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Galectin-8 Up-regulation During Hypopharyngeal and Laryngeal Tumor Progression and Comparison with Galectin-1, -3 and -7

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Abstract. *Aim: To define specific staining patterns for the adhesion/growth-regulatory lectin tandem-repeat-type galectin-8 in hypopharyngeal and laryngeal tumor progression and relate these parameters to galectins 1, 3 and 7 in the quest to explore the galectin network. Materials and Methods: The level of expression of galectin-8 was determined immunohistochemically in a series of 18 and 16 cases of tumor-free epithelium, 24 and 10 cases of low-grade dysplasia, 22 and 15 cases of high-grade dysplasia located in peri-tumoral area of 74 and 37 hypopharyngeal and laryngeal carcinomas. Results: Marked upregulation in galectin-8 staining intensity and immunopositive area in malignancy versus dysplasia was seen in hypopharyngeal cancer ($p<10^{-6}$), in laryngeal cancer for labeling index and high-grade dysplasia/carcinoma ($p<10^{-6}$). No correlation to recurrence was delineated. Conclusion: The presented data revealed a divergence within the galectin-1, -3, -7 and -8 network during tumor progression.*

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The emerging concept of the sugar code viewing glycans as bioactive signalling molecules in cellular communication is currently turning new attention to analyzing glycosylation in malignancy in the search for biomarkers (1). Of note, a recent study on carcinogenesis upon microsatellite instability drew unknown correlations between the reconstitution of distinct genes, such as that for the transforming growth factor-β type 2 receptor, and glycan reactivity to sugar receptors. Intriguingly, a tumor suppressor was found to direct a coordinated modulation of glycan presentation with expression of an endogenous lectin sensitising tumor cells to lectin-dependent induction of anoikis (2, 3). Equally interesting, the interplay of glycans with endogenous lectins is capable of affecting tumor cell proliferation *via* signal cascades which trigger increased production of cyclin-dependent kinase inhibitors p21/p27 (4, 5). Prominent among the studied effectors from the class of endogenous lectins are the galectins, this fact providing a reason to study their expression profiles in cancer immunohistochemically in order to define new disease markers.

Toward this end, we are performing a systematic investigation on galectins in head and neck cancer. Initially, we revealed the proto-type galectin-1 and -7 to be prognostic predictors in laryngeal (LSCC) and hypopharyngeal squamous cell carcinomas (HSCC), respectively (6, 7).

Tumor progression was associated with characteristic changes in staining parameters and shifts between cytoplasmic and nuclear sites as well as stromal presence (8, 9). The course of expression and localization were also different for the chimera-type galectin-3 in comparison between both tumor forms (10). With three members of the galectin family thus studied so far, the next step is to gain further insights into the galectin network. In fact, members of the third galectin subgroup, namely that of the tandem-repeat-type proteins, are known to be expressed by diverse types of human tumor cells as revealed by reverse transcriptase-polymerase chain reaction (RT-PCR) fingerprinting (11). Its functionality as growth regulator noted above, combined with its ability to induce apoptosis *in vitro*, its equipotency to fibronectin in ligating integrins and indications for a regulation of expression upon transformation directed our interest to galectin-8 (4, 12-18). Its binding to pro-matrix metalloproteinase-9 (pro-MMP-9) leading to accelerated processing to the mature form in neutrophils, MMP-9 being an independent prognostic marker in laryngeal and hypopharyngeal cancer, and the discovery that the non-mammalian vertebrate models of galectin evolution harbor galectin-8 as sole tandem-repeat-type galectin underscore the importance of studying this protein in clinical specimens (19-21). We thus raised a polyclonal antibody against the recombinant protein and ensured absence of cross-reactivity to other members of the galectin network to answer the following questions: Is this lectin present in both tumor types? How is expression associated with tumor progression? Do the measured profiles differ? Is any correlation to the clinical course discernible?

Table I. *Clinical data*.

Variable	High-stage HSCC	LSCC	
		Low-stage	High-stage
Age (years)			
Range	40-78	36-88	43-78
Average	55	57	57
Gender (n)			
Male	71	25	12
Female	3		
Site (n)			
Supraglottic		3	9
Glottic		20	
Supraglottic and glottic		2	
Subglottic and glottic			3
Piriform sinus	60		
Postcricoid	12		
Posterior wall	2		
Histological grade (n)			
Well differentiated	40	22	9
Moderately differentiated	23	3	3
Poorly differentiated	11		
Stage (n)			
I		23	
II		2	
IV	74		12
Tumor treatment (n)			
CO_2 laser cordectomy		8	
Frontolateral laryngectomy		1	
Vertical partial laryngectomy		2	
Supracricoid partial laryngectomy		11	1
Supraglottic laryngectomy		3	
Total laryngectomy			11
Partial pharyngolaryngectomy	9		
Total pharyngolaryngectomy	51		
Circular pharyngolaryngectomy	4		
Esopharyngolaryngectomy	10		
Dissection of the neck (n)			
Functional		17	20
Radical			1
Recurrence (n)			
Local	17	1	4
Distant	11	1	7

Materials and Methods

Patients' characteristics. A total of 111 cases, including 74 patients with HSCC and 37 patients with LSCC who underwent surgery aimed at curative tumour resection, were studied (see Table I for clinical data). The patient files were compiled retrospectively (January 1989 to December 2001) from the records of the ENT Department at the Hôpital Claude Huriez (Lille, France) and at the CHU Saint-Pierre (Brussels, Belgium). After common CO2 laser resection, the surgeon nearly invariably performed additional small resection to enable careful analysis of tumor boundaries. Twenty percent of stage IV HSCC and LSCC patients presented positive margins as did 10% of stage I and II LSCC patients. The description of the tumor status was based on the histopathological grade of tumor differentiation (criteria defined in (22, 23) and the TNM staging classification (24). Detailed information on the patient's age, gender, tumor histopathology, type of laryngeal or hypopharyngeal surgery, response to treatment at the primary tumor site and follow-up data up to the last contact with the patient together with the status of the disease at that moment were available for 74 HSCC and 37 LSCC patients (see Table I). All specimens under study were from patients who did not undergo chemotherapy or radiotherapy prior to surgery. All stage IV HSCC and LSCC patients received additional postoperative radiotherapy. For stage I and II LSCC patients, four patients (10%) received postoperative radiotherapy because they presented positive margins. The cohorts of the HSCC and LSCC patients were thus nearly homogeneous in terms of histopathological and clinical criteria. Patients suffering from SCCs localized at other sites of the head and neck area were excluded from the study. This study was approved by the local Institutional Review Board.

Antibody preparation and quality controls. Cloning of the cDNA for human galectin-8 with a linker length of 70 amino acids started from total RNA of human HCT-116 colorectal carcinoma cells (2×10^6 cells) isolated using the RNAeasy kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and reverse transcription. The sequences of the two specific primers were

designed based on published information under the Genbank AF342816 entry (colorectal carcinoma-derived galectin-8 variant II). The PCR amplification was directed by the sense primer 5-CATATGATGTTGTCCTTAAACAACCTAC-3 with an internal *NdeI* restriction site (underlined) and the antisense primer 5-GTCGACCTACCAGCTCCTTAC-3 with an internal *SalI* restriction site (underlined). PCR products were separated by agarose gel electrophoresis. The predicted product was eluted from the respective gel slice by applying a gel extraction kit (Qiagen) and ligated into the *EcoRV*-linearized pET-Blue-1 AccepTor™ Vector (Novagen, Darmstadt, Germany) with single 3'dU overhangs for amplification. Subsequently, the cDNA sequence was ligated into the expression vector pET-12a (Novagen) to direct recombinant production in the *Escherichia coli* strain BL21(DE3)pLysS (Novagen). Lectin purification was performed as described previously for chicken galectin-8, with controls for purity by one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining as well as gel filtration, for lectin activity by hemagglutination, as well as solid-phase and cell-binding assays (20, 25-27). Polyclonal antibodies were then raised in rabbits using repeated booster injections under the control of enzyme-linked immunosorbent assay (ELISA) to monitor the course of serum titer, and immunoglobulin G (IgG) fractions were purified from serum by affinity chromatography on protein-A Sepharose Fast Flow resin (GE Health Care, Munich, Germany) (28). These preparations were routinely subjected to specificity controls by ELISA and Western blots using human galectin-1, -2, -3, -4, -7 and -9. Weak cross-reactivities to galectin-1, -3 and -9 were removed by chromatographic affinity depletion using resin-immobilized galectins (29, 30).

Immunohistochemistry. All tumor samples were fixed 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 (10). Before starting the immunohistochemistry protocol, dewaxed tissue sections were briefly subjected to microwave pretreatment in a 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_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 avoid false-positive staining reactions resulting from endogenous biotin. After thorough washing with PBS, the sections were incubated for 20 min with a solution of 0.5% casein in PBS and sequentially exposed at room temperature to solutions of i) the specific primary anti-galectin-8 antibody; ii) the corresponding biotinylated secondary antibody (polyclonal goat anti-rabbit IgG); and iii) the avidin-biotin-peroxidase complex (ABC kit). Incubation steps were separated by thorough washing steps to remove unbound proteins. Antigen-dependent presence of the peroxidase complex in the sections was visualized by incubation with the chromogenic substrates containing diaminobenzidine and H_2O_2. After rinsing, the sections were counterstained with luxol fast blue and mounted. To exclude antigen-independent staining, the incubation step with primary antibody was omitted from the protocol in controls. In all cases these controls were negative. The biotinylated secondary antibodies and ABC kit came from DakoCytomation (Glostrup, Denmark).

Definition of low- and high-grade epithelial dysplasia. Morphological characteristics of dysplasia include increased cellular density associated with a large number of mitotic figures in the vicinity of the basal layer, irregular maturation, loss of polarity, and dyskeratosis. Cytologically, dysplasia is characterized by an increased ratio of nuclear to cytoplasmic area, anisocytosis, poikilocytosis, nuclear polymorphism, chromatin condensation and large nucleoli, features sometimes associated with atypical mitotic figures. Low-grade dysplasia, comprising mild and moderate dysplasia, presents atypical features extending over the lower or middle third of the epithelium. High-grade dysplasia, including severe dysplasia, and carcinoma *in situ* extend over the entire thickness of the epithelium (23).

Computer-assisted microscopy. Following the immunohistochemical steps, the quantitative features of galectin staining were determined using a computer-assisted KS 400 imaging system (Carl Zeiss Vision, Hallbergmoos, Germany) connected to a Zeiss Axioplan microscope, as detailed previously (8, 10). For each microscopic field, the analyses were focused on neoplastic cells or tumor-free epithelia using computer-assisted morphometry after interactive identification. These tissue areas are delimited precisely with the computer mouse. In each case, 15 fields covering a surface area ranging from 60,000 to 120,000 μm^2 were scanned. The quantitative analysis of the immunohistochemical staining yielded data on the following two variables: i) the labeling index (LI), *i.e.* the percentage of positive cells, and ii) the mean optical density (MOD), *i.e.* staining intensity of positive cells (8, 10). For each type of dysplasia (low- or high-grade), the respective fields within peritumoral areas were defined by one of us (XL) specialized in this diagnostic procedure.

Data analysis. Independent groups of quantitative data were compared using the non-parametric Kruskal-Wallis (more than two groups) or Mann-Whitney *U*-tests (two groups). In the case of more than two groups, *post-hoc* tests (Dunn procedure) were used to compare pairs of groups (in order to avoid multiple comparison effects). A *p*-value of <0.05 was considered as significant. The relationships between the qualitative (or ordinal) variables analyzed were studied by contingency tables. The level of significance of correlations was evaluated by the χ^2 or the exact Fisher test (in the 2×2 cases). The standard survival time analysis was performed using the Kaplan-Meier method and the Gehan generalized Wilcoxon test. A decision-tree approach was systematically applied to disclose threshold values aiming at separation of patient groups with/without recurrence (21). Statistical analyses were carried out using Statistica software (Statsoft, Tulsa, OK, USA).

Results

Galectin-8 and HSCC. Following rigorous specificity controls of the polyclonal antibody preparations by ELISA and Western blotting against human galectin-1, -2, -3, -4, -7 and -9 and chromatographic removal of cross-reactive activity, we proceeded to determine the level of expression and localization profile of galectin-8 immunohistochemically in a series of 18 cases of tumor-free epithelum (designated as TF-E), 24 cases of low-grade dysplasia (Low_D), 22 cases of high-grade dysplasia (High_D) located in peritumoral area of 74 stage IV

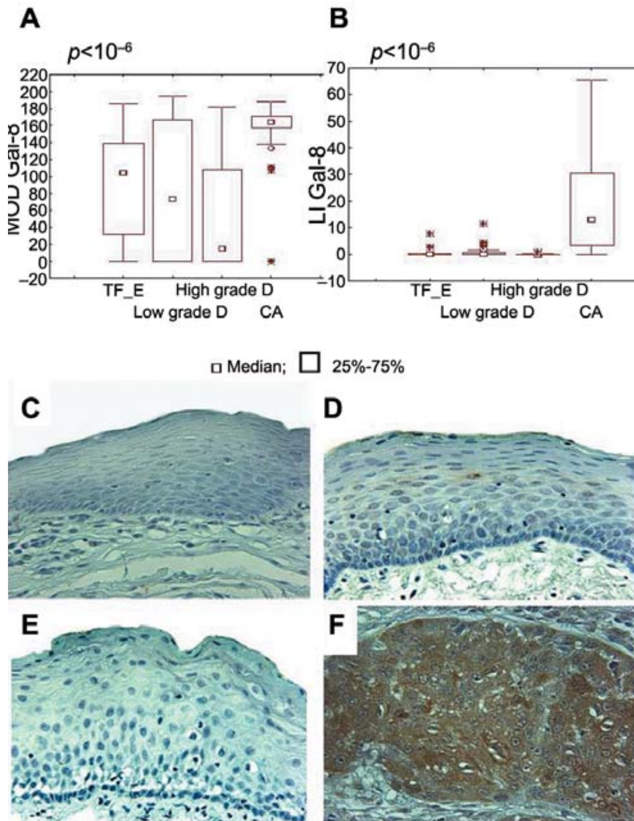

Figure 1. *Quantitative determination (by computer-assisted microscopy) of the intensity of galectin-8-dependent staining (mean optical density, MOD) (A) and of the percentage galectin-8-immunopositive cells (labeling index, LI) (B) in a series of 18 TF_E, 24 Low_D, and 22 High_D specimens as well as 74 stage IV HSCCs (CA). Panels C, D, E and F illustrate galectin-8-dependent staining in TF_E (C), Low_D (D) and High_D (E) specimens in areas surrounding hypopharyngeal cancer (F). Magnification A-F ×320.*

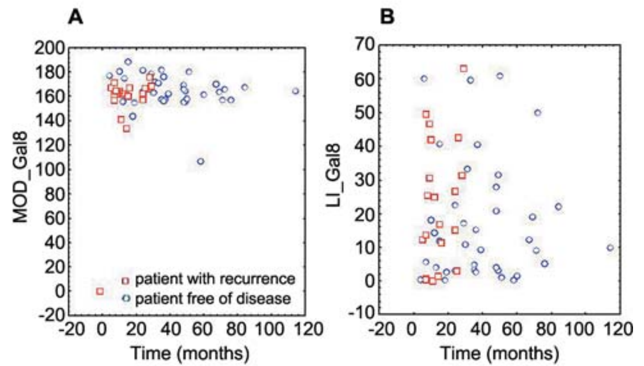

Figure 2. *Quantification of galectin-8 immunodetection by MOD (A) and LI (B) in hypopharyngeal carcinoma specimens from patients with or without recurrence (time is referred to a period of months with or without recurrence).*

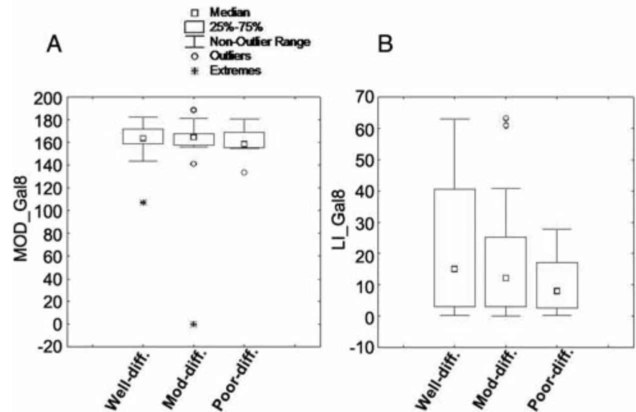

Figure 3. *Quantification of galectin-8 immunodetection (MOD, A; LI, B) in well-, moderately and poorly differentiated hypopharyngeal tumors.*

HSCCs. In TF_E, we observed a low-frequency galectin-8-specific immunolabeling of variable intensity in the cytoplasm of basal and suprabasal layers (3/18 cases of hypopharyngeal TF_E) (Figure 1A-C) upon progression to malignancy; no clear trend was detected for either of the quantitative parameters (Figure 1A, B). At the level of the preneoplastic hypopharyngeal lesions, 3/24 of the Low_D cases and only 1/22 of the High_D cases of remained rather weakly positive for galectin-8 in the cytoplasm (Figure 1A, B, D, E). In contrast, hypopharyngeal carcinomas presented a highly significant increase in both parameters, with 91% of the malignancies showing cytoplasmic positivity (67/74 cases) (Figure 1A, B, F). Compared to tumor-free tissue in hypopharyngeal lesions, both immunohistochemical variables (staining intensity (MOD): *post-hoc* comparison: p=0.009 and percentage of immunopositive area (LI): *post-hoc* comparison: p=0.000001) were higher in carcinoma cases than those in TF_E. This difference continued to be at a high level of statistical significance when comparing the HSCC cases to cases of Low-D (MOD, *post-hoc* comparison: p=0.002; LI, *post-hoc* comparison: $p<10^{-6}$) and of High-D (MOD: *post-hoc* comparison: $p<10^{-6}$; LI: *post-hoc* comparison: $p<10^{-6}$).

Following the analysis of the staining pattern, we next processed the data to discern any clinical correlation with recurrence. By applying a decision-tree methodology, discriminatory thresholds for the two quantitative variables (LI and MOD) were systematically sought for in both studied cohorts (Table I). No significant predictive measure for recurrence in patients suffering from HSCC was delineated (Figure 2A, B). In addition, no statistical correlation to TNM status or tumor grading was observed (Figure 3A, B). In

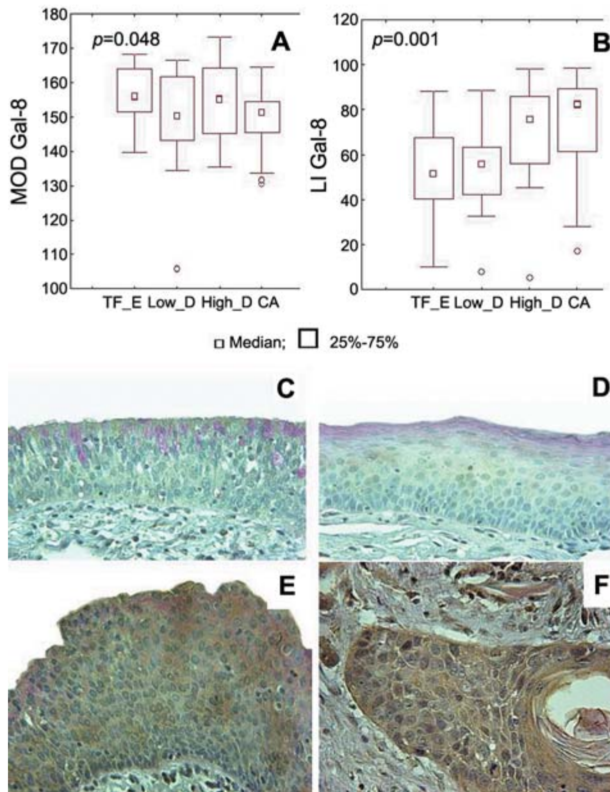

Figure 4. *Quantitative determination (by computer-assisted microscopy) of MOD (A) and LI (B) in a series of 16 TF_E, 10 Low_D and 15 High_D specimens as well as 37 LSCCs (CA; 25 stages I and II, 12 stage IV).*

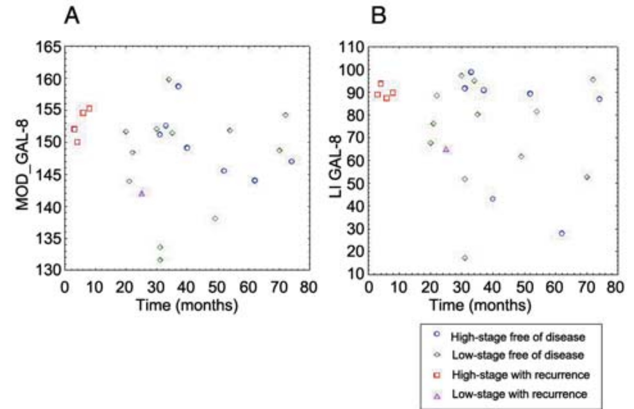

Figure 5. *Quantification of galectin-8 immunodetection by MOD (A) and LI (B) in relation to the recurrence status of patients with low- and high-stage LSCC (time is referred to a period of months with or without recurrence).*

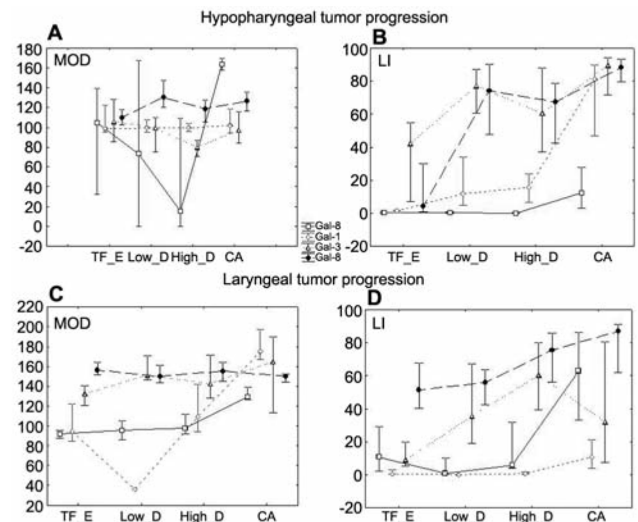

Figure 6. *Patterns of the two quantitative immunohistochemical parameters during tumor progression in HSCC (A, B) and LSCC (C, D) for galectin-8 compared to those of galectin-1 and -7 (8), as well as galectin-3 (10).*

order to answer the question on the expression pattern in laryngeal lesions, our analysis was extended to a panel of respective cases under identical conditions.

Galectin-8 and LSCC. Figure 4A-F illustrates data on galectin-8-specific staining in a series of 16 TF_E, 10 Low_D, 15 High_D and 37 LSCCs (25 stage I and II, 12 stage IV). Galectin-8 presence was detected in this tumor type and quantitative parameters were different to these in hypopharyngeal tissue. In marked contrast to hypopharyngeal cancer, the MOD variable showed no evidence for a significant difference in the four cohorts (Figure 4A). However, the LI parameter was consistently higher in this tumor class than in the hypopharyngeal cases (LI variable, Kruskal-Wallis: $p<10^{-6}$) (Figure 4B). In intercohort comparison, the percentage of galectin-8-immunopositive cells (LI variable) was higher in the carcinoma cases than in High_D, Low_D and TF_E cases (Figure 4B). This difference surpassed the significance threshold when comparing LSCC cases to TF-E (LI, *post-hoc* comparison $p=0.003$) and when comparing them to Low-D (LI: *post-hoc* comparison $p=0.04$). No correlation with recurrence was detected (Figure 5). Overall, the courses of disease progression in hypopharyngeal and laryngeal cancer appeared to be associated with their characteristic profiles of galectin-8 presence.

Discussion

This study explores a new level of complexity of the galectin network in head and neck cancer. Having previously examined the two proto-type galectin-1 and -7 and the

chimera-type galectin-3 in hypopharyngeal and laryngeal carcinomas, we herein initiated monitoring for a tandem-repeat-type galectin under identical conditions, allowing direct comparison. Proteins of this specific subgroup combine two different lectin domains, covalently connected by a linker peptide, so that their ligand selection is distinct from other galectins composed of identical subunits (31). In the case of galectin-8, preferential reactivity to α2,3-sialylated/3'-sulfated *N*-acetyllactosamine/lactose and to poly-*N*-acetyllactosamine chains/ABH histo-blood group epitopes are hallmarks of the specificity of its two lectin modules (32-34). This carbohydrate-binding profile translates into targeting α1-, α3-, α5-, α6- and αvβ1 as well as αM integrins (14, 16, 19, 35, 36). Previously known as prostate carcinoma tumor antigen-1 and as Po66 epitope of human lung squamous carcinoma (12, 13), the production of autoantibodies to galectin-8 has recently been demonstrated in autoimmune rheumatic diseases and in acute inflammation, as well as in *neu* transgenic mice, pointing to an association of this lectin with diverse disease processes (37, 38). Intuitively, its marked binding capacity to integrins gives reason to assume non-redundant aspects of functionality, a central incentive for this study. Indeed, our data provide solid evidence for a characteristic profile of localization and of quantitative parameters of immunohistochemical staining depending on the tumor site as summarized in Figure 6.

Staining parameters increased significantly in hypopharyngeal cancer, this being detectable only for the labeling index in laryngeal carcinomas. Its extent was markedly higher in laryngeal then in hypopharyngeal tumors. For comparison, lung cancer types were invariably positive including squamous metaplasia and pleura tumors (39-41). The way the two quantitative immunohistochemical parameters were associated with the course of tumor progression followed characteristic courses when compiling the data for the two proto-type galectins (8) and the chimera-type galectin-3 (10) to enable direct comparison to those of galectin-8 (Figure 6). Indications for a shift between cytoplasmic and nuclear localization, seen in the cases of galectin-1 and -7 (8), as well as galectin-3 (10), were also not observed, another characteristic feature of the galectin-8-dependent staining profile. Although no direct association of galectin-8 with recurrence was disclosed, in contrast to galectin 1 and -7, (6, 7), it is tempting to relate the accelerated processing of proMMP-9 by its binding to galectin-8 in neutrophils (19) to the positive prognostic effect of MMP-9 (21).

Looking at the data from studies in model systems such as NSCLC, synovial fluid cells and thymocytes, they shape the notion for galectin-8 to trigger growth arrest and apoptosis (4, 14, 17, 18). However, the analysis of the given series of clinical material teaches the salient lesson that a respective activity *in vitro* will not necessarily engender a positive prognostic significance. What is more is that studies of colon, testicular and urothelial cancer even reported a negative prognostic significance, with correlations to staging, grading and muscle-infiltrating growth in bladder carcinomas, and monitoring of cholesteatoma detected no apparent influence on the level of apoptosis, respectively (42-46). This collective evidence reveals a clear cell type-dependent relationship between galectin-8 presence and clinical correlations. Considering this protein in the context of the galectin network, the presented results give further research the direction to proceed to map galectin presence in squamous cell carcinomas in order to accomplish complete fingerprinting of this family of effector proteins for this first tumor system.

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