

Galectin-9 as a Prognostic Factor with Antimetastatic Potential in Breast Cancer

Akemi Irie,¹ Akira Yamauchi,^{2,5} Keiichi Kontani,¹ Minoru Kihara,¹ Dage Liu,¹ Yukako Shirato,⁵ Masako Seki,⁵ Nozomu Nishi,^{3,5} Takanori Nakamura,³ Hiroyasu Yokomise,¹ and Mitsuomi Hirashima^{4,5}

Abstract Purpose: Galectin-9, a member of the β -galactoside-binding galectin family, induces aggregation of certain cell types. We assessed the contribution of galectin-9 to the aggregation of breast cancer cells as well as the relation between galectin-9 expression in tumor tissue and distant metastasis in patients with breast cancer.

Experimental Design: Subclones of MCF-7 breast cancer cells with high or low levels of galectin-9 expression were established and either cultured on plastic dishes or transplanted into nude mice. The tumors of 84 patients with breast cancer were tested for galectin-9 expression by immunohistochemistry. The patients were followed up for 14 years.

Results: MCF-7 subclones with a high level of galectin-9 expression formed tight clusters during proliferation *in vitro*, whereas a subclone (K10) with the lowest level of galectin-9 expression did not. However, K10 cells stably transfected with a galectin-9 expression vector aggregated in culture and in nude mice. Ectopic expression of galectin-9 also reduced MCF-7 cell adhesion to extracellular matrix proteins. Tumors of 42 of the 84 patients were galectin-9 positive, and those of 19 of the 21 patients with distant metastasis were galectin-9 negative. None of the 13 patients with galectin-9-positive tumors and lymph node metastasis up to level II manifested distant metastasis. The cumulative disease-free survival ratio for galectin-9-positive patients was more favorable than that for the galectin-9-negative group ($P < 0.0001$). Multivariate analysis revealed that galectin-9 status influenced distant metastasis independently of and to a greater extent than lymph node metastasis.

Conclusions: Galectin-9 is a possible prognostic factor with antimetastatic potential in breast cancer.

Distant metastasis is a major clinical determinant of the survival of individuals with breast cancer. The number of lymph node metastases (node status) has long been used to predict distant metastasis in breast cancer. The various biological markers proposed for the prediction of distant metastasis in breast cancer include loss of *nm23* expression (1, 2), with increased expression of this gene induced by inhibition of DNA methylation also having been found to prevent distant metastasis (3). The levels of both total and low molecular weight cyclin E, as determined by immunoblot analysis, are also correlated with survival in patients with breast cancer, especially in those with node-negative cancer (4).

However, none of these biological markers is as effective as node status in the prediction of distant metastasis or is suitable as an indicator of the need for adjuvant chemotherapy.

Galectin-9 is a member of the β -galactoside-binding galectin family of proteins (5–8). Among the members of the galectin family, galectin-1 (9) and galectin-3 (10–13) contribute to tissue invasion by and metastasis of several types of cancer cells, including breast cancer cells. Galectin-3 also serves as a marker for preoperative diagnosis of nodular thyroid lesions (14). We have recently shown that a high level of galectin-9 expression in the tumors of individuals with melanoma is associated with a significantly increased survival time and a lower frequency of distant metastasis (15). We now provide evidence that galectin-9 induces the aggregation both *in vitro* and *in vivo* and reduces adhesion to the extracellular matrix of breast cancer cells, and that a high level galectin-9 expression in breast cancer tissue is significantly associated with a low frequency of distant metastasis.

Authors' Affiliations: ¹Second Department of Surgery and Departments of ²Cell Regulation, ³Endocrinology and ⁴Immunology and Immunopathology, School of Medicine, Kagawa University and ⁵GalPharma Co. Ltd., Kagawa, Japan
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Note: A. Irie and A. Yamauchi contributed equally to this work.

Requests for reprints: Akira Yamauchi, Department of Cell Regulation, School of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. Phone: 81-87-891-2403; Fax: 81-87-891-2403; E-mail: yamauchi@kms.ac.jp.

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Materials and Methods

Antibodies. Polyclonal antibodies to galectin-9 were generated in rabbits by injection of a recombinant peptide corresponding to the COOH-terminal domain of the human protein. The antibodies were purified by chromatography on Sepharose 4B (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) conjugated with the antigen. Immunoblot analysis revealed that the antibodies exhibited no cross-reactivity with other galectins, including galectin-1, -3, -7, and -8.

Cell culture and subcloning. The estrogen-dependent breast cancer cell line MCF-7 was obtained from American Type Culture Collection (Rockville, MD) and maintained under 5% CO₂ at 37°C in DMEM supplemented with 2 mmol/L of L-glutamine, 10% fetal bovine serum, and penicillin-streptomycin (ICN Biomedicals, Aurora, OH). Subclones of MCF-7 cells were established by the limiting dilution method. In brief, a cell suspension was distributed into the wells of 96-well round-bottomed culture plates at a cell concentration of 0.5 cell per well. Only wells containing a single cell were selected thereafter, and 12 subclones were obtained. Clone K10 had the lowest level of galectin-9 expression and was used for transfection with galectin-9 cDNA.

Immunoblot analysis. Cells (1×10^6) were harvested and lysed with an ice-cold solution containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, aprotinin (50 trypsin inhibitory units/mL), and leupeptin (50 µg/mL). After centrifugation of the lysate at $16,000 \times g$ for 10 minutes at 4°C, the supernatant was subjected to SDS-PAGE on a 5% to 15% gradient gel in a minigel apparatus (Bio-Rad, Richmond, CA). The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), which was then exposed for 1 hour to 5% skim milk containing 0.05% Tween 20 before consecutive incubations with antibodies to galectin-9 (2 µg/mL) and horseradish peroxidase-conjugated goat antibodies to rabbit IgG (Amersham Pharmacia Biotech). Immune complexes were detected with the ECL system (Amersham Pharmacia Biotech).

Construction of galectin-9 expression plasmids and cell transfection. Expression vectors for human galectin-9S, -9M, and -9L, which are defined by the linker size of galectin-9, were constructed by inserting cDNAs that included the entire coding region plus seven nucleotides upstream of the start codon into the *EcoRI-XhoI* site of pBK-CMV (Stratagene, La Jolla, CA). Cells were transfected with the use of the FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN) and were subjected to selection for 2 weeks with G418 (800 µg/mL).

Assay for cell adhesion. Cells were harvested, washed thrice with PBS, resuspended in serum-free DMEM, and transferred to the wells (5×10^5 cells per well) of 96-well plates that had been coated with type IV collagen, fibronectin, vitronectin, or laminin (Biocoat ELISA plates, Becton Dickinson, San Jose, CA). After incubation for 90 minutes at 37°C, the wells were washed thrice with PBS to remove nonadherent cells and the remaining cells were fixed with 3% paraformaldehyde, stained with 0.4% crystal violet (Sigma-Aldrich, St. Louis, MO) in methanol, and washed with tap water. The number of adherent cells was quantified by measurement of absorbance at 540 nm with a plate reader.

Cell Transplantation. Female KSN nude mice (SLC, Shizuoka, Japan) were maintained under specific pathogen-free conditions and with a 12-hour light, 12-hour dark cycle; they had free access to food and water. At 8 weeks of age, the mice were given s.c. injections into the second left mammary gland of MCF-7 K10 cells (8×10^6 in 0.1 mL of physiologic saline) that had been transfected either with a galectin-9 expression vectors or with the corresponding empty plasmid. The mice were subsequently given i.p. injections of 100 µg of estradiol in 100 µL of physiologic saline (E.P. Hormone Depot; Teikoku Hormone, CITY, Japan) every 2 weeks; they were killed 8 weeks after cell injection, and the resulting tumors were resected. The relation between cell aggregation and galectin-9 expression was evaluated by immunohistochemical staining.

Patients. Eighty-four women ages >35 years with breast cancer were enrolled in the study and provided informed consent. The median age was 54 years and the median observation period was 118 months. Modified radical mastectomy was done on each patient between 1987 and 1992. Adjuvant therapy was administered to 73% of the patients. This study was carried out according to the ethical guidelines of the Declaration of Helsinki, and specific approval was obtained from the Ethics Committee of Kagawa Medical University.

Immunohistochemical analysis. Immunohistochemical staining of sections of formalin-fixed, paraffin-embedded tissue was done with

antibodies to galectin-9 and an EnVision+ Peroxidase Rabbit System (Dako, Kyoto, Japan). In brief, sections (thickness, 4 µmol/L) were heated at 100°C for 16 minutes in 10 mmol/L sodium citrate buffer (pH 6), subjected to paraffin removal, and rehydrated. After quenching of endogenous peroxidase activity with 0.3% hydrogen peroxide, the sections were treated for 2 hours at room temperature with 5% bovine serum albumin to block nonspecific staining. They were then incubated at room temperature first overnight with primary antibodies (5 µg/mL) and then for 1 hour with EnVision+ solution containing horseradish peroxidase-conjugated secondary antibodies. 3,3'-Diaminobenzidine tetrahydrochloride was used as the chromogen. An immunoglobulin fraction isolated from normal rabbit serum (Dako) was used as a negative control. All sections were counterstained with Mayer's hematoxylin solution. The percentage intensity of stained tumor cells in each section was determined independently by two observers. The staining intensity was graded as 0 when no staining was detectable, 1 when staining was weak, 2 when staining was clearly positive, and 3 when staining was strongly positive.

The immunohistochemical evaluation incorporating both the percentage and intensity of stained cells [histochemical score (HSCORE)] was used (15), and HSCORE was calculated by the following formula.

$$\text{HSCORE} = \sum P_i (i)$$

where $i = 1, 2, 3$ and P_i varies from 0% to 100%. An HSCORE >80 was defined as positive staining.

Statistical analysis. The relations between the expression of galectin-9 in primary lesions and either distant metastasis, node status, estrogen receptor status, clinical stage, histopathologic grade, or adjuvant therapy were assessed with the χ^2 test and Fisher's exact test. Disease-free survival curves were generated by the Kaplan-Meier method and were analyzed either with the log-rank test or with the χ^2 test for groups with no recurrence. Multivariate analysis with Cox's proportional hazards regression model was done to examine the effects of different variables on the occurrence of distant metastasis. All P values were based on two-tailed statistical analysis, and values <0.05 were considered statistically significant.

Results

Induction of cell aggregation by galectin-9. We established 12 subclones of MCF-7 cells, which we divided into two groups based on whether cell proliferation was accompanied by pronounced cell aggregation. The level of expression of galectin-9, as revealed by immunoblot analysis, was higher in all subclones that exhibited pronounced aggregation than in the subclones that did not (Fig. 1A-C). The subclone K10 did not aggregate and exhibited the lowest level of galectin-9 expression; however, stable transfection of K10 cells with an expression vector for each of three different size of galectin-9s, but not with the empty vector, resulted in the formation of tight cell clusters on proliferation *in vitro* (Fig. 1D and E). Subcutaneous injection of K10 cells transfected with the galectin-9 vector into the mammary glands of nude mice resulted in the formation of round-margined tumors with large nests, whereas injection of cells transfected with the empty vector resulted in scattered growth of the ectopic cells with the formation of small nests resembling scirrhous carcinoma (Fig. 1F and G). These results suggested that galectin-9 might contribute to the aggregation of breast cancer cells.

Reduced adhesion of breast cancer cells to extracellular matrix proteins induced by galectin-9 expression. Adhesion of cancer cells to the extracellular matrix is an essential step in tumor cell invasion. Transfection of MCF-7 cells with expression vectors for three different forms (S, M, and L) revealed that the cells

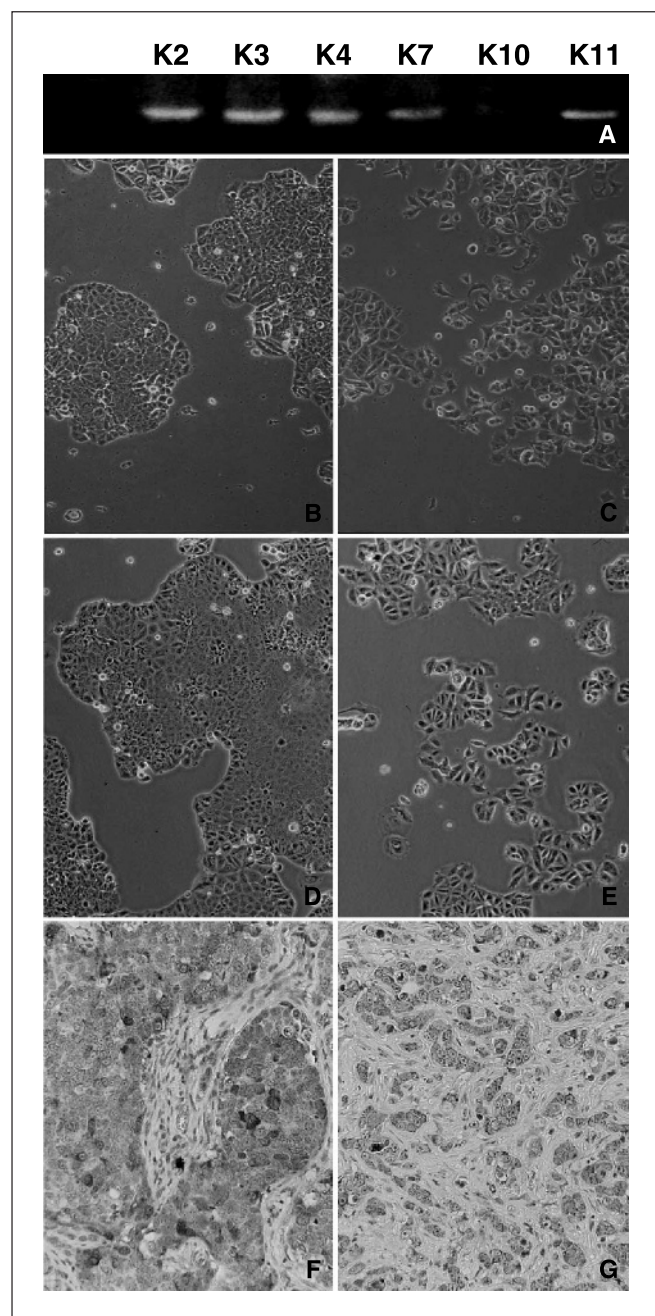


Fig. 1. Relation between galectin-9 expression and cell aggregation in MCF-7 subclones. *A*, immunoblot analysis of galectin-9 in MCF-7 subclones that exhibited pronounced aggregation during proliferation (K2, K3, K4) and in those that did not (K7, K10, K11). *B* and *C*, phase-contrast images of MCF-7 subclones K4 (*B*) and K10 (*C*), which exhibited both galectin-9 expression and cell aggregation at high levels and at low levels, respectively. Cells were seeded at a density of 50,000 cells/mL in 30-mm plastic dishes and were observed after culture for 5 days. Magnification, $\times 400$. *D* and *E*, phase-contrast images of MCF-7 subclone K10 after stable transfection either with an expression vector for galectin-9 (showing marked cluster formation (*D*)) or with the empty vector (showing a low level of aggregation (*E*)). Cells were plated and cultured as in *B* and *C*. *F* and *G*, immunostaining of galectin-9 in tumors formed in nude mice by MCF-7 subclone K10 cells that had been stably transfected with a galectin-9 expression vector (*F*) or with the empty vector (*G*). The cells transfected with the galectin-9 vector proliferated to form large nests, whereas those transfected with the control vector formed small nests. Magnification, $\times 400$.

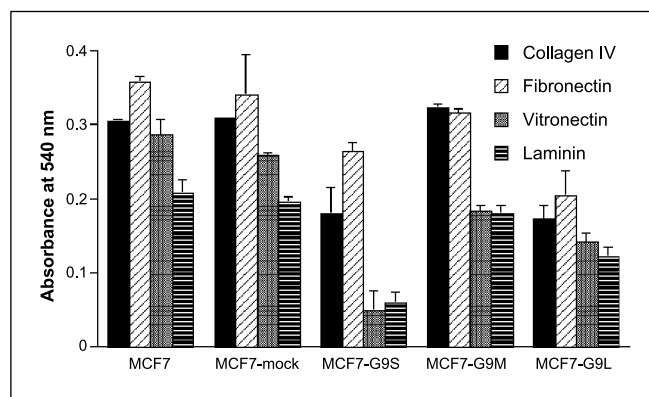


Fig. 2. Assay for cell adhesion. Adhesion of galectin-9-transfected MCF-7 cells were examined. MCF-7 mock, transfectants by empty vector; MCF-7-G9S, -G9M, and -G9L, transfectants by expression vectors of galectin-9S, M, and L, respectively.

expressing the S and L types of galectin-9 exhibited reduced adhesion to type IV collagen, fibronectin, vitronectin, or laminin *in vitro* (Fig. 2). These results thus suggested that galectin-9 might inhibit tumor cell invasion to extracellular matrix and attachment to vascular endothelium, considering that type IV collagen also expresses on vascular endothelial cell surface.

Expression of galectin-9 in breast cancer tissue and its relation to prognosis. We examined galectin-9 expression in tumor tissue of 84 patients with breast cancer by immunohistochemical staining. Galectin-9 was detected in the cytoplasm but not in the nucleus of the cancer cells (Fig. 3). Tumors from 42 of the 84 patients (50%) were positive for galectin-9. Galectin-9 expression was not detected in tumors from 19 of the 21 patients with distant metastasis ($P < 0.0001$), indicating that galectin-9 expression was inversely associated with distant metastasis. Galectin-9 expression was correlated with histopathologic grade, but not correlated, however, with node status, estrogen receptor status, clinical stage, or adjuvant therapy (Table 1). By the analysis, in which galectin-9 is not involved, either node status or stage was significantly correlated with distant metastasis ($P = 0.0010$ and 0.0007 , respectively; data not shown).

The cumulative disease-free survival ratios for patients with galectin-9-positive or galectin-9-negative tumors were 95% and 44%, respectively, and disease-free survival curves are shown in Fig. 4A. Patients with galectin-9-positive tumors had a more favorable disease-free survival than those with galectin-9-negative tumors ($P < 0.0001$). The same results were obtained both in node-negative ($P = 0.010$; Fig. 4B) and in node-positive cases ($P < 0.0001$; Fig. 4C). During the period of follow-up, none of the 13 patients with both galectin-9 expression in tumor tissue and lymph node metastasis up to level II manifested distant metastasis (Fig. 4D). It was thus possible to stratify disease-free survival by node status in the galectin-9-negative group (Fig. 4E) but not in the galectin-9-positive group (Fig. 4F).

Multivariate analysis with Cox's proportional hazards regression model showed that galectin-9 and node status were significant predictive factors for distant metastasis of breast cancer, and that low galectin-9 expression was associated with a higher relative risk for metastasis in patients with breast cancer than was node status (Table 2). Furthermore, a stepwise selection method revealed that the influence of galectin-9 status on the development of distant metastasis was independent of that of node status (Table 3).

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Discussion

We have showed a contribution of galectin-9 to the aggregation of breast cancer cells both *in vitro* and *in vivo*. Galectins exhibit a variety of biological functions including mediation of cell aggregation. We have previously shown that exogenously added recombinant galectin-9 induced the aggregation of red blood cells (16) and of eosinophils (17). In melanoma cells, galectin-9 at the cell surface, but not that in the cytoplasm, participates in cell aggregation (15). Exogenously added recombinant galectin-9 also induced melanoma cell aggregation in a manner that was sensitive to lactose, which competitively inhibits the interaction between galectin-9 and β -galactoside. These observations suggest that the interaction of galectin-9 on the surface of melanoma cells with its ligand is required for cell aggregation.

In the present study, we found that the aggregation of MCF-7 cells was associated with the expression of galectin-9 in the cytoplasm. We detected little or no difference in the expression levels of other galectins, including galectin-1, -3, and -8, among MCF-7 subclones (data not shown), indicating that the role of galectin-9 in the aggregation of these cells is specific. Galectin-9 was not detected at the surface of MCF-7 cells, even of those in aggregates. However, we cannot exclude the possibility that a low level of galectin-9 expression at the cell surface is sufficient to induce aggregation of MCF-7 cells. This apparent discrepancy between breast cancer and melanoma cells may be attributable to the difference in cell origin (epithelial versus nonepithelial). Additional studies are required to clarify the functional role of galectin-9 in the cytoplasm as well as the relation between the expression of galectin-9 in the cytoplasm and that at the cell surface.

Cancer cells likely detach from tumor tissue individually during migration into lymphatic or blood vessels and metastasis

Table 1. Correlation between galectin-9 expression in breast tumor tissue and clinical features

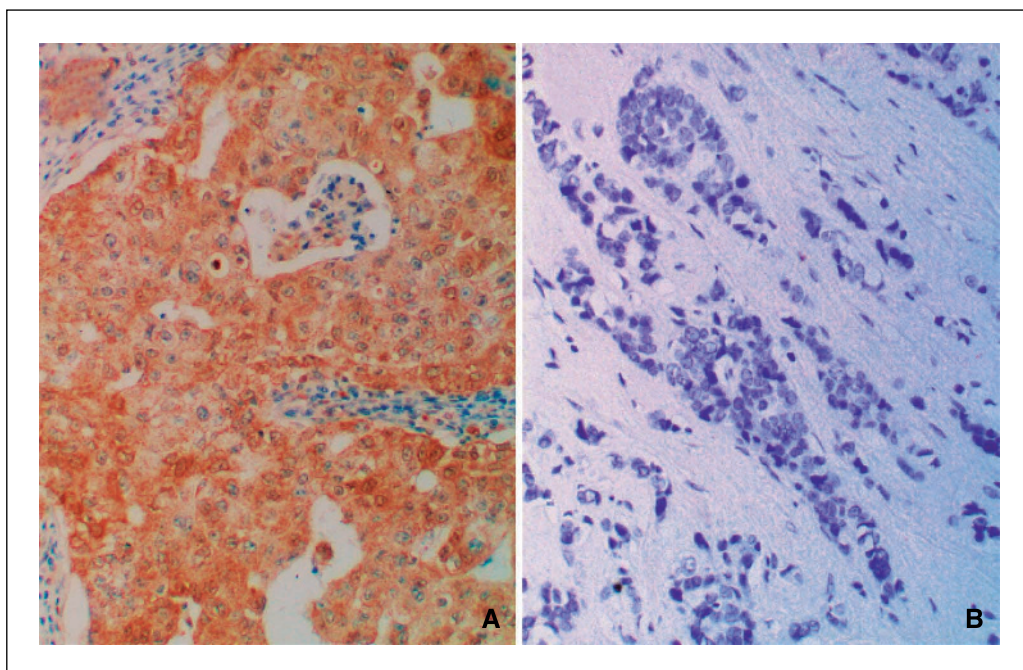
	n	Galectin-9		P*
		Positive	Negative	
Distant metastasis				<0.0001
Positive	21	2	19	
Negative	63	40	23	
Lymph node metastasis				>0.9999
Positive	38	19	19	
Negative	46	23	23	
Estrogen receptor				0.071
Positive	53	22	31	
Negative	31	20	11	
Stage				0.964
I	47	23	24	
II	19	10	9	
III	18	9	9	
Histopathologic grade				0.034
I	24	17	7	
II	34	16	18	
III	26	9	17	
Adjuvant therapy				0.139
None	23	15	8	
Hormone therapy [†]	25	13	12	
Chemotherapy [‡]	36	14	22	

* Analysis by the χ^2 test and Fisher's exact test.

[†] Tamoxifen was administered in all cases.

[‡] Adriamycin-containing regimen, 8 cases; cyclophosphamide-containing regimen, 16 cases; mitomycin, 22 cases; tegafur-uracil, 9 cases. Combination with tamoxifen, 30 cases.

Fig. 3. Immunohistochemical staining of breast cancer tissue for galectin-9 expression. Representative galectin-9–positive (A) and galectin-9–negative (B) tumors are shown. Galectin-9 was detected in the cytoplasm but not in the nucleus of positive cells. Magnification, $\times 400$.



to distant organs. Given that galectin-9 mediates cancer cell aggregation, we hypothesized that it might prevent metastasis. Our present clinical data now show that breast cancer patients with tumors that expressed galectin-9 at a high level had a significantly lower frequency of distant metastasis than did those with tumors with a low level of galectin-9 expression, and this relation was apparent in both node-negative and node-positive cases. In addition to galectin-9, various other biological factors have been shown to correlate with distant metastasis in breast cancer. Expression of HER-2/neu (18–20) or p53 (21, 22) as evaluated by immunohistochemical staining has thus been found to be predictive of metastasis, and measurement of

total cyclin E and its low molecular weight component by immunoblot analysis has been shown to be informative for prediction of survival in node-negative cases but not in node-positive cases (14). However, none of these factors was more effective than was node status in prediction of relative risk. More recently, DNA microarray analysis has revealed a strong correlation between gene expression profiles and distant metastasis in breast cancer (23, 24). Our present results suggest that it is possible to identify patients who need adjuvant therapy after mastectomy on the basis of immunohistochemical determination of galectin-9 status, although chemotherapy is commonly recommended for all node-positive patients.

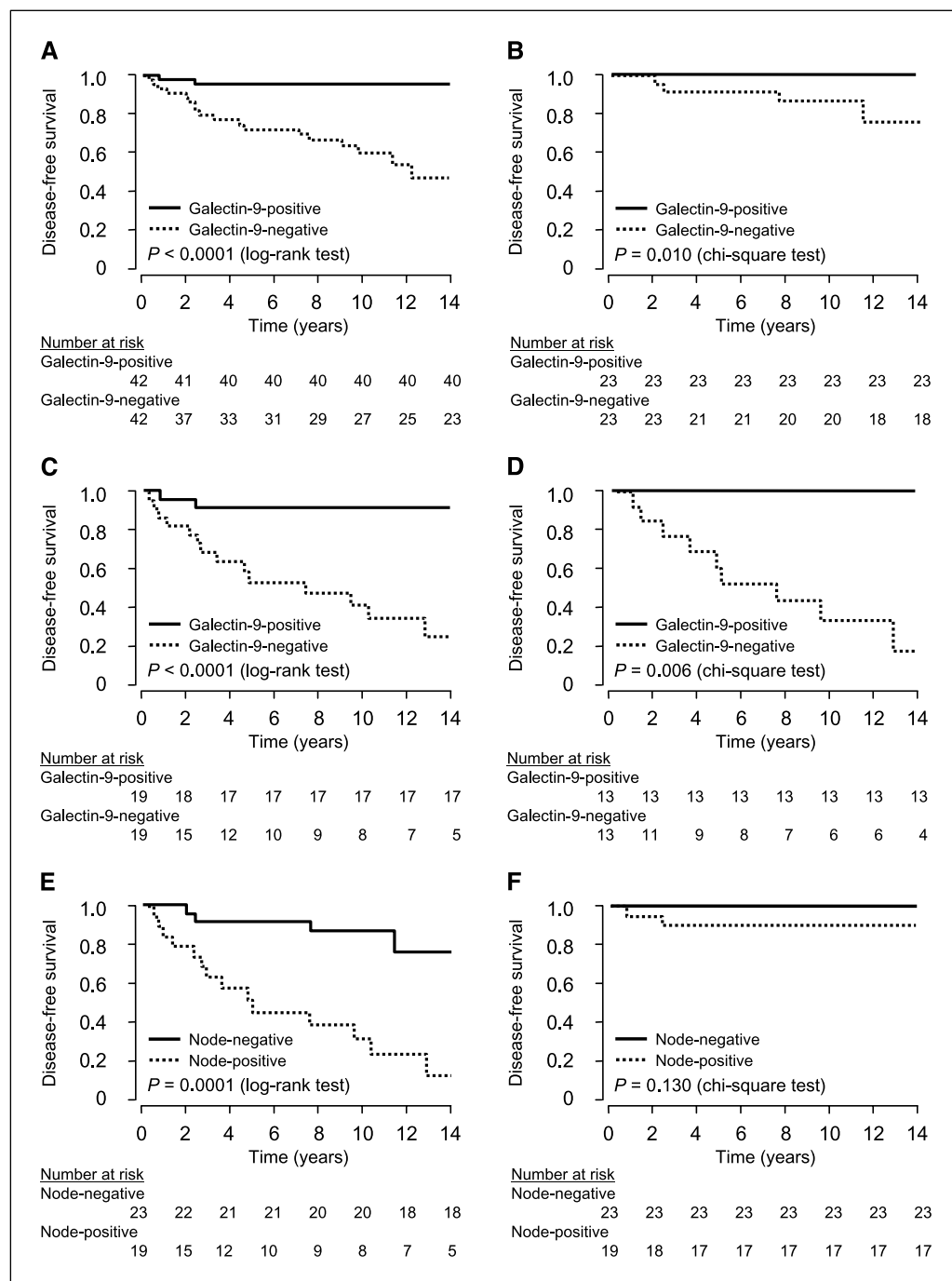


Fig. 4. Disease-free survival curves generated by Kaplan-Meier analysis. A, all cases. B, node-negative cases. C, node-positive cases. D, node-positive cases with axillary lymph node metastasis up to level II. E, galectin-9-negative cases. F, galectin-9-positive cases.

Table 2. Multivariate analysis with Cox's proportional hazards model for prediction of distant metastasis in patients with breast cancer

	Assigned score	Relative risk (95% CI)	P
Galectin-9			
Positive	0	25.496 (5.219-124.557)	<0.0001
Negative	1		
Lymph node metastasis			
Positive	1	7.911 (2.666-23.477)	0.0009
Negative	0		
Estrogen receptor			
Positive	0	2.467 (0.908-6.703)	0.105
Negative	1		

Abbreviation: CI, confidence interval.

Table 3. Variables with independent influence on distant metastasis calculated by a stepwise selection procedure

Step 2*	Assigned score	Relative risk	P
Galectin-9			
Positive	0	18.361	0.0002
Negative	1		
Lymph node metastasis			
Positive	1	6.528	0.0004
Negative	0		

*Galectin-9, lymph node metastasis, and estrogen receptor status were submitted in this analysis.

Galectin-9 expression was correlated with histopathologic grade and inversely correlated with the occurrence of distant metastasis, but not with other clinical features including lymph node metastasis. This suggests that galectin-9 expression changes according to the degree of differentiation, and that poorly differentiated tumors with low galectin-9 expression exhibit metastatic potential. Although two patients with high galectin-9 expression manifested distant metastasis during the follow-up period, these individuals already had lymph node metastasis at level III at the time of operation. Furthermore, the influence of galectin-9 on distant metastasis was independent of that of node status. These results suggest that galectin-9 expression is not associated with node status in patients with breast cancer, in contrast to patients with malignant melanoma, in whom low galectin-9 expression is significantly associated with positive node status (15). The reason for this difference between breast cancer and melanoma remains to be determined.

Galectin-9 has been found to induce apoptosis in T cells (25, 26) and malignant melanoma cells (15). Exogenously added

galectin-9 thus induces both aggregation and apoptosis in melanoma cells, suggesting that both cell adhesion and apoptosis are required for galectin-9-induced suppression of melanoma. In contrast with melanoma cells, exogenously added galectin-9 induced apoptosis in MCF-7 cells at most 25%, and did not induce aggregation. This discrepancy may also be attributed to the difference in cell origin. We also show that transfection by galectin-9S or -9L inhibited the adhesion of MCF-7 cells to the molecules on extracellular matrix (fibronectin, vitronectin and laminin) and on endothelial cells (collagen type IV). Taken together, galectin-9 suppresses metastasis in multisteps by inhibiting invasion to extracellular matrix, detachment from tumors, and attachment to vascular endothelium. Our data thus suggest that galectin-9 expression is a new and useful prognostic factor with antimetastatic potential in patients with breast cancer. It may also prove to be a prognostic factor for other malignant tumors, especially malignant melanoma.

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