primary invasive ductal or lobular breast cancers at Malmö University Hospital, May 1998 to May 1999. Reviewing hospital charts, we found that there was insufficient tumor material, or pathological reports were missing for 13 of the patients. Furthermore, 4 of the patients had metastases at the time of diagnosis and were therefore not suitable for our study. Tumors from the remaining 113 women were stained and evaluated for Wnt-5a expression. However, the results for 17 of the tumors could not be included for the reasons mentioned below (see “Evaluation of the Immunostaining”). In all, evaluable staining results were obtained for tumors from 59 DCA and 37 LCA patients.

In the second part of the study (Tables 2–4; Fig. 6), 153 consecutive cases were found by searching the patient database comprising females who had had surgery for primary invasive ductal breast cancer at Malmö University Hospital during two periods: January to August 1980 and January to September 1985. After reviewing hospital charts, we excluded 22 of the 153 patients because of insufficient tumor material or missing pathological reports. The following subgroups were also deemed unsuitable for inclusion: patients with inadequate follow-up, because they had died within 2 years of surgery for reasons other than their breast malignancies or follow-up was done at another hospital ($n = 17$); and patients who had received preoperative treatment ($n = 10$) or had metastases at the time of diagnosis ($n = 10$). The tumors from

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³ The abbreviations used are: DCA, ductal carcinoma; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; LCA, lobular carcinoma; PgR, progesterone receptor; RFS, recurrence-free survival; CI, confidence interval.

Table 1 Association between loss of Wnt-5a protein expression and clinicopathological features of invasive ductal carcinomas (1998/99)

	No.	Weak (+/-)	Normal (++++)	P
Age				
<50	7	2	5	0.45
≥50	52	24	28	
Histological grade				
I	13 (22%)	3 (23%)	10 (77%)	0.01
II	24 (41%)	9 (38%)	15 (62%)	
III	22 (37%)	14 (64%)	8 (36%)	
ER ^a				
Negative	13	11 (85%)	2 (15%)	0.003
Positive	36	12 (33%)	24 (67%)	
PgR ^a				
Negative	21	14 (67%)	7 (33%)	0.02
Positive	28	9 (32%)	19 (68%)	
Tumor Size				
T1 ≤20 mm	30	12 (40%)	18 (60%)	0.96
T2 >20 ≤ 50 mm	22	12 (55%)	10 (45%)	
T3 >50 mm	7	2 (29%)	5 (71%)	
Lymph node status ^a				
Negative	34	14 (41%)	20 (59%)	0.79
Positive	25	12 (48%)	13 (52%)	

^a All tumors were not subjected to all of the indicated analyses.

the remaining 94 women were analyzed for Wnt-5a expression. It was necessary to exclude the results for 11 of the tumors because of staining problems (see "Evaluation of the Immunostaining"), leaving a total of 83 evaluable tumors. Reviewing data on the last follow-up visit, we found recurrent disease in 32 of 83 (39%) of the patients of whom two developed loco-regional recurrence, whereas the remainder developed distal metastases. The follow-up period started on the day of primary surgery, and the end point for patients with recurrence was the date of diagnosis. This time variable is henceforth referred to as RFS. The follow-up time for patients living without any detected recurrence was 14 years.

Surgical treatment included mastectomy or breast-conserving surgery. None of the included patients had received radiation treatment or hormonal therapy before resection, and they were clinically devoid of distant metastatic disease. The patients used for analysis of RFS (Tables 2–4) were postoperatively either not treated at all or treated with radiation therapy, chemotherapy, antioestrogens, or various combinations of the three. There was no difference in the postoperative treatment between the Wnt-5a-negative and Wnt-5a-positive groups. This is most likely explained by the fact that the treatment was mainly based on lymph node status and tumor size, neither of which is related to the presence or absence of Wnt-5a (Table 2).

A small piece of all of the tumors was frozen in liquid nitrogen immediately after surgery and stored at -80°C for receptor analyses. In parallel, tumor specimens were also fixed in 10% formalin and embedded in paraffin.

Histology. Tumor size, lymph node involvement, and mode of invasion were defined according to the Tumor-Node-Metastasis classification of the International Union against Cancer. The histological grading of the tumors was performed using the modified criteria of Bloom and Richardson, as described by Elston and Ellis (14) and the WHO system (15).

Cell Cultures. *Mycoplasma*-free human mammary epithelial cells, HB2, were cultured as described elsewhere (16). Briefly, the cells were grown in DMEM supplemented with 10% fetal bovine serum, 10 µg/ml bovine insulin, and 5 µg/ml hydrocortisone, incubating at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. HB2 cells overexpressing erbB-2 were generously provided by Dr. Dan Baeckström (University of Gothenburg, Gothenburg, Sweden). The C57 MG (mouse mammary) and NIH 3T3 (fibroblast) cell lines were obtained from American Tissue Culture Collection (Rockville, MA) and were cultured according to the recommendation of the supplier.

Analysis of Steroid Receptors. The content of ERs and PgRs in the tumors was determined using an immunohistochemical method reported previously (17, 18). Analysis of ER content was determined with the mouse monoclonal anti-ER antibody M 7047 (1:100; Dakopatts, Copenhagen, Denmark), whereas the PgR content was determined with the mouse monoclonal antibody M 3529 (1:10; Dakopatts). A staining intensity of < 10% compared with positive control (100%) was used as a cutoff point for classifying a tumor as negative.

Generation of Anti-Wnt-5a Antibody. Wnt-5a polyclonal antiserum was generated in rabbits immunized with a synthesized peptide corresponding to amino acids 275–290 at the COOH-terminal region of human and mouse Wnt-5a protein (Ref. 19; accession no. A48914). The antiserum was purified in two steps: first, all of the IgGs in the antiserum were collected using a protein A-Sepharose column; thereafter the eluted fraction was additionally purified on a Sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden) containing the immobilized peptide used for immunization. The affinity-purified antibody was then eluted with 0.1 M glycine.

The anti-Wnt-5a antibody was used in Western blot screening of several cell lines for expression of the Wnt-5a protein. We found that NIH 3T3 cells were negative, and HB2 (normal human mammary epithelial cells) and C57 MG cells (normal mouse mammary cells) positive for expression of Wnt-5a (Fig. 1), which is consistent with previous reports indicating that Wnt-5a mRNA is expressed in the latter two of these cell lines (6, 16).

To additionally confirm the antibody-antigen specificity, we performed peptide-blocking experiments in which the initial molar ratio was 1:1 (peptide:antibody). The antibody was then incubated with increasing amounts of synthesized peptide (1:2–1:10) against which the antibody was developed. In subsequent Western blot analysis, the intensity of the Wnt-5a signal declined with increasing molar ratio of peptide:antibody and was eventually undetectable at a 10-fold excess of the peptide.

Protein Preparation and Western Blot Analysis. Western blot analysis of expression of Wnt-5a was done to confirm the specificity of the anti-Wnt-5a antibody. Briefly, experimental cells were lysed, and their protein content was determined before separation by gel electrophoresis, as described previously (8). The proteins were subsequently transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were incubated, first for 1 h with the anti-Wnt-5a antibody (1:2000) and then for 30 min with a mouse-antirabbit peroxidase-conjugated secondary antibody. The antibody-antigen complex was detected using an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

Immunohistochemistry. Immunohistochemical analysis was performed on 4-µm-thick paraffin-embedded tumor sections placed on slides coated with poly-L-lysine, according to a protocol published previously (20). Deparaffinized sections were preincubated with 1% BSA to block nonspecific binding and then incubated with anti-Wnt-5a antibody (1:500) for 1 h. A biotinylated

Table 2 The relationship between Wnt-5a expression and other clinicopathological features (1980/85)

	No.	Weak (+/-)	Normal (++++)
Patients with recurrence			
Age			
<50	3	2 (67%)	1 (33%)
≥50	29	23 (79%)	6 (21%)
Histological grade			
I	7	7 (100%)	0 (0%)
II	7	3 (43%)	4 (57%)
III	18	15 (83%)	3 (17%)
Tumor Size			
T1 ≤ 20 mm	14	11 (79%)	3 (21%)
T2 >20 ≤ 50 mm	15	12 (80%)	3 (20%)
T3 > 50 mm	3	2 (67%)	1 (23%)
Lymph node status ^a			
Negative	8	7 (87%)	1 (13%)
Positive	18	14 (78%)	4 (22%)
Recurrence-free patients			
Age			
<50	10	6 (60%)	4 (40%)
≥50	41	12 (29%)	29 (71%)
Histological grade			
I	9	4 (44%)	5 (56%)
II	14	3 (21%)	11 (79%)
III	28	11 (39%)	17 (61%)
Tumor Size			
T1 ≤ 20 mm	33	8 (24%)	25 (76%)
T2 > 20 ≤ 50 mm	18	10 (56%)	8 (44%)
T3 > 50 mm	0	—	—
Lymph node status ^b			
Negative	33	12 (36%)	21 (64%)
Positive	13	5 (38%)	8 (62%)

^a Data available for 26 of 32 patients.

^b Data available for 46 of 51 patients.

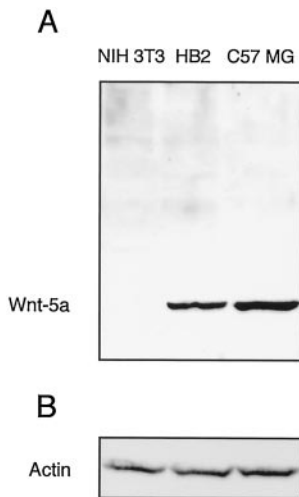


Fig. 1. *A*, testing the specificity of the anti-Wnt-5a antibody. Lane 1 shows the absence of Wnt-5a protein in a lysate of NIH 3T3 cells (negative control); Lanes 2 and 3, show the presence of Wnt-5a in lysates of HB2 and C57 MG cells, respectively. *B*, the membrane was stripped and reprobbed with an antiactin antibody to confirm equal loading. The molecular weight of Wnt-5a was estimated to *M_r* 39,000 by comparison with molecular weight standards.

mouse antirabbit antibody was used as the secondary antibody, and immunoreaction was visualized by using a streptavidin/biotin-peroxidase complex kit (Vectastain; Vector Laboratories, Burlingame, CA). The sections were counterstained with hematoxylin.

Stained slides were evaluated by two independent observers (M. J. and J. D.) with no knowledge of the clinical outcome. Tumor cells were examined in at least five microscopic fields of view, with a $\times 10$ objective, and the intensity of the staining was scored and expressed as a percentage of the endogenous Wnt-5a signal in the immunoreactively entrapped epithelial cells of normal ducts or lobules (100%). The sum of the scores for each section was then used to grade the staining of the tumor as normal (+++; > 75%), moderate (++; 30–75%), weak (+; < 30%), or negative (-). The most intense staining (+++) was designated normal, not high, because it represents endogenous Wnt-5a expression, that is, the level expressed in normal cells.

Tumors that lacked normal ducts or lobules or could not be assigned to a specific category because of heterogeneous staining were excluded. A total of 12 ductal and 5 lobular tumors were excluded in the first part of the study, and 11 ductal tumors in the second part.

The same scoring system was used to evaluate Wnt-5a expression in tumor cells that had disseminated into lymph nodes. In these experiments, a section of a positive lymph node and a section of the corresponding primary tumor were placed side by side on a slide and stained simultaneously.

Statistical Analyses. For statistical analysis, the tumors were divided into two categories: those displaying normal (staining +++/++) and those exhibiting weak (staining +/-) expression of Wnt-5a, referred to as loss of Wnt-5a expression.

Fisher's exact test or, when appropriate, χ^2 tests (21) were used to evaluate the relationship between Wnt-5a expression and other clinicopathological and biological variables, such as histological grade, tumor size, lymph node status, ER and PgR status, and age of patient at the time of cancer diagnosis. Univariate analysis of RFS was performed using the Kaplan-Meier method and log-rank tests (22). Kaplan-Meier estimates were curtailed when <5 individuals were at risk. A Cox regression model was used for multivariate analysis (23), and Schoenfeld's test was applied to check proportional hazard assumptions. Stata 6.0 software was used for all of the statistical analyses.

RESULTS

Analysis of Wnt-5a Expression. It has been reported that Wnt-5a is endogenously expressed in normal mouse breast tissue (24, 25), and in normal human and mouse breast cell lines (8, 26). In the present study, we found that the epithelial cells of normal ducts and lobules in

the tumor samples expressed Wnt-5a endogenously, regardless of the age of the patients. Examples of different levels of Wnt-5a staining in DCA tumors are shown in Fig. 2. The cytoplasm of normal epithelial cells was uniformly stained for Wnt-5a protein. However, there was no staining of the myoepithelial and stromal cells, which suggests that Wnt-5a expression is confined to the epithelial cells of the breast tissue. Immunoreactivity classified as normal staining/expression was as strong as that seen in the epithelial cells of normal ducts (Fig. 2, *a* and *b*), whereas intermediate staining was slightly less than the normal expression (Fig. 2, *c* and *d*). To be able to classify tumors as "Wnt-5a negative" (exhibiting loss of Wnt-5a expression), information we had obtained previously was used to determine the cutoff percentage of staining. In those experiments, we demonstrated that 10–40% inhibition of Wnt-5a endogenous expression, achieved by an antisense approach, was associated with a reduced ability of the cells to attach to collagen, and that the Wnt-5a antisense cells also failed to undergo phosphorylation of a collagen-binding receptor referred to as the discoidin domain receptor 1 (8). On the basis of these findings, we considered a reduction of Wnt-5a expression to < 30% of the normal level to indicate loss of Wnt-5a protein function. Tumors classified as showing weak Wnt-5a expression (+) exhibited much lower immunoreactivity than those displaying intermediate expression (+++; Fig. 2, *e* and *f*). By comparison, a complete loss of Wnt-5a expression and a staining intensity comparable with the background staining were seen in tumors classified as negative (-; Fig. 2, *g* and *h*). Thus, throughout the present study, we refer to the weak and negative staining groups together (+/-) as representing loss of Wnt-5a expression. At present, we do not know the molecular mechanism responsible for these reduced expression levels of Wnt-5a. However, recent work in our laboratory clearly show that the lack of Wnt-5a expression in human mammary MCF-7 cells is not attributable to a mutation,⁴ suggesting that an epigenetic mechanism is responsible for the loss of Wnt-5a expression in human mammary epithelial cells.

During the course of our study, we observed an increased expression of Wnt-5a in one-third of the DCIS (Fig. 3), whereas the other DCIS exhibited normal Wnt-5a expression levels. Notably we could also see such up-regulations of Wnt-5a in intraductal components of invasive DCAs that had lost their expression of the protein (Fig. 4). However, because of the low number of DCIS, we could not additionally analyze these tumors.

Loss of Wnt-5a Expression in Relation to Other Prognostic Factors. The first part of the present study demonstrates that Wnt-5a expression was lost in 44% of the DCA tumors. Statistical analysis of the relationship between expression of Wnt-5a and other prognostic factors, such as age of patient at time of cancer diagnosis, histological grade and size of tumor, and lymph node status, revealed a significant association between Wnt-5a negativity and increasing histological grade. A more detailed analysis of the histological features revealed no correlations between loss of Wnt-5a expression and tubule formation ($P = 0.25$) or nuclear grade ($P = 0.06$), whereas we found a strong correlation between a high mitotic index and loss of Wnt-5a ($P = 0.006$). Furthermore, loss of Wnt-5a protein was significantly associated with absence of ER and PgR. Wnt-5a expression in relation to the investigated clinicopathological and biological variables is shown in Table 1.

Because Wnt-5a and lymph node status are independently related to the aggressive potential of invasive ductal breast carcinomas (Table 1), we also investigated the expression of this protein in the primary tumors and their corresponding lymph nodes. The anti-Wnt-5a antibody stained the infiltrating tumor cells in the lymph nodes to differ-

⁴ M. Jönsson and T. Andersson, unpublished observations

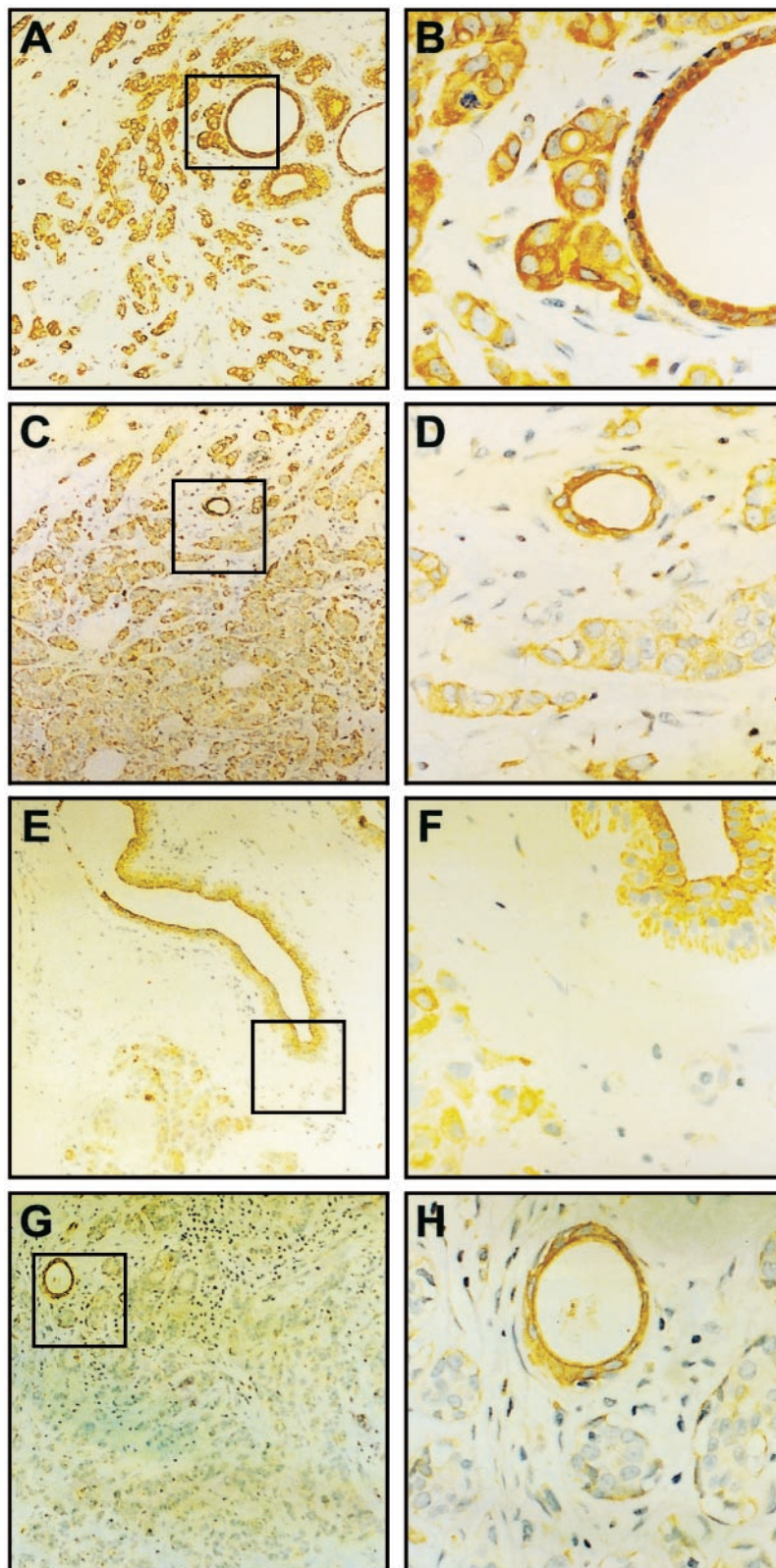


Fig. 2. Immunostaining of Wnt-5a protein in four representative invasive DCAs. A, Wnt-5a in an invasive ductal tumor showing an expression level similar to that of an entrapped normal duct. B, detail of A: to the *right*, the epithelium of a normal duct expressing Wnt-5a protein; to the *right*, the infiltrating tumor cells containing a similar expression of the protein (+++). C, Wnt-5a in an invasive ductal tumor showing an intermediate staining (++) compared with that of an entrapped normal duct. D, detail of C. E, expression of Wnt-5a in an invasive ductal tumor showing a reduced Wnt-5a protein expression (+) compared with that of the normal duct. F, detail of E: to the *right*, a normal duct expressing Wnt-5a protein; to the *left*, infiltrating tumor cells containing a reduced level of the protein. G, expression of Wnt-5a in an invasive ductal tumor showing loss of Wnt-5a expression (-) compared with that of an entrapped normal duct. H, detail of G. Magnifications: $\times 100$ in A, C, E, and G; $\times 400$ in B, D, F, and H.

ent degrees but none of the other cell types (Fig. 5). By a direct comparison on the same glass, we found that positive lymph nodes showed a similar degree of Wnt-5a staining as its corresponding primary tumor (data not shown).

In regard to evaluation of LCA tumors included in the first part of the study, a normal expression of Wnt-5a protein was found in 76%

of tumors, which is greater than the corresponding value obtained for the DCA tumors (56%; $P = 0.05$). The difference was even more pronounced when Wnt-5a scores (-, +, ++, +++) were taken into account ($P = 0.006$). Moreover, completely negative Wnt-5a expression was found in 20% of the DCA tumors but in none of the LCA tumors (data not shown). Analyzing loss of Wnt-5a expression in

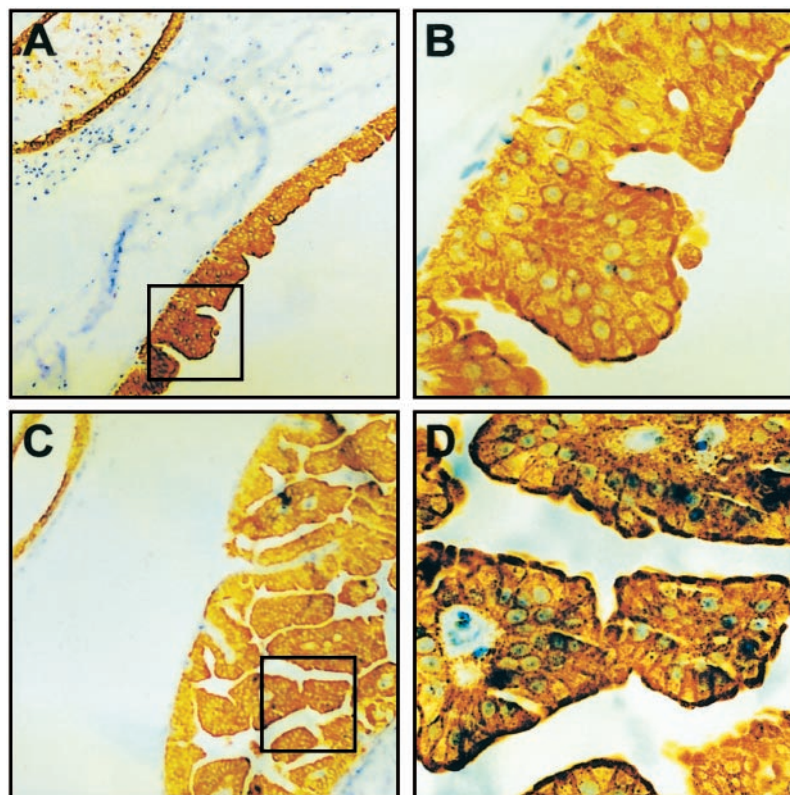


Fig. 3. Elevated expression of Wnt-5a protein in DCIS. A, expression of Wnt-5a in a normal duct (to the left) and the elevated expression in a DCIS (to the right). B, detail of A showing that expression of Wnt-5a is most marked at the apical surface of the epithelial cells, whereas there is no staining of the underlying myoepithelial cells. C, expression of Wnt-5a in a normal duct (to the left) and the elevated expression in a less-differentiated DCIS (to the right). D, detail of C. Magnifications: $\times 100$ in A and C; $\times 400$ in B and D.

LCAs in relation to the other prognostic factors, we noted no association between loss of Wnt-5a and lymph node metastasis, histological grade, ER and PgR status, age, or tumor size.

Loss of Wnt-5a Expression in Relation to Recurrent Disease.

The finding that loss of Wnt-5a expression was significantly associated with higher histological grade of DCA and absence of ER and PgR, which are characteristics of an aggressive tumor phenotype, prompted us to investigate the relationship between the loss of Wnt-5a protein and recurrence outcome. Analyzing Wnt-5a expression in 83 primary invasive ductal tumors, we found normal Wnt-5a expression in only 22% of the tumors from the patients with recurrent disease ($n = 32$) compared with 65% of the tumors from recurrence-free patients ($n = 51$). In other words, loss of Wnt-5a in tumors was twice

as common in recurrent as in recurrence-free patients (78% versus 35%). Statistical analysis showed a strong association ($P < 0.001$) between loss of Wnt-5a in primary tumors and subsequent recurrence, indicating that a patient lacking expression of Wnt-5a in a primary tumor is at a risk of relapse.

As seen in Table 2, several patients developed distant metastases, although no disseminated tumor cells were found in their lymph nodes. Comparing the primary tumors of the lymph-node-negative patients with recurrent disease with those from the recurrence-free women, we observed that loss of Wnt-5a expression was 2.5-fold greater in the former group than in the latter (87% versus 36%; $P = 0.02$). Furthermore, the distribution of histological grade between these two groups of patients is very similar (Table 2), but, despite that,

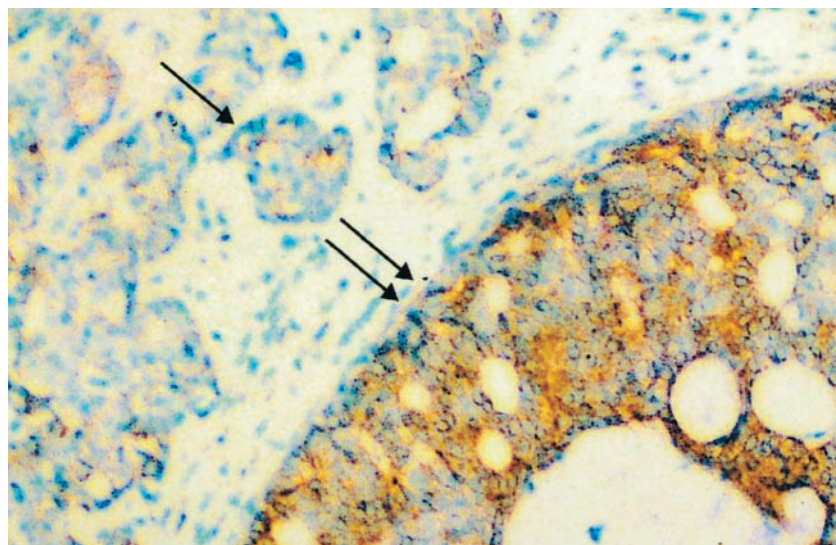


Fig. 4. Expression levels of Wnt-5a protein in a heterogeneous DCA. The single arrow designates invading DCA cells with a low expression of Wnt-5a; double arrows designate an intraductal component of the carcinoma with a relatively high expression of Wnt-5a. Magnification: $\times 400$.

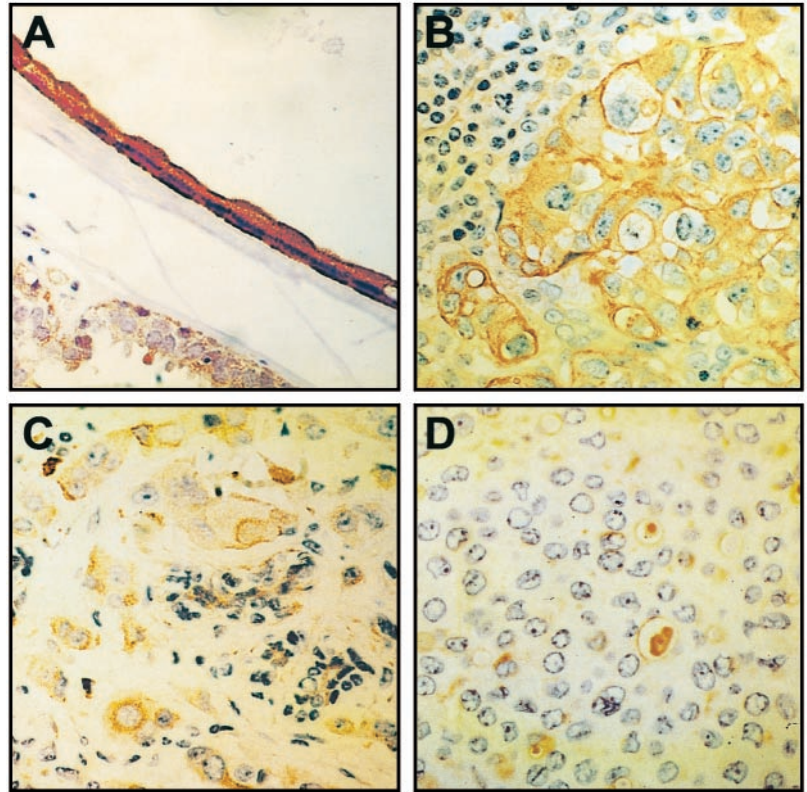


Fig. 5. Expression levels of Wnt-5a in lymph node metastases. A, Wnt-5a protein expression in a section of a normal duct (control) placed next to a section of a metastatic lymph node. B, a representative lymph node with tumor cells expressing an intermediate level of Wnt-5a protein (++). C, a lymph node with tumor cells exhibiting a weak expression of Wnt-5a protein (+). D, a lymph node with tumor cells with no expression of Wnt-5a protein (-). Magnifications: $\times 100$ in A; $\times 400$ in B, C, and D.

loss of Wnt-5a expression is twice as common in grade III primary tumors from patients with recurrent disease as in those from recurrence-free patients. The same was true for grade I and II tumors, indicating that loss of Wnt-5 is independently related to recurrence of the histological grade and lymph node involvement at the time of primary operation.

Loss of Wnt-5a in Relation to RFS. Kaplan-Meier estimates of the 5-year recurrence rate were 56% (95% CI = 40–70) and 89% (95% CI = 74–96) for the Wnt-5a-negative and the Wnt-5a-positive group, respectively (Fig. 6). A log-rank test revealed highly significant differences between the recurrence rates over time. As summarized in Table 3, lymph node status and tumor size also proved to be significant prognostic factors for RFS. However, histological grade (II versus I; III versus I) and age at time of cancer diagnosis (<50 versus ≥ 50 years) had no prognostic significance in univariate analyses (data not shown).

The independent prognostic value of loss of Wnt-5a expression and the interrelationships with other prognostic factors were also exam-

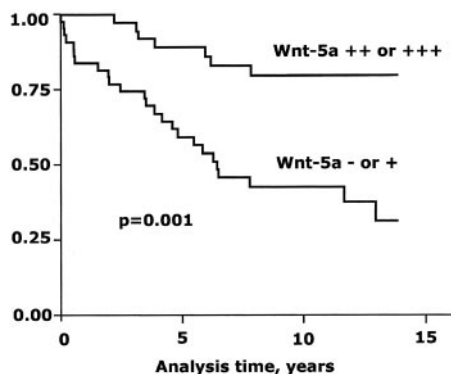


Fig. 6. Kaplan-Meier curve showing RFS stratified by Wnt-5a expression (1980/1985).

Table 3 Univariate analyses of RFS (1980/85)

Variables	Hazard ratio	95% CI	P-value
Tumor size			
>20 vs. ≤ 20 mm	2.0	1.0–4.1	0.05
Continuous	1.04 ^a	1.02–1.05	<0.001
Lymph node status			
- vs. +	5.3	2.3–12	<0.001
Wnt-5a expression			
+/- vs. +++/+++	4.5	1.9–10	<0.001

^a Per mm.

ined by multivariate analyses. We performed Cox regression analysis after adjusting for lymph node status and tumor size, and found Wnt-5a-negativity to be an independent risk factor with a hazard ratio of 4.8. As seen in Table 4, lymph node status (- versus +) and tumor size (continuous) were also highly significant. A subgroup analysis revealed significantly poorer RFS in the Wnt-5a-negative than in the Wnt-5a-positive group for both lymph-node-negative and lymph-node-positive patients (data not shown).

DISCUSSION

The present study provides information that helps to clarify the hitherto apparently contradictory roles (6, 7, 9–11) of Wnt-5a in induction and progression of breast cancer. Furthermore, it elucidates the significance of Wnt-5a expression for features assumed to be associated with aggressive tumor phenotype and disease recurrence. Our results show that many of the invasive ductal, but not the lobular, carcinomas we studied had lost the ability to express Wnt-5a protein. Different expressions in lobular and ductal breast carcinomas have been shown previously for other tumor-related proteins, including E-cadherin (12). This suggests that different gene programs are activated during induction/progression of invasive DCAs and LCAs.

According to our results, the loss of Wnt-5a protein expression is

Table 4 Multivariate analysis of RFS based on the Cox model (1980/85)

Variables	Hazard ratio	95% CI	P
Tumor size			
Continuous	1.03 ^a	1.01–1.05	0.003
Lymph node status			
– vs. +	3.7	1.6–9.0	0.003
Wnt-5a expression			
+/- vs. +++/++	4.8	1.8–13	0.002

^a Per mm.

associated with grade III tumors and the absence of ERs and PgRs, which are indicative of tumors undergoing autonomous growth. This agrees with but certainly does not prove the notion that Wnt-5a is related to tumor suppression (1). Furthermore, the loss of Wnt-5a expression seen in these grade III DCAs is in accordance with previous studies showing that this protein can inhibit cell transformation (5), normal growth, and migration (6–8). It is possible that activation of growth-promoting factors inhibits Wnt-5a expression and thereby facilitates proliferation and/or migration of these tumor cells. That possibility is suggested by the finding that transfection of mouse mammary cells with *neu/c-erbB-2* can be associated with down-regulation of Wnt-5a mRNA expression (6). However, unpublished data from our laboratory show that a similar overexpression of *erbB-2* has no effect on the endogenous protein expression of Wnt-5a in human breast epithelial cells (HB2). These different observations could possibly be attributable to the use of cells from different species and/or techniques, but most likely they reflect the use of different cell types, because the cells used in the former study clearly have a myoepithelial and not epithelial origin (6).

Interestingly, during our investigation we observed that Wnt-5a was markedly overexpressed in several DCIS. This finding is consistent with results published by Lejeune *et al.* (9), showing elevated Wnt-5a expression in benign proliferating breast diseases. These authors suggested that the up-regulation of Wnt-5a in benign tumors is attributable to an up-regulation in stromal cells, because there is a much greater proportion of such cells than of normal epithelium in fibroadenomas (8). However, our finding that Wnt-5a protein is produced only in epithelial cells refutes that suggestion and implies that such overexpression of Wnt-5a could instead play a functional role in breast carcinomas *in situ*.

In light of the findings that Wnt-5a protein is involved in regulation of cell migration (27) and that Wnt-5a was lost in the invasive ductal tumors, we extended our investigation to include patients with recurrent ductal breast cancer. We found a strong statistical association between loss of Wnt-5a protein in primary tumors and recurrent breast disease, which suggests that this protein affects the metastatic process by influencing cell adhesion and migration. This assumption is supported by the observation that Wnt-5a participates in inhibition of cell migration in *Xenopus* embryos (24), and by our previous results showing that loss of Wnt-5a in normal human mammary epithelial cells is associated with altered adhesion to and increased invasion of collagen gels (8). Furthermore, in our earlier study, we noted that Wnt-5a nonexpressing MCF-7 breast cancer cells that were transfected with a Wnt-5a-containing vector exhibited increased adhesion and decreased invasion of collagen gels (8).

Notably, we also observed that loss of Wnt-5a in the primary tumors was not associated with metastasis to regional lymph nodes. Instead, by performing multivariate analysis, we found that loss of Wnt-5a protein expression was a powerful predictor of recurrence, independent of lymph node status, and tumor size. Yet, when positive lymph nodes were detected they showed Wnt-5a staining levels similar to that seen in the primary tumors. These observations are compatible with the well-known fact that up to 30% of lymph-node-

negative patients who undergo surgery for invasive breast carcinoma suffer recurrence and die within 10 years. Notwithstanding the obvious clinical potential of the observed lack of association between loss of Wnt-5a and metastasis to regional lymph nodes, the molecular mechanism(s) responsible for this phenomenon is not yet known.

Despite the indications that loss of Wnt-5a protein promotes cell dissemination and metastasis via its effects on cell adhesion and motility, it cannot, of course, be excluded that loss of Wnt-5a is also involved in regulation of other cellular processes. One possibility being that loss of Wnt-5a could affect cell survival and, thus, the resistance of DCAs to therapy. Although transfection of mammary cells with antisense Wnt-5a does not affect the population doubling time (5), additional studies are needed to assess whether Wnt-5a also contributes to the development of drug resistance.

Consequently, our results confirm the importance of standard prognostic factors such as histological grade and tumor size (28, 29), but they also indicate that determining loss of Wnt-5a protein expression may aid in improving prognostication and identification of women at high risk of recurrence, because loss of Wnt-5a in primary DCAs is an independent predictor of relapse. Following confirmation of the present findings in a more extensive prospective study, it may be possible to use Wnt-5a as a clinical marker of an aggressive tumor phenotype and disease recurrence. More speculatively, reconstitution of the Wnt-5a signaling pathway in ductal breast cancers (which lack Wnt-5a protein expression) might provide a therapeutic means of reducing the risk of cancer cell dissemination and metastasis.

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