

# Adhesion/Growth-regulatory Tissue Lectin Galectin-1 in Relation to Angiogenesis/Lymphocyte Infiltration and Prognostic Relevance of Stromal Up-regulation in Laryngeal Carcinomas

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**Abstract.** *Background:* Galectin-1 has been found to modulate lymphocyte invasion in inflammation and to be involved in angiogenesis in models, thus prompting examination of its clinical relevance in laryngeal cancer. *Patients and Methods:* Immunohistochemical processing of tissue sections ( $n=53$ ) from patients with stage I/II ( $n=35$ ) and stage IV ( $n=18$ ) laryngeal squamous cell carcinoma (LSCC) with a specific anti-galectin-1 antibody and monitoring of CD45/CD31 positivity was combined with quantitative morphometric analysis. *Results:* Lectin presence in the tumor and endothelial cells was positively correlated, while a negative relationship to the number of CD45-positive lymphocytes was demonstrated. No association was seen with the extent of neovascularization. The mean optical density (MOD) of lectin-dependent staining in the tumor stroma was significantly increased compared to normal stroma. *Conclusion:* Galectin-1 was not associated with angiogenesis in the studied cohort while galectin-1 in endothelial cells may negatively influence lymphocyte invasion and the mean optical density for the stromal galectin-1 signal is up-regulated in tumors.

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Tumor cell behaviour is necessarily determined by the expression of effectors and the reactivity to microenvironmental factors, for example from the stroma. Due to the increasing realization that the glycan part of cellular glycoconjugates is a rich source of biochemical signals, the basis of the concept of the sugar code, increasing attention is being directed to delineate the relationship of these properties to tumor features (1). In this context, endogenous lectins act as “translators” and elicit potent biosignaling after contact formation with suitable docking sites (1-3). What had been put forward as an attractive hypothesis, that is a functional role of tumor cell lectins (4, 5), has been convincingly validated, so that the monitoring of distinct sugar receptors has become an active research field.

The example of galectin-1 in glioblastoma, initially detected by virtue of its sugar-specific binding using neoglycoproteins, and then defined as a prognostic marker with an impact on tumor invasion, has set an instructive example for the dynamics of the field (6, 7). Tumor models *in vitro* have delineated galectin-1 activity as a tumor growth modulator, for example in hepatocellular carcinoma and neuroblastoma cells (8, 9). The orchestration of glycan remodeling and galectin-1 up-regulation by the tumor suppressor p16<sup>INK4a</sup> in pancreatic carcinoma cells to reconstitute susceptibility to anoikis underscores the potential and tight control of this lectin (10). Additionally, its ability to bind to intracellular ligands such as oncogenic H-ras (11, 12), has broadened the spectrum of activities, making the lectin an attractive study object in immunohistopathology. Prognostic relevance has been inferred, for instance in colon carcinoma, and muscle-

infiltrating growth in urothelial carcinoma was related to lectin presence (13, 14). Structurally, this lectin is well suited for *cis*-crosslinking and transient *trans*-bridging operative in signaling and cell contacts, and its extended binding site is used for exquisitely selective ligand binding (15-18). When presented by endothelial cells, lectin also has a role in lymphocyte trafficking in inflammation and on angiogenesis in model systems, directing interest to the monitoring of lectin expression in vessels in tumors *in situ* (19-24). Having given analysis of the expression of galectins in head and neck cancer special emphasis and noting a relationship to recurrence and dismal prognosis in laryngeal and hypopharyngeal tumors (25-27), the focus herein was on galectin-1 in laryngeal squamous cell carcinomas (LSCCs). By studying galectin-1 presence quantitatively as well as monitoring the density of CD45-positive lymphocytes and CD31-positive vessels we herein address the following issues. Is endothelial positivity for galectin-1 associated with lymphocyte infiltration? Is vessel density related to galectin-1 presence, and is it of prognostic value? Does stromal positivity depend on tumor cells, and is it of prognostic value?

## **Patients and Methods**

**Characteristics of the patients.** Specimens from a total of 53 patients with LSCC (35 cases of stages I and II, 18 cases of stage IV), who had undergone surgery aimed at curative tumor resection, were studied (Table I). In this laryngeal carcinoma series, the 53 specimens included 37 cases from the glottic location, 14 cases from the supraglottic and 2 from the subglottic site. The mean age was 57 (with a range between 36 and 88) years. These cases were collected by retrospective compilation (January 1989 to December 2001) from the records of the ENT Department of the Hôpital Claude Huriez (Lille, France). The description of the tumor status was based on the histological stage of tumor differentiation (criteria given in (28)) and the TNM classification (29). Patients suffering from SCCs located at other sites of the head and neck area were deliberately excluded from this study. All the tumor specimens came from patients who had not undergone chemotherapy and/or radiotherapy prior to their surgery. As a consequence, this series represented a homogeneous collection of laryngeal SCCs in terms of histopathological and clinical criteria.

**Immunohistochemistry.** All the tumor samples were processed under identical conditions. The specimens were maintained for 24 h in 10% buffered formaldehyde, dehydrated and routinely embedded in paraffin. Immunohistochemistry was performed on 5 µm-thick sections mounted on silane-coated glass slides, as detailed elsewhere (25-27). In the first step, the dewaxed tissue sections were briefly subjected to microwave pretreatment in 0.01 M citrate buffer (pH 6.0) for 2×5 min at 900 W. The sections were then incubated with a solution of 0.4% hydrogen peroxide for 5 min to block endogenous peroxidase activity, rinsed in phosphate-buffered saline (PBS; 0.04 M Na<sub>2</sub>HPO<sub>4</sub>, 0.01 M KH<sub>2</sub>PO<sub>4</sub> and 0.12 M NaCl, pH 7.4) and successively exposed for 20 min to solutions containing avidin (0.1 mg/ml in PBS) and biotin (0.1 mg/ml in

Table I. *Clinical data.*

Variable	Low-stage (I, II) LSCC 35 cases	High-stage (IV) LSCC 18 cases
Age (years)		
Range	36-88	43-78
Average	57	57
Gender (cases)		
Male	35	18
Female	-	-
Site (cases)		
Supraglottic area	5	9
Glottic area	24	1
Supraglottic and glottic areas	6	6
Subglottic and glottic areas	-	2
Histological grade (cases)		
Well-differentiated	33	10
Moderately differentiated	2	8
Poorly differentiated	-	-
TNM stage (cases)		
T1N0M0	31	-
T2N0M0	4	-
T4N0M0	-	9
T4N1M0	-	5
T4N2M0	-	6
Tumor treatment (cases)		
CO <sub>2</sub> laser cordectomy	8	-
Frontolateral laryngectomy	2	-
Vertical partial laryngectomy	3	-
Supracricoid partial laryngectomy	19	3
Supraglottic laryngectomy	3	-
Total laryngectomy	-	15
Treatment of the neck (cases)		
Functional neck dissection	19	23
Radical neck dissection	-	2
Histology (cases)		
Positive margins	3	-
Larynx cartilage invasion	-	11
Positive node/capsular effraction	3/-	13/5
Recurrence (cases)		
Local	3	4
Nodal	2	1
Distant	-	6
Follow-up		
Range (months)	2-130	5-74
Average (months)	43	30

PBS) to avoid false-positive staining reactions resulting from endogenous biotin. After a thorough washing step with PBS, residual protein-binding sites were non-specifically saturated by incubation with a solution of 0.5% casein in PBS for 20 min. Following these steps the sections were sequentially treated at room temperature with solutions of i) the specific primary anti-galectin-1 antibody (produced in house, Pr. Gabius) rigorously checked for the absence of cross-reactivity to other galectins (30); ii) the corresponding biotinylated second-step antibody (polyclonal goat anti-rabbit IgG) and iii) the avidin-biotin-peroxidase complex (ABC kit reagents). The incubation steps were routinely alternated

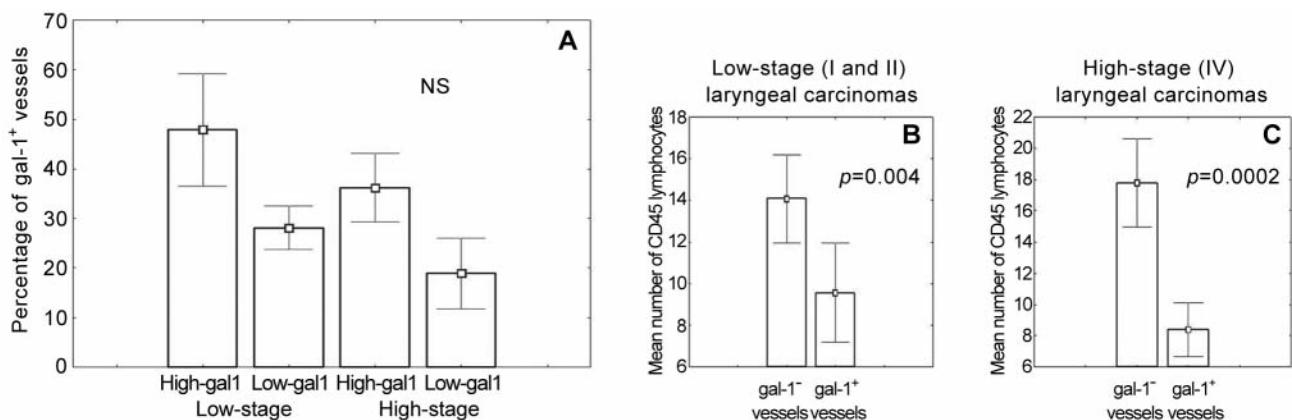


Figure 1. Relationship between level of galectin-1 expression in low- and high-stage tumors and percentage of galectin-1-positive vessels (A). Mean number of CD45-positive lymphocytes (per 16,500  $\mu\text{m}^2$  of tissue area) around galectin-1-positive and -negative vessels in 32 low-grade laryngeal carcinomas (B) and 18 high-grade laryngeal carcinomas (C); p-values computed by the Mann-Whitney non-parametric test.

with thorough washing steps to remove unbound marker proteins. The antigen-dependent presence of the peroxidase complex in the sections was visualized by incubation with the chromogenic substrates diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. After rinsing, the sections were counterstained with luxol fast blue and mounted with a synthetic medium. To exclude antigen-independent staining, the incubation step with primary/secondary antibodies, respectively, was omitted from the protocol in controls. In all cases, these controls were negative. The biotinylated secondary antibody and ABC kit reagents were purchased from DakoCytomation (Glostrup, Denmark). Additionally, the mouse monoclonal anti-CD45 antibody (Neomarkers, Fremont, Westinghouse, USA) and the CD-31 antibody (DakoCytomation, Glostrup, Denmark) were used to detect the presence of lymphocytes and the number of vessels in the LSCC tissue, respectively.

**Computer-assisted microscopy.** After immunohistochemical processing, the galectin-dependent signals were quantitatively evaluated by using a computer-assisted KS 400 imaging system (Carl Zeiss Vision, Hallbergmoos, Germany), as detailed previously (25-27). In each case, 15 fields corresponding to a surface area ranging between 60,000 and 120,000  $\mu\text{m}^2$  were scanned. The computer-assisted morphometric analysis of the parameters of immunohistochemical expression of each marker was quantitative and concerned the labelling index (LI), which referred to the percentage of cells stained by the antibody, and the mean optical density (MOD), which corresponded to the staining intensity (25-27).

**Data analysis.** Independent groups of quantitative data were compared using the non-parametric Mann-Whitney U-test (two groups). Correlation between numerical variables was analyzed by means of the non-parametric Spearman correlation test. The standard survival-time analyses were performed by computing Kaplan-Meier graphs and running the Gehan-generalised Wilcoxon test. Standard Cox regression analysis was also used to fit to the survival data the explanatory models generated on the basis of the variables analyzed in the study (*i.e.* mean number of CD-31-positive vessels). The statistical analyses were carried out using Statistica software (Statsoft, Tulsa, USA).

## Results

**Evidence of negative control of lymphocyte infiltration by galectin-1.** Galectin-1 positivity was found to be distributed in the squamous cell carcinoma and also the endothelial cells, T lymphocytes and macrophages. Of note, a significant number of peritumoral endothelial cells belonged to the immunoreactive cell population. In the low-stage LSCCs with a high level (LI>55%) of galectin-1 presence, about 48% of the endothelial cells (vessels) expressed galectin-1, whereas endothelial-cell positivity decreased (27%) for tumors harboring less galectin-1 (Figure 1A). Similar findings were observed in the high-stage tumor cases (about 35% of endothelial cells with galectin-1-specific immunostaining in tumors presenting high levels of galectin-1 versus 18% of galectin-1-positive endothelial cells in tumors presenting low levels (LI≤55%) of galectin-1) (Figure 1A).

In the sub-series of 32 LSCCs of stages I and II, the mean number of CD45-positive lymphocytes around the lectin-positive vessels was significantly lower than the mean number of CD45-positive cells around the lectin-negative endothelia (Mann-Whitney: p=0.004) (Figure 1B, Figure 2). Similar results were shown in the series of 18 stage IV LSCCs (Figure 1C; Mann-Whitney: p=0.00002). Evidently, the extent of lymphocyte infiltration was negatively associated with the presence of this lectin.

**Lack of evidence of galectin-1 effector function on angiogenesis.** No statistically significant differences were obtained with respect to the MOD or LI variables for galectin-1 presence and vessel staining (correlation between the MOD variable and the mean number of CD31-positive vessels: Spearman r=0.10, p=0.91; correlation between the LI variable and the mean number of CD31-positive vessels:

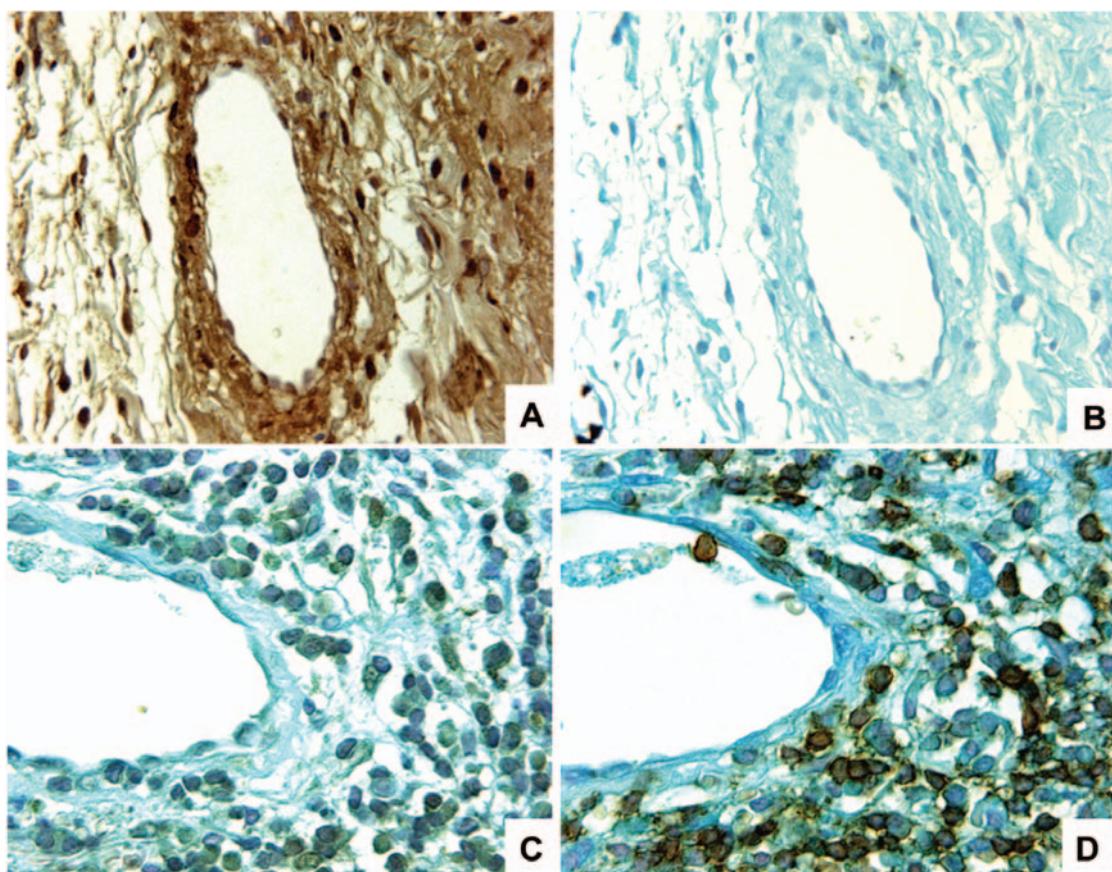


Figure 2. A galectin-1-positive vessel in a high-stage laryngeal carcinoma (A), with low density of CD45-positive lymphocytes (B), and a galectin-1-negative vessel in a high-stage laryngeal carcinoma (C) with surrounding CD45-positive lymphocytes (D). Magnification A-D:  $\times 320$ .

Spearman  $r=0.41$ ,  $p=0.67$ ). By multivariate Cox regression analysis, the tumor stage was found to have significant prognostic value (Table II). In the low-stage LSCCs, 51% of the cases presented more than two CD31-positive vessels (mean number) per field. In the high-stage tumors, 78% of the cases had more than two CD31-positive vessels (mean number) per field. By Kaplan-Meier plot with the threshold of two CD31-positive vessels per field, the difference in the risk of disease recurrence almost reached significance (Wilcoxon test:  $p=0.06$ , Figure 3). Thus, lectin presence in the tumor cells had no discernible influence on the vessel density, but the vessel density itself may have a bearing on tumor recurrence.

**Up-regulation of galectin-1 in tumor stroma.** Different patterns of immunolabelling were revealed in normal and tumor stroma. In normal stroma, galectin-1 was weakly detectable in a small number of fusiform cells (Figure 4A). In contrast, intense signals were seen in the stroma of laryngeal carcinomas (stages I, II, and IV) in the fusiform cells, as

Table II. Cox regression models.

Model/ Variable	$\beta$	$\exp(\beta)$	p-value
Survival analysis p=0.02			
Tumor stage	1.30	3.67	0.04
Number of CD31-positive vessels	0.37	1.46	0.1

The “Model/p-value” defines the overall level of significance of the model. The equation at the basis of the Cox regression model is an exponential function of a linear combination of the variables considered, where  $\beta$  indicates the coefficient of each variable in the linear combination and  $\exp(\beta)$  its exponential value. The p-value is a measure of the level of significance of the contribution of each variable to the model (and leads to the conclusion that  $\beta$  is significantly different from zero).

exemplarily shown in Figure 4B. In quantitative terms, the MOD variable was significantly increased in the tumor stroma compared to normal stroma (Mann-Whitney:  $p=0.005$ ) (Figure 4C). Data for the LI variable gave no indication of a

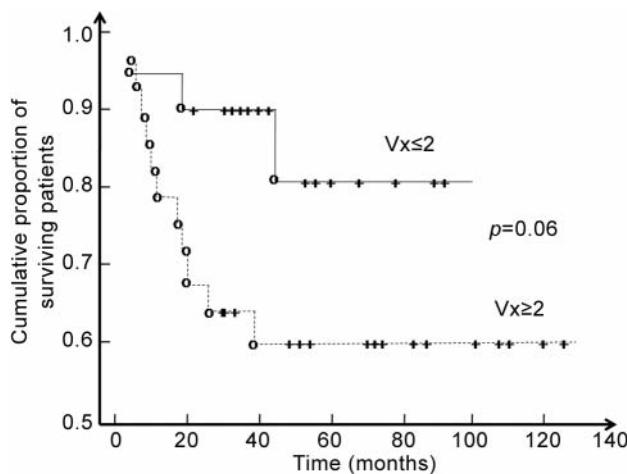


Figure 3. Survival curves of patients with less than or more than a mean of two CD31-positive vessels ( $Vx$ ) per  $16,500 \mu\text{m}^2$  of tissue area.

significant difference. Manual data selection revealed that both quantitative variables (MOD and LI) describing the level of galectin-1 expression in the cancer-associated stroma did not have a significant prognostic value for this group of patients.

## Discussion

Based on initial findings of a negative influence of galectin-1 on trans-endothelial migration of polymorphonuclear leukocytes and T-cell homing to activated endothelium (22, 24), herein *in situ* evidence of a similar role in tumor endothelium is reported. With the focus on tumor-associated lymphocytes galectin-1 presence in melanomas was not correlated to apoptosis of tumor-associated T lymphocytes, whereas CD7-positive T leukemic cells appeared as potential targets of galectin-1 during progression of the Sézary syndrome (31, 32). Regarding the currently ongoing discussion on galectin-1 as an emerging target for interfering with angiogenesis (23) the present data provided no indication of such activity in this group of patients, whereas angiogenesis itself had prognostic relevance.

In addition to the tumor cell properties intense signals were also found in the stroma as evidence of significant up-regulation, as for example detected previously for galectin-3 and breast cancer (33). However, no prognostic value was seen in this case. Nonetheless, the significant increase of stromal lectin expression prompts further investigation of the mechanisms by which tumor cells may be regulated by stromal lectin, such as by monitoring accessible binding sites using labelled tissue lectin, a histochemical step towards functional glycomics (34-36). Should lectin contact to distinct ligands prove to be crucial, the development of blocking compounds against particular

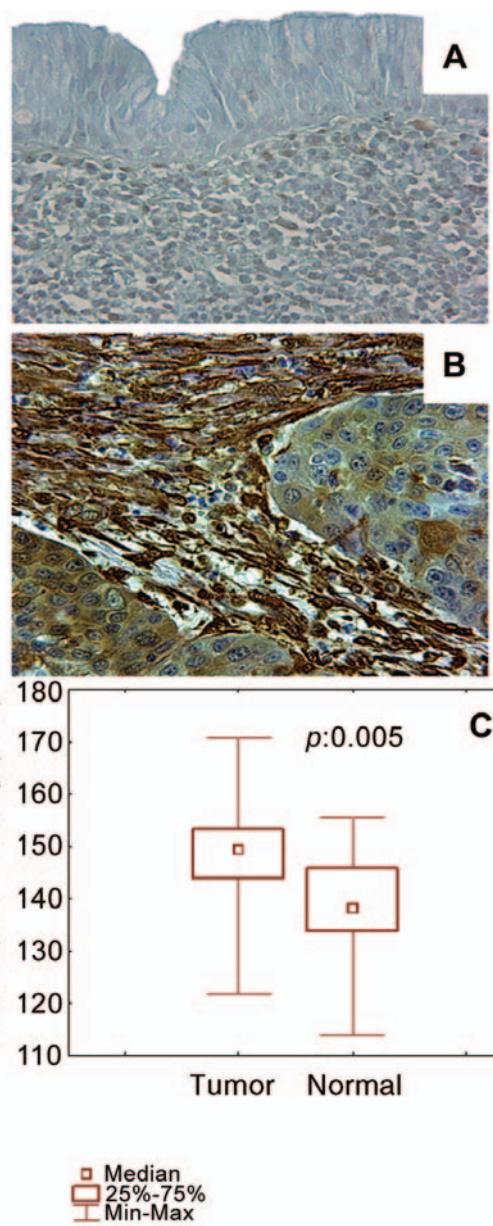


Figure 4. Lack of galectin-1 expression in normal stroma (A) and very strong galectin-1-dependent immunolabelling in tumor-associated stroma (B). Statistical analysis of the MOD data in the two groups (Mann-Whitney;  $p=0.005$ ) (C). Magnification A, B:  $\times 320$ .

galectins, such as by library approaches combined with optimal glycocluster design (37-40), could then provide a therapeutic approach.

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