Growth/Adhesion-regulatory Tissue Lectin Galectin-3: Stromal Presence but not Cytoplasmic/Nuclear Expression in Tumor Cells as a Negative Prognostic Factor in Breast Cancer

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Abstract. *Background: The endogenous lectin galectin-3 can regulate cell adhesion and proliferation in vitro, thus prompting the examination of its clinical relevance in breast cancer. Materials and Methods: Immunohistochemical processing of tissue sections (n=273; drop-out rate 20.4%) was used for the assessment of galectin-3 expression. Cytoplasmic/nuclear staining and presence in the tumor stroma were analyzed in human breast cancer patients. Results: A weak correlation with positive steroid receptor status was revealed for cytoplasmic positivity. Nuclear staining was correlated to the lobular type of invasive carcinoma, and tumor stroma expression to high-grade malignancy. Multiple testing of cut-off points to divide the cases into groups based on different levels of immunopositivity combined with univariate Kaplan-Meier survival analysis and computations following the multivariate Cox regression model disclosed no prognostic correlation to either cytoplasmic or nuclear expression of galectin-3. The presence of galectin-3 in the stroma, however, indicated an unfavorable prognosis. Prediction of overall survival was feasible using a model consisting of stage and c-erbB2 status. Conclusion: These data signify that caution should be exercised in extrapolating from the anti-apoptotic/prometastatic activity of galectin-3 in model systems to the clinical situation.*

Malignant transformation is accompanied by alterations in cell surface glycosylation which so far have been interpreted as aberrations in the glycosylation machinery (1, 2). Due to the recent realization of the unsurpassed capacity of carbohydrates to store biological information, the detection of endogenous lectins and the emerging delineation of the functional interplay between lectins and distinct carbohydrate epitopes relevant for growth/adhesion regulation this situation has changed considerably (3, 4). In fact, the terminal, spatially accessible galactose epitopes in particular are now considered to act as potential docking sites for tissue lectins. Fittingly, the enzymatic machinery to generate these sites is variegated, enabling notable structural diversity, and several lectin families are involved with targeting them. Among them, the galectins are receiving special attention for two reasons: their expression in normal and tumor tissue, and their proven activity as modulators of proliferation/apoptosis, adhesion, migration and other cellular activities such as mediator release (5-7). Intriguingly, in addition to their carbohydrate-mediated functionality certain galectins also harbor the capacity for salient protein-protein interactions en route acting as effectors of cell growth or presumed regulators of gene transcription/pre-mRNA splicing (8-11).

Prominent in this respect, the only chimera-type galectin (the carbohydrate recognition domain is associated with a collagenase-sensitive stalk and an amino-terminal region of twelve amino acids controlling intracellular targeting) is distinguished from the other family members by its distinct multimodular assembly. It accounts for localization at nuclear, cytoplasmic and extracellular sites and characteristic aggregation properties when in contact with multivalent ligands (8, 12, 13). Following its detection in breast cancer tissue by immunohistochemistry, reverse transcription-polymerase chain reaction and Western blotting, galectin-3 has been reported to exert strong effects on both non-tumorigenic and tumorigenic cell lines and to protect breast cancer cells from drug-induced apoptosis or to enhance their sensitivity to TRAIL-induced cytotoxicity (10, 14-19). Although the *in vitro* work renders it likely that

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intra- and extracellular galectin-3 is involved in disease progression, this certainly does not automatically prove clinical relevance and prognostic significance. Therefore, it is pertinent and timely to monitor galectin-3 expression in tissue sections and relate these findings to the clinical data of breast cancer patients in order to define any potential of galectin-3 detection for routine histopathological application. In a broader context, the comparison of conclusions from work on cell models and clinical specimens will be informative to gauge the predictive value of models for the clinical situation in this case. Toward this aim, immunohistochemical analysis of 273 samples from an initial group of 343 patients was completed.

Materials and Methods

Patients and tumor material. Three hundred and forty-three female patients with breast carcinoma identified from the routine files of the Department of Pathology, Robert-Bosch-Krankenhaus (Stuttgart, Germany) were included in the study. The cases were diagnosed between 1986 and 1997. The computerized database contained information about the age of the patients, pre- or postmenopausal status, histological type (ductal or lobular invasive carcinoma or specified types of breast carcinoma), grading, number of positive lymph nodes, size of the tumor, stage of the disease at diagnosis, expression of progesterone and estrogen receptors (PR, ER), and c-erbB2 expression, as well as the oncological treatment regimen. All data on TNM and grading were retrieved from the reports of the Department of Pathology. Information about the clinical outcome was obtained by hospital chart review or direct telephone interview with the patients' family doctors resulting in 273 cases available for Kaplan-Meier survival analysis. The drop-out number of 70 cases (giving a drop-out rate 20.4%) was due to patients without follow-up information (N=42), cases without sufficient availability of tumor sections (N=14) and cases where the quality of the tumor tissue was inadequate for reliable analysis (N=14). Biological tumor classification was accomplished by routine immunostaining methods in the cases of ER, PR and c-erbB2. For c-erbB2 detection, the commercial CB-11 antibody (Novocastra, UK) was used. The intensity of c-erbB2 immunostaining was scored from 0-3. Scores 2 and 3 were classified as positive following standard recommendations. Fifty patients received cyclophosphamide, methotrexate and 5-fluorouracil, fifty-four patients were treated with anthracyline-containing treatment regimens, seventy-four patients were treated with tamoxifen and one hundred and thirty-three were treated with radiotherapy.

Immunostaining technique. Immunohistochemical processing was performed on 5 μm-thick sections of formalin-fixed and paraffin-embedded tissues. For antigen retrieval, the sections were treated by microwaves twice for 5 minutes in a citrate buffer (pH antigen retrieval). The endogenous peroxidase activity was blocked by exposing the sections to a 0.03% H_2O_2 solution containing 0.1% sodium azide. After brief washing with PBS buffer, the sections were incubated with the primary mouse monoclonal antibody (dilution 1:100, Novocastra) at 25˚C for 60 minutes. Following further washing steps with PBS buffer to remove unbound antibody, the sections were treated with the biotinylated secondary antibody followed by incubation with the avidin-biotin complex. Staining was completed by a 10 minute incubation with the chromogen 3,3'-diaminobenzidine/hydrogen peroxide. Development of the chromogenic product by peroxidase activity used the chromogen solution as given above. Final staining was performed in hemalaun for 1 min. The controls included staining for cytokeratin 18 (positive control), and omission of the incubation step with the probe to pinpoint antigen-independent staining. Endogenous biotin staining was excluded by comparing positive immunostains with and without the biotin blocking kit and the envision staining kit.

Assessment of immunostaining. The extent of staining in the population of breast cancer cells was expressed as a percentage in each sample, resulting in five groups: 0 corresponding to 0%, 1 correponding to 1%-10%, 2 corresponding to 11%-50%, 3 corresponding to 51%-80% and 4 corresponding to 81%-100% of the carcinoma cells. The intensity of staining was scored on a semi-quantitative scale from 0 to 3. This evaluation is similar to the accepted scoring of ER or PR in breast cancer. The two scores of extent and intensity were multiplied as in the steroid receptor method to obtain the total immunoreactive score (IRS score for galectin-3) of the carcinoma cells for each case (20). The stroma expression of galectin-3 was scored as absent/weak or moderate/strong. The nuclear galectin-3 expression was scored 0 (<5% positive tumor nuclei) or 1 (nuclear staining >5%) independently of the galectin-3 scoring in the tumor cell cytoplasm. The staining features of the normal breast tissue, surrounding stroma, leukocytes and blood vessels were also assessed by a score ranging from 0 to 3. The results of immunohistochemical staining were independently evaluated by a pathologist (P.F.) and two physicians. In cases of differences between results, the assessment of the pathologist was used. The tumor stroma was defined as the mesenchymal cells either between tumor cells or surrounding the tumor. Only galectin-3 staining in fibrocyctes or fibroblasts was assessed for scoring expression of galectin-3 in the tumor stroma.

Statistical analysis. Included and excluded patients were compared by the Chi-square test, Fisher's exact test and the *t*-test. A level of $p>0.05$ was considered to indicate that the characteristics of both populations did not differ. Co-linearity was tested by the Chi-square test ($p>0.05$ signifiying no co-linearity) or Fishers' exact test (when Chi-square could not been applied). Different cut-off points were used for separating galectin-3-positive from negative tumor cases as shown in Table II. For each cut-off point the significance was calculated by log-rank tests in the Kaplan-Meier analysis. The survival analysis was performed using the Kaplan-Meier test and the Cox hazard model. All parameters that were significant in univariate analysis were included in the Cox model. Inter- and intra-observer variability was calculated by the kappa statistics (21) measuring the extent of agreement statistically surpassing chance. A kappa value of >0.8 denotes excellent, >0.6 good, and >0.4 moderate concordance.

Results

Patient characteristics and controls. A drop-out rate of 20.4% was observed. Comparison of the characteristics showed no significant differences between the groups of included and excluded drop-out patients except for the menopausal status (weakly significant; data not shown). Thus no notable bias

Table I. *Prognostic evaluation of the tested panel of parameters of the patients (N=273).*

Parameter	Number (%)	Missing cases	Log-rank	*p*
Premenopausal	50 (26.2)	82	0.18	0.1676
Postmenopausal	141 (73.8)			
Invasive ductal	218 (79.8)	0	3.8	0.152
Invasive lobular	25 (9.2)			
Not specified	30 (11.0)			
pT1	74 (27.5)	5	27.8	<0.00001
pT2	130 (48.3)			
pT3	30 (11.2)			
pT4	35 (13.0)			
G1	12 (4.5)	5	3.7	0.155
G2	169 (63.1)			
G3	87 (32.5)			
pN0	117 (43.5)	4	37.6	<0.00001
pN1	132 (49.1)			
pN2-N3	20 (7.4)			
M0	256 (95.8)	6	45.3	<0.00001
M1	11 (4.2)			
Stage				
I	49 (18.6)	9	25.4	<0.00001
II	141 (53.6)			
III	62 (23.5)			
IV	11 (4.2)			
Estrogen receptor				
positive	163 (62.4)	14	1.51	0.22
negative	95 (36.6)			
Progesterone receptor				
positive	154 (60.4)	18	0.3	0.59
negative	101 (39.6)			
c-erbB2				
negative	195 (80.3)	29	6.8	0.009
positive	48 (19.7)			
Cytoplasmic galectin-3				
absent	96 (35.2)	0	4.0	0.136
low-moderate	149 (54.5)			
strong	28 (10.3)			
Nuclear galectin-3				
absent	153 (75.0)	69	0.0	0.86
present	51 (25.0)			
Galectin-3 in tumor stroma				
absent	146 (73.7)	75	3.8	0.05
present	52 (26.3)			

Table II. *Galectin-3 positivity of breast cancer specimens.*

Scoring	Cytoplasmic galectin-3 expression in tumor cells		
0	96	35.1%	absent 35.1%
1	32	11.7%	low to moderate 54.6%
2	31	11.4%	
3	8	2.9%	
4	39	14.3%	
6	39	14.3%	
8	7	2.6%	high 10.3%
9	8	2.9%	
12	13	4.8%	
Nuclear galectin staining in tumor cells: absent *versus* present			
Absent	153	75.0%	
Present	51	25.0%	
Galectin-3 in tumor stroma			
Absent or weak[a]	146	73.7%	
Moderate or strong	52	26.3%	

[a]Only specimens with sizeable tumor stroma and sufficient tumor area were assessed.

was introduced by the reduction of the group size of patients. The mean age of the studied patients was 56.84 years (SD±13.03, median 56). The mean follow-up for all patients (survivor and non-survivor) was 76.59 months (SD±44.14, median 69.13) with a range of 185 months. The mean follow-up for survivors was 90.61±42.17 months. The mean disease-free survival was found to be 65.74 months (SD± 46.14, median 57.0). One hundred and sixty-six patients were alive and 18 patients had suffered from fatal non-tumor-related diseases. Eighty-eight patients (32.24%) had died because of the tumor disease. The controls during immunohistochemical processing (omitting galectin-3 antibody, second antibody or ABC complex) were negative demonstrating an antigen-independent background. Inter-observer variability (assessment of staining results by blind observers) was moderate (kappa=0.50, $p<0.001$).

Immunohistochemical staining pattern in non-malignant tissue. Galectin-3 was expressed in non-malignant tissue, as shown in Figure 1a-d. The staining of the breast tissue was heterogeneous, often displaying stronger reactivity than in carcinoma cells. There was no significant difference in the staining intensity between the lobuli and ductuli of breast tissue. The blood vessels showed variable staining patterns, most of them being strongly positive (see Figure 1c). When the tumor stroma was analyzed for galectin-3 expression, 26.3% of all tumors were found to be moderately or strongly positive (Table I, Table II). In several specimens fibroblasts or fibrocytes in the tumor stroma revealed nuclear staining for galectin-3. Non-tumor stroma failed to be reactive. Stromal staining for galectin-3 was positively related to nuclear staining (Chi-square=7.1, $p=0.0076$). Inflammatory cells were positive, most of them being monocytes or histiocytes (see Figure 1d). Lymphocytes, plasma cells and neutrophil leukocytes were negative.

Staining pattern in tumor tissue. The histopathological data of the patients is summarized in Table I and galectin-3-dependent results for cytoplasmic and nuclear localization

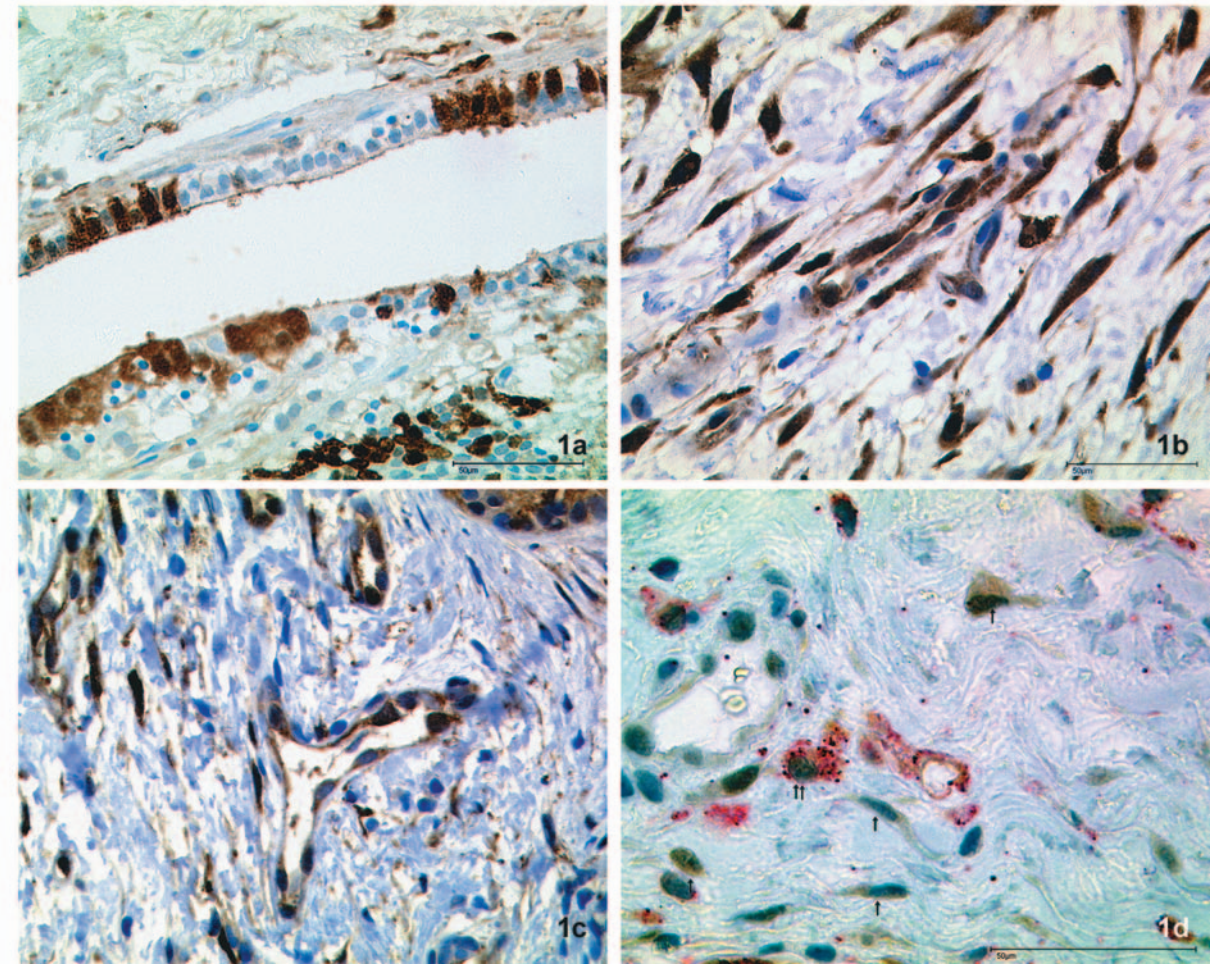

Figure 1. *Galectin expression in non-tumoral breast tissue. a) Normal breast lobuli. b) Galectin-3-positive histiocytes in dilated ductulus. c) Galectin-3 expression in breast ductulus. d) Galectin-3-positive vessel endothelium.*

and tumor stroma are detailed in Table II. Examples of galectin expression in breast cancer tissues for processed cases are documented in Figure 2. The staining of primary breast cancer cells was often heterogeneous, showing strongly and weakly stained areas of the tumor in the same section. Of the breast cancer cases 10.3% were characterized by a high level of cytoplasmic galectin-3 expression (score >8), whereas 35.1% of the cases were devoid of any detectable galectin-3, as shown in Table II. Staining was found in both the cytoplasm and the nuclei of tumor cells, cytoplasmic staining being predominant. Galectin-3-positive tumor nuclei (>5%) were found in 25% of patients. Galectin-3 presence (cytoplasmic, nuclear and/or in the tumor stroma) was not related to any of the monitored variables such as T (T1 *versus* T2-4) (p=0.14), N (p=0.80), M (p=0.93), tumor stage (p=0.82), tumor grade (p=0.49), histological type of breast cancer (p=0.50), age (p=0.96), menopausal status (p=0.53) or to c-erbB2 status in the Chi-square test (p=0.41). Hormone receptor status for the estrogen (p=0.06), but not the progesterone receptor (p=0.64) appeared to correlate to a noteworthy, albeit not significant extent with cytoplasmic expression of galectin-3. Nuclear staining for galectin was more frequent in invasive lobular carcinoma than in invasive ductal carcinoma (p=0.001), and galectin-3 expression in tumor stroma was related to a high grade of malignancy (p=0.03) and N (p=0.03).

Kaplan-Meier curves for univariate analysis. Stage, pT, pN, M, and c-erbB2, but not cytoplasmic or nuclear expression of galectin-3 were shown to predict overall survival (OS) and disease-free survival (DFS) in univariate analysis (Figure 3, 4, Table I). These results were obtained regardless of whether all different IRS were considered (Table II) or whether cases with a low level of galectin-3 expression were compared with those expressing high levels of galectin-3.

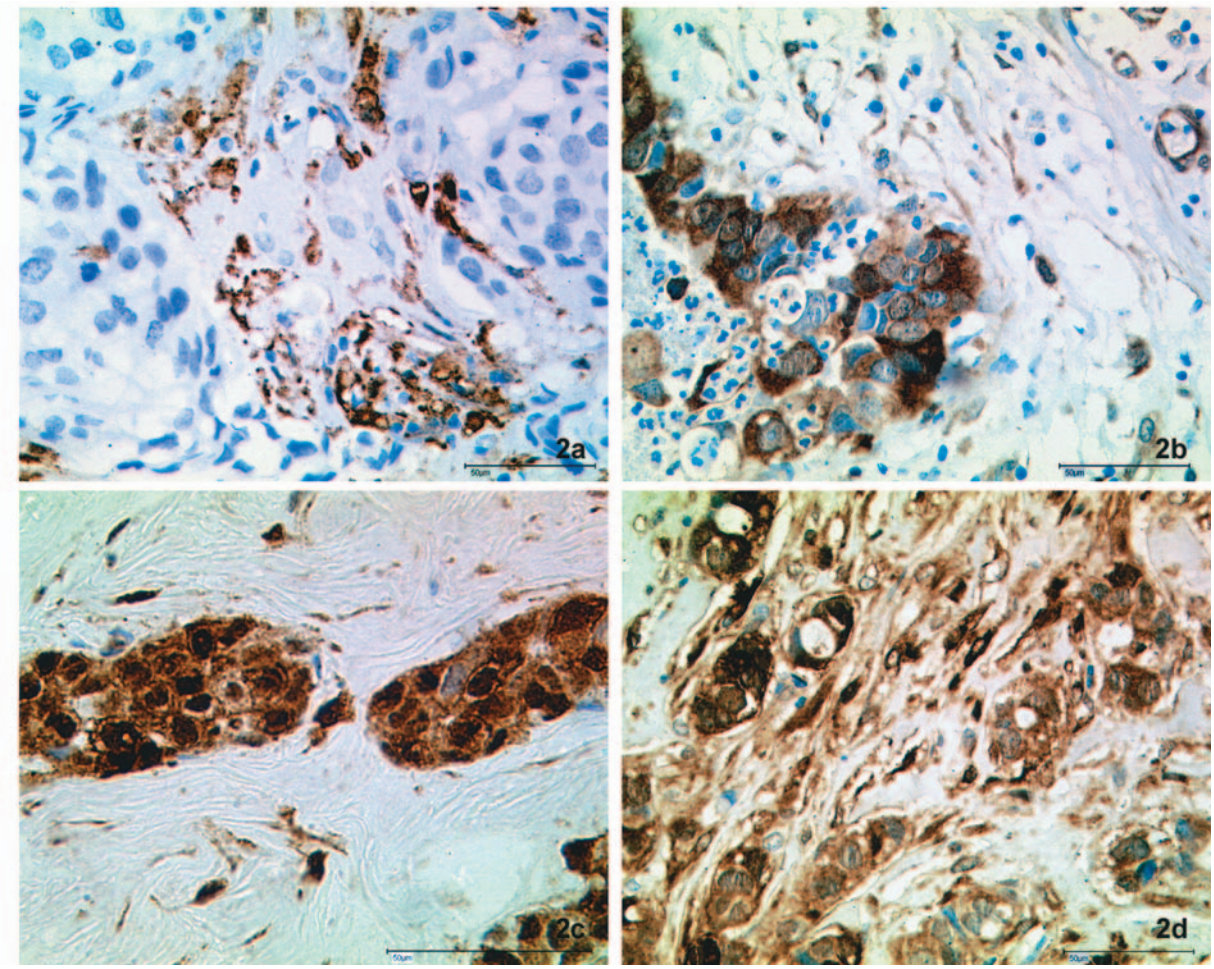

Figure 2. *Galectin expression in breast cancer. a) Invasive ductal breast cancer without cytoplasmic galectin-3 expression. b) Invasive ductal carcinoma with strong cytoplasmic galcetin-3 expression. c) Nuclear galectin-3 expression in tumor cells. d) Galectin-3 expression in tumor stromal cells*

Nuclear staining for galectin-3 in tumor cells was not related to disease outcome. The data for tumor stroma expression of galectin-3 demonstrated a negative correlation to prognosis ($p=0.05$) (Figure 5). One-year OS was 95.9% for the galectin-3-negative tumor stroma *versus* 94.2% for the cancer specimens expressing galectin-3 in the tumor stroma. Three-year survival was 82.4% *versus* 78.6%, the 5-year survival 74.8% *versus* 54.9% and the 10-year survival 59.0% *versus* 40.4% for the galectin-3-negative *versus* -positive tumor stroma respectively.

Cox regression model for multivariate analysis. As shown in Table III (listing only the significant results), cytoplasmic galectin-3 was not correlated to prognosis in a multivariate analysis using the Cox regression model for prediction of OS or DFS as far as nuclear or cytoplasmic expression was concerned. The final parameter set for predicting OS in this patient group consisted of c-erbB2 expression, stage, and stromal galectin-3 expression. For DFS, only stage was prognostic in the Cox model, while galectin-3 expression did not reach significance ($p=0.099$).

Discussion

The example of plant lectins commonly used as mitogens signifies that protein-carbohydrate interactions can elicit a variety of physiological responses (22). In addition to serving as mitogens for B- or T-cells, tumor cells too, can directly or indirectly be stimulated, as was shown, for example, for a mistletoe lectin (23, 24). While it is a routine histochemical technique to monitor the glycosylation of cells (glycomic profile) with plant lectins (25), the detection of endogenous lectins is a key step toward evaluation of the functionality of glycans.

Firstly, the high frequency of galectin-3 expression which was noted in previous studies has been confirmed (15, 26-

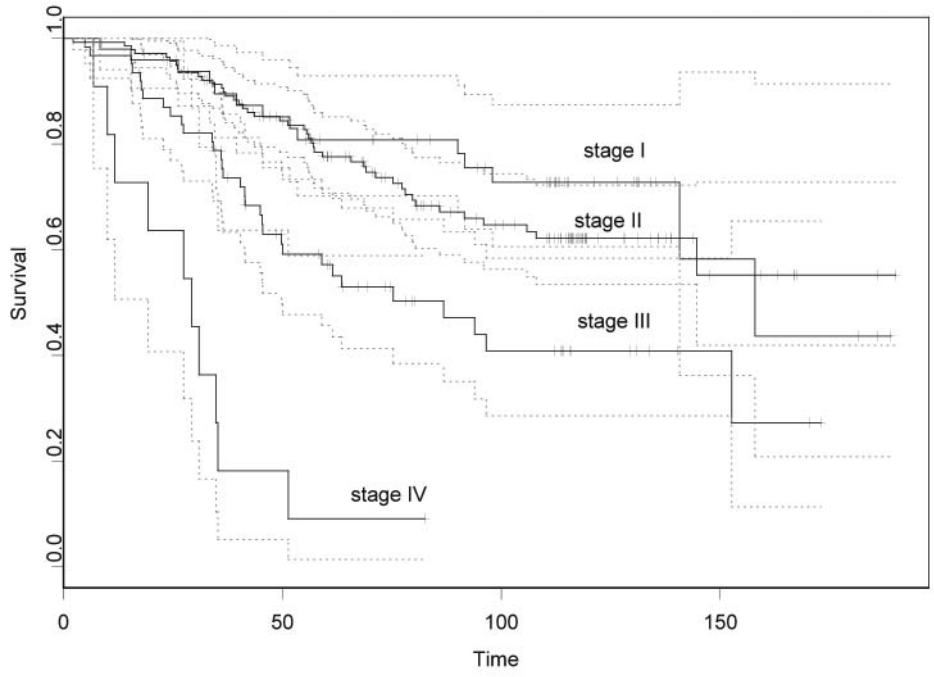

Figure 3. *OS and tumor stage ($p<0.00001$).*

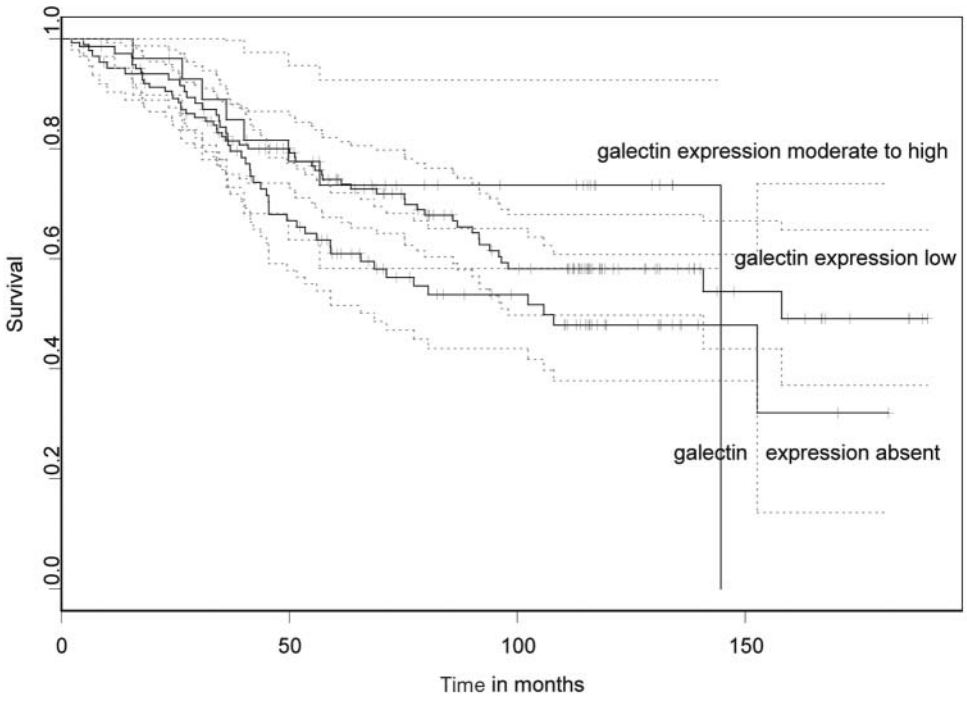

Figure 4. *OS and cytoplasmic galectin-3 expression ($p=0.136$).*

30), with 64.9% of the breast cancer patients revealing at least weak immunostaining for galectin-3. Gene expression profiling of immortalized human mammary luminal epithelial cells with different levels of c-erbB2 have indicated down-regulation of galectin-3 expression (31). In our study, however, the c-erbB2 status and galectin-3 expression were not correlated ($p=0.41$). Next, galectin-3 expression in tumor cells (cytoplasmic or nuclear presence)

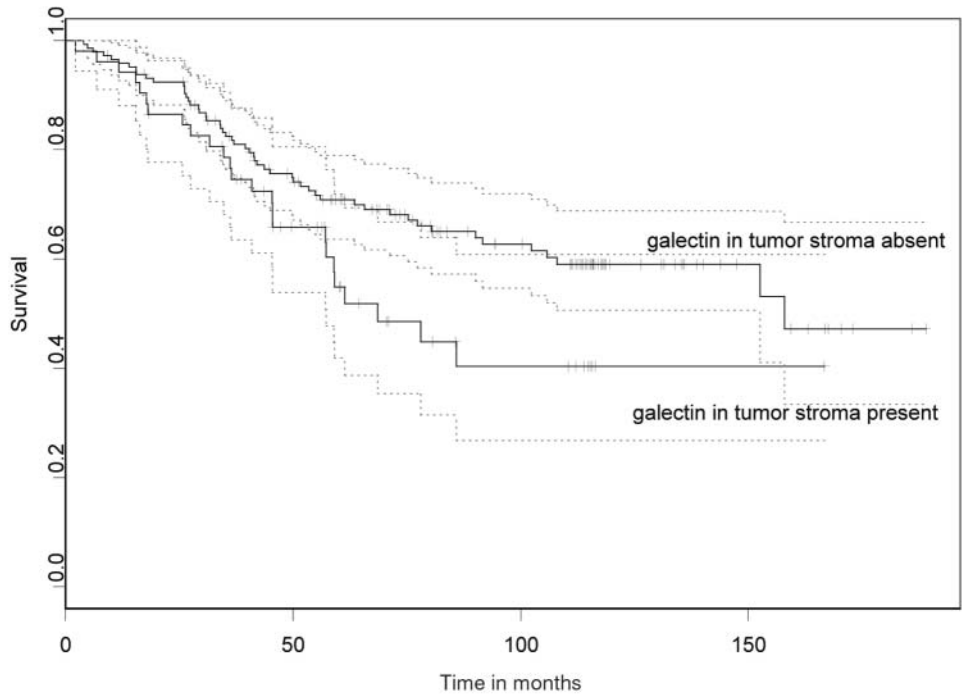

Figure 5. *Galectin-3 expression in tumor stroma (fibrocytes and fibroblasts) (p=0.05).*

Table III. *Cox regession analysis for OS and DFS.*

Variable	Event	B	SD	Wald z-value	*p*-value	Exp(B) (RR)	Confidence interval 95% lower-upper
Stage	OS	0.657	0.207	3.17	0.0015	1.929	1.285-2.900
c-erbB2	OS	0.301	0.310	1.936	0.053	1.359	0.996-1.8501
Stromal galectin-3	OS	0.745	0.1587	2.4908	0.013	1.405	1.156-3.892
Stage	DFS	0.709	0.215	3.296	0.00098	2.031	1.332-3.10
Stromal galectin-3	DFS	0.482	0.293	1.620	0.0990	1.648	0.9131-2.870

Likelihood ratio test: 30.2, $p=0.000792$; Wald test 29.1; $p=0.00119$.

provided no prognostic information, a result not readily reconcilable with the data from model systems where galectin-3 expression was a feature of the malignant phenotype, or from a recent tissue-array investigation on nuclear positivity as a negative indicator (16, 30). In contrast, the presence of galectin-3 in the stroma negatively affected survival, a potential functional factor being the enhancement of migration (32). Since the extrapolation of galectin-3-dependent parameters from one tumor class to other entities is not justified, the relevance of cytoplasmic expression in tongue carcinoma and nuclear expression in adenocarcinoma and squamous cell carcinoma of the lung for disease recurrence and of galectin-3 presence during progression of melanoma and colorectal carcinoma deserves attention (33-36).

The galectins form a complex, currently not yet precisely defined, network of effectors (37-39). Looking at evidence from RT-PCR and immunohistochemistry, galectins-1, -2, -4, -7, -8 and -9 could be expected to be co-expressed in breast cancer with galectin-3 to a currently unknown level (14, 40), and a study on colon cancer highlights the importance of extending the studies of a few selected galectins to comprehensive fingerprinting (41). In fact, functional divergence was noted for galectins-1, -2 and -7 *versus* galectin-3 in a tumor model at the level of ligand selection; in this case the pentasaccharide of ganglioside GM_1 served as a high-affinity ligand (42-45). Now that the respective reagents are available, it is possible to envisage that the fingerprinting of clinical tumor samples as well as the scrutinization of cell models can provide clues to explain the reported data. In addition, the availability of labeled

galectins as tools to monitor the presence and localization of binding sites histochemically warrants their exploitation in this respect (15, 40, 48-52).

Acknowledgements

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