High level of galectin-1 expression is a negative prognostic predictor of recurrence in laryngeal squamous cell carcinomas

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Abstract. Monitoring of gene-expression profiles is assumed to refine tumor characterization of laryngeal squamous cell carcinomas (LSCCs) with a therapeutic perspective. This is especially expected for adhesion/growth-regulatory effectors such as galectins, a class of endogenous lectins. Using computer-assisted microscopy, we investigated the prognostic value contributed by the quantitative determination of the immunohistochemical levels of expression of galectin-1, -3 and -7 in a series of 62 LSCCs including 42 low- and 20 high-stage LSCCs. As galectin-1 may have a key role leading to a tumor escape from immune surveillance, we also investigated whether or not the level of galectin-1 expression correlated with lymphocyte infiltration in LSCCs. The immunohistochemical determination of expression of galectin-1 is of prognostic value in human squamous laryngeal cancers. LSCCs that display high levels of galectin-1 have worse prognoses than laryngeal cancers with low levels of galectin-1 expression. Elevation of galectin-1 levels in laryngeal cancers can contribute to the process of tumor immune escape by killing the activated T-cells and other protumoral activities such as promoting motility or activity of oncogenic H-Ras proteins. The quantitative determination of galectin-1 in LSCCs is an independent prognostic marker when opposed to TNM staging. It has the potential to identify patients unlikely to benefit from T-cell-mediated immunotherapy, although the definitive effector function from its pro- and antitumoral activity profile has not been delineated.

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Introduction

In Europe laryngeal cancer accounts for 2-5% of all malignancies (1). Of special note for prioritizing development of new treatment modalities, this tumor class (together with cancers of the uterine corpus) do not show an improvement in 5-year survival rates in the United States over the last 30 years (2). Laryngeal squamous cell carcinomas (LSCCs) comprise the vast majority (+95%) of laryngeal malignancies (2). The loco-regional extent of the disease is a key factor for success of treatment (1). Surgery and radiotherapy constitute main therapeutic options (1). The choice between these two procedures is often controversial, and Almadori *et al* accordingly state that a change in the approach to address this disease clinically is required to reduce laryngeal cancer-related mortality (2). Taking advantage of the ongoing correlation of gene expression profiles with development and prognosis of laryngeal cancers, these insights are expected to provide new targets for therapy (3). In detail, these advances from basic science will probably: i) define the molecular characteristics of the cascade leading to laryngeal cancer; ii) provide rational markers for screening, staging, and surveillance; iii) design approaches for targeted therapy, including gene transfer and small-molecule therapy directed at specific molecular pathways involved in neoplasia; and iv) identify high-risk laryngeal cancer patients (3). Extending current gene-expression profiling monitoring gene-expression characteristics can then be directed to distinct protein classes, which have proven their role as cellular effectors. Our report follows this strategy. Due to the emerging importance of glycans of glycoconjugates as biochemical signals and their recognition by lectins for regulating cell activities as diverse as adhesion, angiogenesis, invasion and proliferation/apoptosis we have focused on a special class of endogenous lectins, which in all mentioned cases serve as

potent effectors (4-6). In fact, previous pilot studies on laryngeal cancer have underscored the potential of these proteins in the quest to define markers for the clinical outcome of LSCC patients (7,8). In this respect, studies on aberrations of glycosylation and glycoprotein signatures can acquire a functional dimension, when the function of glycans as ligands for tissue lectins is considered. In this respect, it is noteworthy to mention a prognostic correlation of a mucin, a potential ligand for several members of the galectin family. Paleri *et al* have shown that there is a survival advantage for patients with advanced-stage non-metastatic LSCCs when the mucin 4 gene is expressed (9). In order to test the hypothesis that galectins may influence the clinical route of LSCCs, we herein determine expression patterns of three members of this family. They are known from clinical and laboratory studies to mediate adhesion/growth regulation (10-12). With respect to head and neck squamous cell carcinomas (HNSCCs), the extents of the expression of galectin-3 and the galectin-3-reactive sites correlate significantly with an increasing level of clinically detectable HNSCC aggressiveness (7,8,13,14). To illustrate that galectins likely operate in a network requiring monitoring of more than one family member it is noteworthy that a significant decrease in galectin-8 expression is observed in larynx when malignant tissue is compared to normal tissue and/or benign tumors (15). Moreover, we recently reported that high levels of galectin-7 expression were associated with rapid recurrence rates and dismal prognoses in 81 stage IV hypopharyngeal SCCs, a feature not observed with galectin-3 and only weakly, if at all, with galectin-1 (16). Initially designated as p53-induced gene 1 (*PIG1*), galectin-7 is a regulator of apoptosis in HeLa/DLD-1 cells through JNK activation and mitochondrial cytochrome c release and can act either positively or negatively on tumor development, depending on the histological type of the tumor (17). Evidently, galectin-1, -3 and -7 are associated with the course of disease in different manners in stage IV hypopharyngeal SCCs, and we therefore decided to determine the immunohistochemical levels of their expression (by computer-assisted microscopy) in a series of 62 LSCCs, including 42 low- (T1N0M0 and T2N0M0) and 20-high stage (T4N0-2M0) LSCCs. In view of the facts that galectins can modulate immune and inflammatory responses in general and that galectin-1 in particular might play a key role in helping tumors, including HNSCCs, to escape immune surveillance in a tumor model, albeit not readily transferable to the clinical level, we also examined whether or not the levels of expression of galectin-1, -3 and -7 correlated with lymphocyte infiltration in LSCCs (18-20).

Materials and methods

Patients' characteristics. We retrospectively reviewed the records of previously untreated patients with laryngeal SCCs examined in the ENT Department of the Hôpital Claude Huriez (Lille, France) between January 1989 and December 2001. Patients were deliberately excluded if they had previously suffered from SCCs at other sites in the head and neck area. The present study therefore is based on a series of 62 LSCC patients (1 female and 61 males) who underwent surgery aimed at curative tumor resection. Table I details the clinical

Table I. Clinical data.

Variable	Low-stage LSCCs (stages I and II), 42 cases	High-stage LSCCs (stage IV), 20 cases
Age (years)		
Range	36-88	43-78
Average	57	57
Sex		
Male	41	20
Female	1	-
Site		
Supraglottic area	5	9
Glottic area	31	1
Supraglottic and glottic areas	6	7
Subglottic and glottic areas	-	3
Histological grade		
Well differentiated	35	12
Moderately differentiated	6	8
Poorly differentiated	1	-
TNM stage		
T1N0M0	31	-
T2N0M0	11	-
T4N0M0	-	9
T4N1M0	-	5
T4N2M0	-	6
Tumor treatment		
Co_2 laser cordectomy	8	-
Frontolateral laryngectomy	2	-
Vertical partial laryngectomy	3	-
Supracricoid partial laryngectomy	26	3
Supraglottic laryngectomy	3	-
Total laryngectomy	-	17
Treatment of the neck		
Functional neck dissection	19	23
Radical neck dissection	-	2
Histology		
Positive margins	3	-
Larynx cartilage invasion	-	11
Positive node/ capsular effraction	3/-	13/5
Recurrence		
Local recurrence	3	4
Nodal recurrence	3	1
Distant recurrence	-	6
Follow-up (months)		
Range	2-130	5-74
Average	43	30

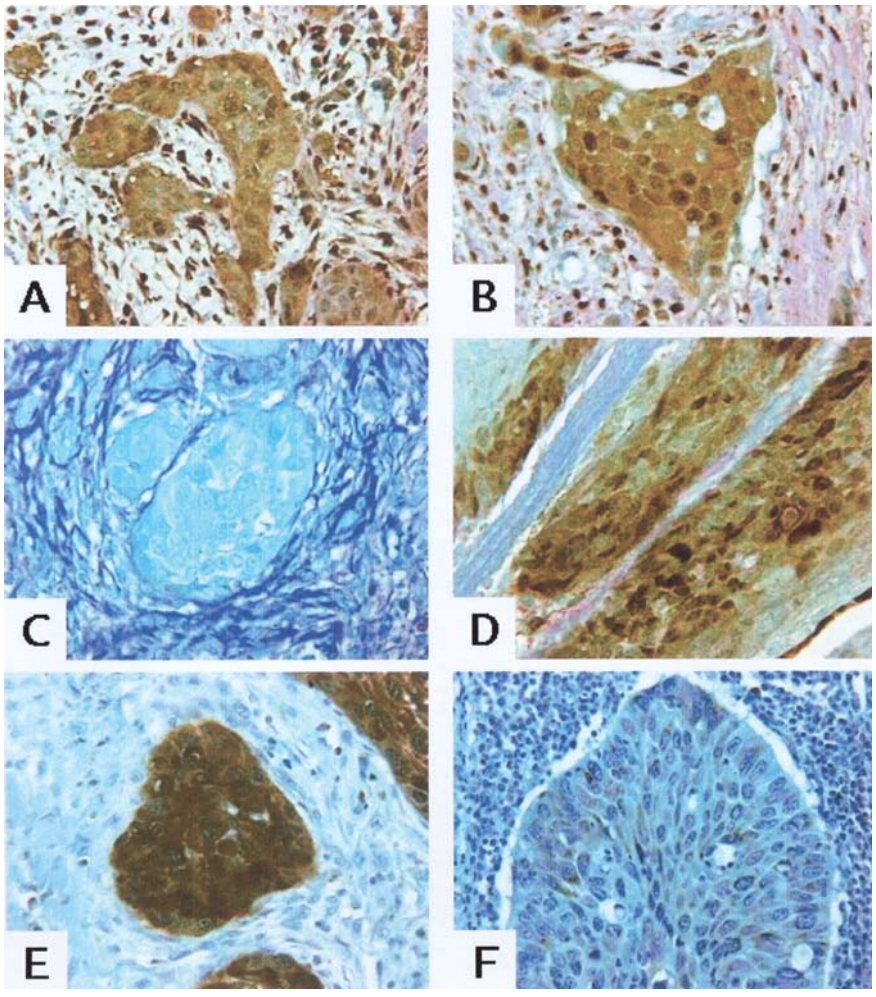

Figure 1. Immunohistochemical staining specific for galectin-1, -3 and -7, respectively, in sections of stage I and II (A, C and E) and stage IV (B, D and F) tumors. In detail, galectin-1 (A), -3 (C) and -7 (E) were localized in stage I and II laryngeal SCCs, as were galectin-1 (B), -3 (D) and -7 (F) in stage IV laryngeal SCCs. Magnification x320.

data of these 62 LSCC patients. Information on the tumor status includes histological tumor differentiation (established on the basis of criteria described by Hyams *et al*) and site as well as TNM classification (21). Details of the treatment and response include the extent of surgical resection and the pathological response at the primary site and the neck. The follow-up data obtained cover the period up to the last contact and the status of the disease at that time.

Immunohistochemistry. All the LSCC specimens were fixed for 24 h in 4% formaldehyde, dehydrated and routinely embedded in paraffin. Immunohistochemistry was performed on 5 μm-thick sections mounted on silane-coated glass slides as detailed recently (16). Briefly, before starting immunohistochemistry dewaxed tissue sections were subjected to microwave pretreatment in a 0.01 M citrate buffer (pH 6.0) for 2x5 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_2HPO_4, 0.01 M KH_2PO_4 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 PBS) to saturate endogenous biotin in order to avoid false-positive staining reactions. After rinsing with PBS, the sections were incubated for 20 min with a solution of 0.5% casein in PBS and sequentially exposed at room temperature: i) to the specific primary anti-galectin antibodies (see below); ii) to the corresponding biotinylated secondary antibody (polyclonal goat anti-rabbit IgG antibody or monoclonal mouse anti-goat IgG antibody); and iii) to the of avidin-biotin-peroxidase complex (ABC kit). The antigen-dependent presence of labeled peroxidase on the sections was visualized by incubation with the chromogenic substrate mix containing diaminobenzidine and H_2O_2. After careful rinsing, the sections were counter-stained with luxol fast blue and mounted with a synthetic medium. For control to exclude antigen-independent staining, the primary antibodies were either omitted or replaced by non-immune antisera. In all cases these controls were negative. The biotinylated secondary antibodies and ABC kit came from DakoCytomation (Glostrup, Denmark).

The preparation of anti-galectin-1, anti-galectin-3 and anti-galectin-7 antibodies and their controls have been detailed previously (16). We furthermore used the mouse monoclonal anti-CD45 antibody (Neomarkers, Fremont, USA) and the rabbit polyclonal anti-CD3 antibody (Cell Marque, Hot Spring, USA) to detect the presence of lymphocytes in LSCC tissue.

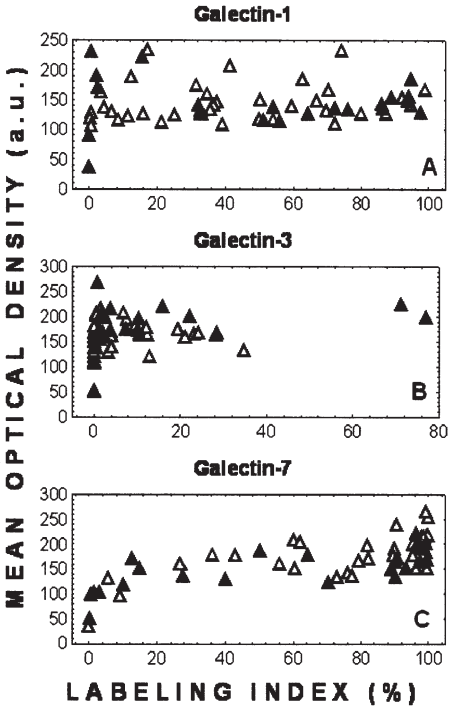

Figure 2. Quantitative determination (by means of computer-assisted microscopy) of the percentages of galectin-immunopositive cells (the labeling index variable on the x-axis) and the extent of galectin presence detected immunohistochemically (the mean optical density variable on the y-axis) in a series of 62 larynx HNSCCs. Each panel illustrates the two-dimensional distribution of the percentage of galectin (-1, -3 or -7)-immunopositive cells (x-axis) vs. the specific staining intensity for galectins (-1, -3 or -7) (y-axis) in relation to the tumor stage, i.e. low (stage I or II, open triangles) and high (stage IV, black triangles).

Computer-assisted microscopy. After immunohistochemistry, the levels of galectin expression were quantitatively determined by using a computer-assisted KS 400 imaging system (Carl Zeiss vision, Hallbergmoos, Germany), as detailed previously (16). For each case we scanned 15 fields corresponding to surfaces ranging between 60,000 and 120,000 μm^2. The computer-assisted morphometric analysis of the parameters of immunohistochemical expression of each marker quantitatively concerned the following two variables: i) the labeling index (LI) which refers to the percentage of cells positively stained for a given marker; and ii) the mean optical density (MOD), which corresponds to the staining intensity of positive cells (16).

Data analysis. Data obtained from the independent groups were compared by the non-parametric Kruskal-Wallis (more than two groups) or Mann-Whitney U tests (two groups). The standard survival time analyses were performed using the Kaplan-Meier curves and the Gehan generalized Wilcoxon test. As previously described, we applied a decision-tree-based technique to determine the threshold values required to separate groups of patients with very different clinical courses such as deceased patients as opposed to patients with no recurrences after a period of 24 months post-surgery (16,22). For each variable this technique investigates the possible splits aimed at spotting the one yielding the largest improvement in differentiating between the two groups of cases defined (16,22). 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

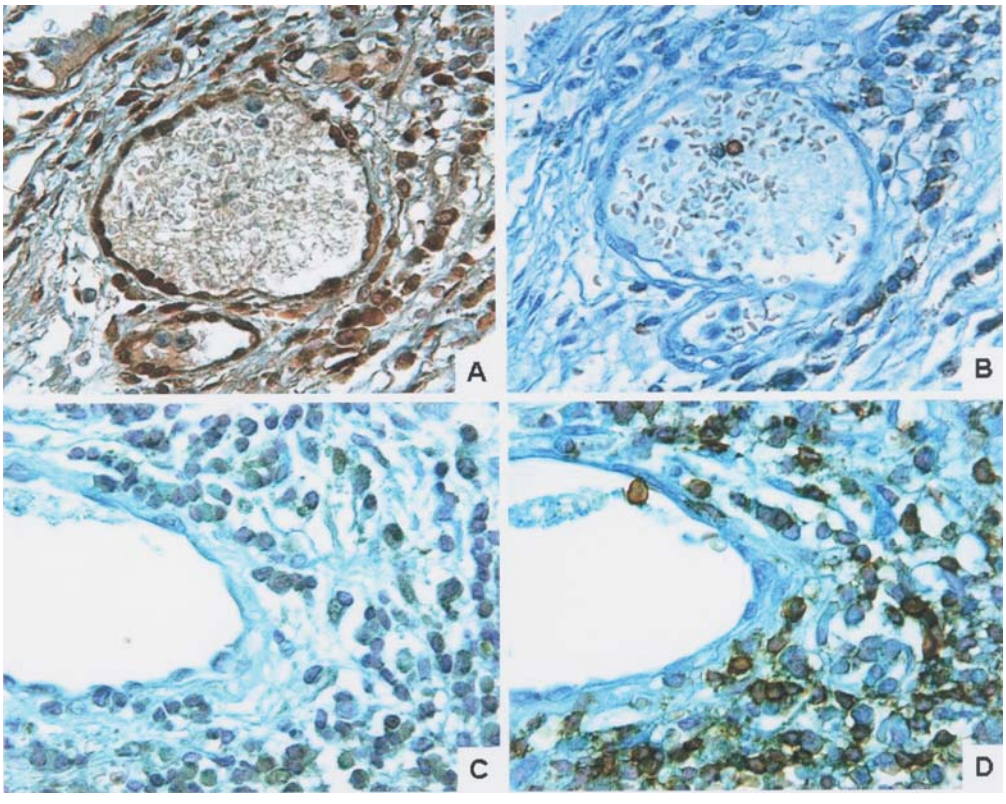

Figure 3. Illustration of the density of CD45-positive lymphocytes (B) around a galectin-1-positive vessel in a high-stage LSCC (A) as opposed to CD45-positive lymphocyte density (FD) around a galectin-1-negative vessel in a high-stage LSCC (C). Magnification x320.

(i.e., the galectin-dependent LI and MOD quantitative variables). This protocol enabled to probe for any possible simultaneous influence of several variables on the survival period. The statistical analysis was performed by using the software Statistica (Statsoft, Tulsa, USA).

Results

Immunohistochemical characterization of expression of galectin-1, -3 and -7 in the low versus the high clinical stages of LSCC. Fig. 1 illustrates typical patterns of galectin-1, -3 and -7 expression in the low (left-hand illustrations) as opposed to the high (right-hand illustrations) clinical stages of LSCC. While the three micrographs on the left side of Fig. 1 document typical patterns of immunostaining for galectin-1 (A), -3 (C) and -7 (E) in stage I LSCCs, the three illustrations on the right side of Fig. 1 depict immunostaining for the three lectins in stage IV LSCCs.

When each case had been subjected to the quantitative analysis, the distribution of the levels of expression of galectin-1, -3 and -7 in low- (I and II) and high- (IV) stage LSCCs (open and black triangles, respectively) were able to be compiled (Fig. 2). These data are presented as percentage areas of immunostained tissue [the labeling index (LI) variable on the x-axis] and the immunostaining intensity [the mean optical density (MOD) variable on the y-axis]. The data presented in Fig. 2 reveal that a large range of variations for both variables (except for the case of galectin-3-dependent LI values) was observed, thus precluding any unambiguous differentiation between low- and high-stage LSCCs.

Relationship between lymphocyte presence in LSCCs and galectin expression. Using a sub-series of 56 cases (including 36 low- and 20 high-stage LSCCs), we next analyzed the relationship between galectin-1, -3 and -7 expression and the number of CD45-positive lymphocytes. No statistically significant data were obtained from these analyses with respect to galectin-3 and -7 (data not shown). In contrast, we observed a significant negative correlation between the number of CD45-positive lymphocytes and the percentage of galectin-1-immunopositive tissue (all cases: Spearman r=-0.39, p=0.003). It is important to note the difference between the low-stage (Spearman r=-0.48, p=0.003) and the high-stage tumors not reacting (Spearman r=-0.24, p=0.3). Whereas the correlation between the number of lymphocytes and the intensity of the galectin-1 immunostaining (all cases: Spearman r=-0.34, p=0.01) was particularly due to the high-stage tumors (Spearman r=-0.48, p=0.03), the correlation was not significant in the case of the low-stage tumors (Spearman r=0.24, p=0.2). The differences in the levels of significance associated with these correlations can likely be due to the different number of cases in each tumor group (36 low-stage vs. 20 high-stage tumors). To document a representative case, Fig. 3 illustrates the density of CD45-positive lymphocytes (B) around a galectin-1-positive vessel in a high-stage LSCC (A) as opposed to the CD45-positive lymphocyte density (D) around a galectin-1-negative vessel in a high-stage LSCC (C).

In view of the significant correlation that we observed between the density of CD45-positive lymphocytes and the levels of galectin-1 expression in LSCCs we also analyzed

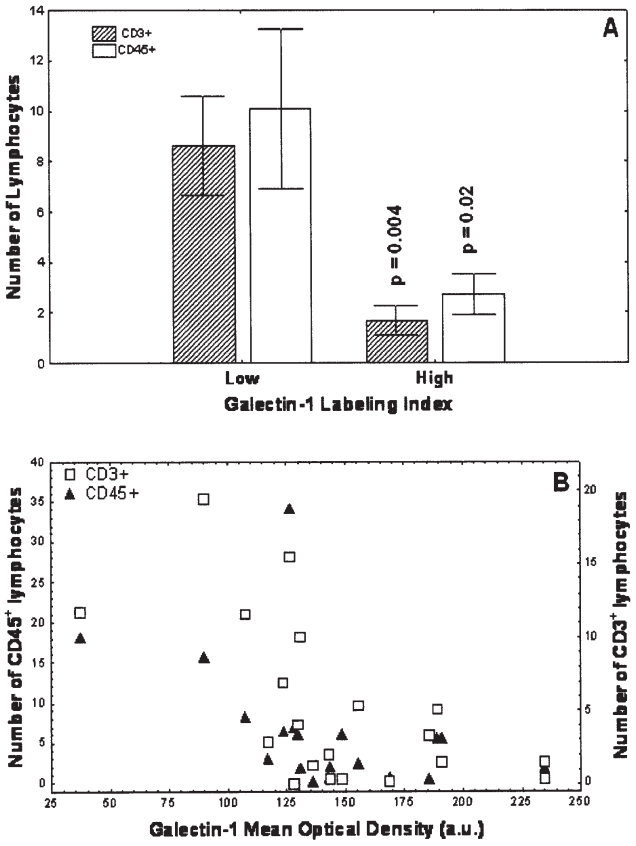

Figure 4. Illustration of the numbers of CD3-positive and CD45-positive lymphocytes counted per 16,500 μm^2 of tumor tissue compared to galectin-1 expression. (A) The data (mean ± SEM) in a group of 10 LSCCs with a low galectin-1 labeling index (<40%) in comparison with the data in a group of 10 LSCCs with a high galectin-1 labeling index (>70%). The open and hatched columns represent the numbers of CD3-positive and CD45-positive lymphocytes, respectively, with p-values computed by means of the Mann-Whitney non-parametric test. (B) The data distribution obtained in relation to the mean optical density of galectin-1 staining. These two plots evidence significant negative correlations between the numbers of CD3-positive (squares) and CD45-positive (triangles) lymphocytes and the galectin-1-dependent MOD values.

the relationship between galectin-1 expression and the number of CD3-positive lymphocytes on a sub-series of 20 cases with low to high galectin-1 expression levels. In terms of the galectin-1 labeling index the series of 20 cases could easily be split into two groups, i.e. one of 10 cases with labeling indices <40% and another of 10 cases with labeling indices >70%. Fig. 4A shows that these two groups are associated with very different numbers of CD3-positive and CD45-positive lymphocytes, both of which being significantly lower in the case of high galectin-1 labeling indices. These results are confirmed by the galectin-1 MOD values illustrated in Fig. 4B that, in turn, display the distribution of the number of CD3-positive lymphocytes and CD45-positive lymphocytes as a function of the galectin-1 MOD variable. These data demonstrate statistically significant negative correlations for both distributions (Spearman r=-0.65, p=0.002 in the case of CD45-positive lymphocytes; Spearman r=-0.57, p=0.009 in the case of CD3-positive lymphocytes).

Contribution of levels of expression of galectin-1, -3 and -7 and lymphocyte density to the prognosis of LSCC recurrence

Table II. Cox regression models.

	Model/p-value	Variable	ß	exp(ß)	p-value
Recurrence without lymphocyte analysis	p=0.0002	Gal1_MOD	0.028	1.028	0.004
		Gal1_LI	0.026	1.027	0.008
		Tumor stage	1.412	4.105	0.01
Recurrence with lymphocyte analysis	$p<10^{-6}$	Tumor stage	2.240	9.397	0.002
		Lym_Nb	-0.489	0.615	0.003
		Gal1_MOD	0.019	1.019	0.04
		Gal1_LI	0.014	1.014	0.1
Survival without lymphocyte analysis	p=0.002	Tumor stage	1.713	5.543	0.007
		Gal1_LI	0.024	1.025	0.008
		Gal1_MOD	0.014	1.014	0.2
Survival with lymphocyte analysis	p=0.0004	Tumor stage	2.250	9.486	0.002
		Gal1_LI	0.018	1.018	0.06
		Lym_Nb	-0.182	0.833	0.08
		Gal1_MOD	0.004	1.004	0.6

The 'model/p-value' indicates the overall level of significance of the model. The variables related to galectin-1 expression are the mean optical density (MOD) and the labeling index (LI). The equation at the basis of the Cox regression model is an exponential function of a linear combination of the variables considered, where ß indicates the coefficient of each variable in the linear combination and exp(ß) 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 ß is significantly different from zero. When $p<0.05$, the feature is associated with significant prognostic values independently of the other parameters taken into account.

and patient survival. First, we tested whether galectin expression levels when related to tumor recurrence could provide any prognostic value. Taking into consideration the tumor stages and the six quantitative variables (LI and MOD values for three galectins) characterizing the immunohistochemical data, we used multivariate Cox regression analysis to identify those independent variables, which might have a significant influence on relapse-free periods. The best explanatory model obtained by this method is described in the top part of Table II. From the seven variables considered (the six quantitative immunohistochemical variables and tumor stage) only three (i.e. the tumor stages and galectin-1 LI and MOD values) were identified to harbor significant and independent prognostic value. Galectin-1 LI and MOD values were associated with higher significant prognostic values than the tumor stages (top of Table II). The prognostic value of galectin-1 expression in LSCCs was confirmed in terms of survival analysis, as is shown in the second part of Table II. However, in this case only galectin-1 LI values provided a prognostic value independently of the tumor stages.

Fig. 5 illustrates the compiled data on galectin-1 immunolabeling in the case of the disease-free patients and those suffering from recurrences. In order to further refine the analysis of the remarkable influence of galectin-1 expression on the risk of tumor recurrence (Table II) we analyzed the data distribution with the aim of discriminating between patients with and without recurrences. Application of a decision-tree technique enabled us to define statistically significant thresholds on the basis of the two quantitative variables (labeling index and mean optical density) characterizing galectin-1 expression in each LSCC (see Materials and methods and ref. 16). We then used Kaplan-Meier analyses to validate the determined thresholds. While Fig. 5A and B document the results obtained in the case of the low-stage LSCCs, Fig. 5C and D illustrate those for the high-stage ones. Fig. 5A and B show that a threshold corresponding to 55% of the galectin-1-immunostained LSCC tissue (LI variable) led to the identification of two groups of patients (both suffering from low-stage LSCCs) associated with significantly different risks of disease recurrence (5B). In the case of the high-stage tumors an efficient level of differentiation required a more complex decision rule (identified by the decision-tree technique) based on two quantitative variables, namely a combination of the thresholds of 80% of galectin-1-immunostained LSCC tissue (the LI variable) on the one hand and 200 arbitrary units of immunostaining intensity (MOD variable; Fig. 5C) on the other. Fig. 5D shows that the patients suffering from high-stage LSCCs characterized by a low expression level of galectin-1 [i.e. below 80% of immunostained tissue (the LI variable) and below 200 arbitrary units of immunostaining intensity (the MOD variable)] were associated with a significantly lower risk of recurrence than the others.

In order to validate the prognostic value of galectin-1 we analyzed a series of 11 additional patients suffering from low-stage LSCCs of whom 10 patients had not suffered from a recurrence (during a period of at least 26 months) and 1 had (8 months after surgery). We observed that the threshold value of 55% of galectin-1 LI (established for low stage LSCCs) enabled us to reliably predict the recurrence status of all the patients correctly.

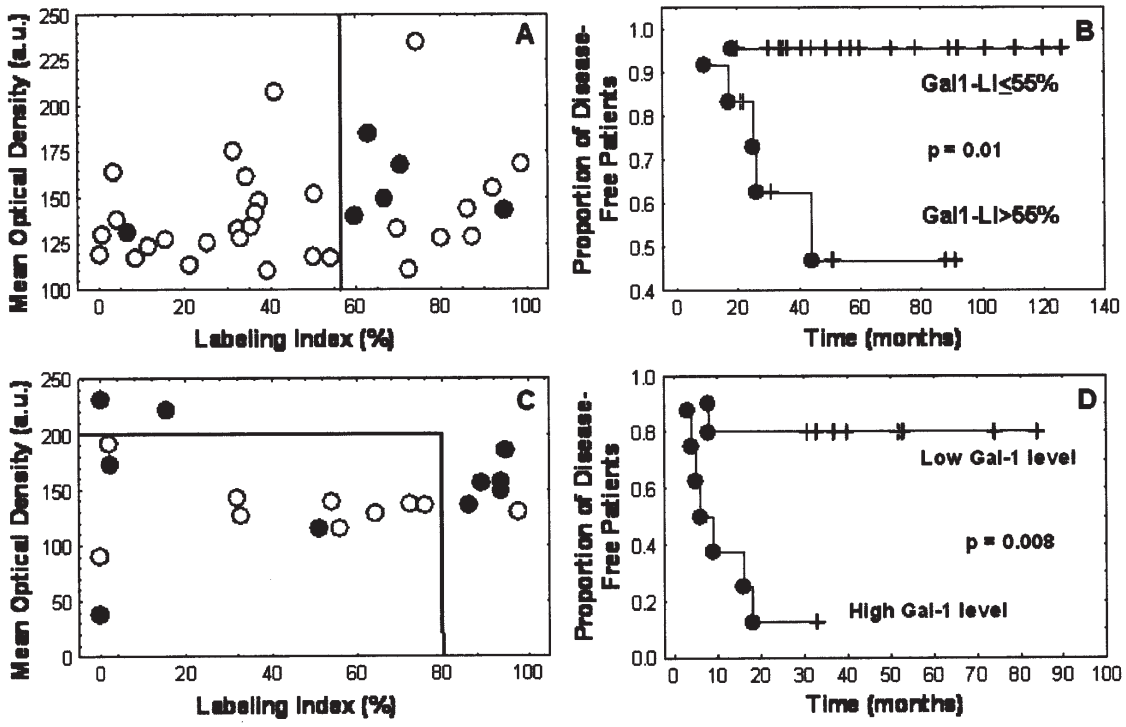

Figure 5. (A) The two-dimensional distribution of the percentages of galectin-immunopositive cells (x-axis) and the extent of galectin expression detected immunohistochemically (y-axis) determined in the low-stage tumors in comparison with the patients' recurrence status (open/black dots, patients without/with recurrence). The vertical line indicates a threshold value (found by means of a decision-tree analysis) to distinguish between patients with and without recurrence. This line thus separates the patients into two groups as a function of the immunohistochemical level of galectin-1-specific staining in their tumors (above or below the threshold value of 55% of immunopositive cells). (C) Results of a similar analysis carried out on the series of high-stage tumors where the horizontal and vertical lines both differentiate between patients with recurring and non-recurring malignancies. The group of patients with recurrence pathologies exhibited a high level of galectin-1-specific staining in their tumors (i.e. above 80% of immunopositive cells or above a mean optical density of 200 a.u.). (B and D) The remission curves associated with the two groups of patients identified on the basis of the level of the staining intensity for galectin-1 measured in their tumors, as indicated in A and C, respectively. In B and D the patients with tumor recurrences are defined by means of black dots and those without by crosses. The p-values were computed by means of Gehan's generalized Wilcoxon test.

We then analyzed the impact of these correlations when combining the galectin-1-associated prognostic values (described above) and lymphocyte density values. Table II presents the results obtained by multivariate Cox analyses when the number of lymphocytes and the galectin-1-related variables were taken into consideration. In its analysis of the recurrence-free periods, the model in Table II indicates that the addition of the lymphocyte numbers greatly increased the predictive value of the model (model p-value) while seriously decreasing the independent contributions of the two galectin-1-related variables. The survival analysis (Table II) gave relatively similar results. Thus, the level of galectin-1 expression appeared to be markedly associated with the lymphocyte density in LSCC tissue, and these two biological variables are statistically associated.

Discussion

Despite numerous advances in treatment modalities involving surgery, radiation and chemotherapy over the last 30 years the 5-year survival period for HNSCC patients in general, and for LSCC patients in particular, has remained below 50% primarily due to local recurrences (23,24). The possibility of developing immunotherapeutic approaches as a treatment for HNSCC patients has consequently attracted recent attention, mainly because the development of HNSCC is notably influenced by the host immune system (23-29). Indeed, the given recent evidence for presence of functional defects and apoptosis of tumor-infiltrating and circulating T-cells in patients with HNSCCs points to the potential that anti-tumor responses might be compromised (23-29). While effective immunological antitumor responses likely involve various parts of the immune system, T lymphocytes certainly continue to be considered as critical immune cells involved in anti-HNSCC immunity (30). HNSCC-cell-derived factors or mediators produced by normal cells in the local microenvironment favor HNSCCs and disable HNSCC-infiltrating lymphocytes (TILs) (30). Cytokines such as interleukin-6 may act as a local factor to further tumor progression (31). Looking at TILs, they appear like activated T-cells, but these cells can be functionally compromised (32). The induction of T-cell immunity following vaccination with a recombinant vaccinia virus expressing interleukin-2 (rvv-IL-2) in an orthotopic murine HNSCC model induces tumor-specific CD8-positive CTL and CD4-positive Th1-type helper T-cells (33). They may establish targets of a cytocidal effect of galectin-1 secreted by cancer cells, as detailed below.

Currently, galectins are attracting the attention of tumor immunologists as potent regulators of immune anti-tumor response. The first evidence of the activity of a galectin as regulator of cellular immune activity came by studies with a galectin-1-like protein in an animal model and the link

between apoptosis induction in CEM leukemia cells and galectin-1 expression (34,35). Next, human leukaemia T cells transfected with galectin-3 cDNA were shown to have high rates of proliferation and are protected against apoptosis by the galectin-3 acting intracellularly (36). Regarding galectin-1 apoptosis is promoted for activated, but not resting immune cells (reviewed in ref. 20). In aggregate, this lectin has been shown to inhibit T-cell effector functioning by promoting T-cell apoptosis, blocking T-cell activation, and inhibiting the secretion of proinflammatory cytokines, explicitly of interleukin-2 (IL-2), and favoring the secretion of the anti-inflammatory cytokine interleukin-10 (IL-10) (reviewed in ref. 20). Of interest, the targeted inhibition of galectin-1 expression *in vivo* renders mice resistant to melanoma challenge in a model study, a process requiring an intact CD4- and CD8-positive T-cell response (19). However, ensuing study of clinical melanoma failed to delineate the assumable correlation, which was actually observed with galectin-3, underscoring the complexity of galectin-mediated regulation *in situ* (37). Nonetheless, the amount of galectin-1 physiologically secreted appeared sufficient to kill T-cells when galectin-1 is presented in the context of extracellular matrix glycoproteins (38). The signal transduction events that lead to cell death caused by galectin-1 in activated T-cells involve a number of intracellular mediators including the induction of distinct transcription factors (i.e., NFAT, AP-1), the activation of the Lck/ZAP-70/MAPK signaling pathway, the modulation of Bcl-2 protein production, the depolarization of the mitochondrial membrane potential and cytochrome c release, the activation of caspase-3, -8 and -9 and the participation of the ceramide pathway (reviewed in ref. 20). These events occur when galectin-1 binds to galectin-1 receptors such as CD3, CD7, CD43 or CD45 present on the T-cell surface (reviewed in ref. 20).

A previous study of HNSCCs has connected staining for galectin-1 with decreased presence of T-cells (as assessed by CD3 staining), suggesting that galectin-1 can be a negative regulator of T-cell activation and survival (18). These authors also observed that both galectin-1 and CD3 expression are independent survival predictors in HNSCC patients, analyzing a series of 101 HNSCCs, but only 8 LSCCs (18). Our present study is based on the analysis of 62 LSCCs. The main goal of our current study has been to investigate which protein from the set of galectin-1, -3 and -7 is associated with the greatest prognostic value in the case of LSCCs in view of previous reports: i) by Le *et al* (18) on the prognostic value of galectin-1 in HNSCCs in general; ii) by Piantelli *et al* on the prognostic value of galectin-3 in laryngeal cancers (13); and iii) by our group on the prognostic value of galectin-7 in hypopharyngeal cancers. Piantelli *et al* showed that 42 of the 73 patients expressed galectin-3 and that galectin-3 expression was positively associated with tumor keratinization and histological grade (13). A significant correlation was found between galectin-3 tumor positivity and extended relapse-free and overall survival (13). By univariate analysis the parameters of high-grade (grade 3 or 4) tumors, non-keratinizing tumors and galectin-3-negative tumors were associated with a significantly increased risk of relapse and death (13). By multivariate analysis only galectin-3 expression retained an independent prognostic significance for both relapse-free and overall survival, and Piantelli *et al* therefore concluded that the lack of galectin-3 presence is an independent negative prognostic marker in laryngeal SCC patients (13). Our current data show that by Cox regression analysis the best explanatory model obtained when using seven variables (i.e. the six quantitative immunohistochemical variables characterizing expression of galectin-1, -3 and -7, and tumor stage), only three variables (i.e. the tumor stages and the galectin-1 LI and MOD values) were identified to have any significant and independent prognostic value. Galectin-1 LI and MOD values are associated with higher significant prognostic values than the tumor stages (Table II). The prognostic value of galectin-1 expression in LSCCs was confirmed in terms of survival analysis, as is shown in the second part of Table II.

Collectively, these data indicate that the immunohistochemical determination of expression of galectin-3 and their ligands is an independent prognostic marker per se in LSCCs (13,14), but when more than one galectin is taken into account (here galectin-1, -3 and -7), it is galectin-1 which is associated with the greatest prognostic value in LSCCs (our current study). The fact that galectin-1 can play this role in LSCCs may be explained by: i) its role as a hypoxia-regulated protein in HNSCCs, bearing in mind that hypoxia confers cellular resistance on conventional chemo-radiotherapy and accelerates malignant progression (18); ii) its activity as inducer of activated T-cell apoptosis (reviewed in ref. 20); and iii) its involvement in the protumoral role of oncogenic *H-ras* suggesting an intracellular function of galectin-1 in line with its cytoplasmic presence detected immunohistochemically (reviewed in ref. 20).

In our previous study of 81 stage IV hypopharyngeal cancers, we observed that high levels of galectin-7 expression were associated with rapid recurrence rates and dismal prognoses in these 81 stage IV hypopharyngeal SCCs, a feature not observed with galectin-3 and weakly, if at all, with galectin-1 (16). Thus, while in the case of laryngeal cancers galectin-1 is associated with a greater prognostic value than galectin-3 and -7, this role appears to be confined to galectin-7 in hypopharyngeal cancers (16). These apparent differences might be explained by disparities in the embryological origins of larynx and hypopharynx tissue, these differences translating directly into significantly different patterns of galectin expression and clinical behavior profiles seen for laryngeal and hypopharyngeal cancers. Indeed, nearly 75% of the patients with glottic carcinomas, which is the most common site for primary tumors of the larynx, had a localized type of the disease (stage I or II) at the time of diagnosis in contrast to nearly 80% of the patients with hypopharyngeal carcinomas, who had advanced levels (stage III and IV) at presentation. Moreover, hypopharyngeal carcinomas have a rich lymphatic network that favors early dissemination of primary lesions to the regional lymph nodes whereas glottic carcinomas are practically devoid of lymphatic networks and thus rarely present with regional lymphatic metastasis at the time of diagnosis.

In conclusion, the present study shows that the determination of the immunohistochemical levels of expression of galectin-1 is of prognostic value in human squamous laryngeal cancers. In detail, laryngeal cancers that have high levels of galectin-1 presence are characterized by worse

prognoses than laryngeal cancers with low levels of galectin-1 expression. The high levels of galectin-1 in laryngeal cancers and their concomitant dismal prognoses could relate to the fact that galectin-1 contributes to the process of tumor immune escape by killing activated T-cells which would otherwise contribute to cellular defence against laryngeal cancers. However, the question is still open on the roles of galectin-3 and -7, both also active as inducers of T-cell apoptosis *in vitro*. Thus, it cannot conclusively be excluded that the special significance of galectin-1 rests upon other effector mechanisms. Further *in vitro* and *in vivo* studies are therefore warranted to delineate galectin-1 functionality.

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