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Galectin-3 – an emerging prognostic indicator in advanced head and neck carcinoma

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Abstract

Galectin-3, is a multifunctional effector. It is the only chimera-type member of the galectin family of endogenous lectins, which share specificity with β-galactosides and have a jelly-roll-like folding pattern. It's activity profile includes modulation of cell–cell and cell–extracellular matrix interactions and the regulation of proliferation and apoptosis/anoikis. While lectin histochemistry with plant/invertebrate proteins is routine practice and immunohistochemical analysis of endogenous lectins has been thoroughly examined, the application of an endogenous lectin as a marker is presently primarily a promising concept. The aims of our study were to test galectin-3 as a technical probe and to correlate staining by the tissue lectin, localising accessible ligands *in situ*, to clinicopathological characteristics and the prognosis of patients (relapse-free and overall survival) in advanced head and neck squamous cell cancer. We measured galectin-3-dependent staining in 53 surgically resected oropharyngeal and laryngeal cancer specimens (stage III or IV). Patients were divided into two groups based on a threshold of 5% positivity in the tumour cell population. The patient's degree of positivity was significantly correlated with their level of differentiation and keratinisation and lack of lymph node involvement ($P = 0.0001$, $P = 0.0007$ and $P = 0.0224$, respectively). Periods of relapse-free and overall survival were significantly shortened when the tumour population failed to meet the positivity criterion, i.e. to harbour ligands for the endogenous lectin ($P = 0.0039$ and $P = 0.0259$, respectively). We conclude that (a) studies with an endogenous lectin as a marker are technically feasible and (b) detection of accessible galectin-3-specific ligands is an independent prognostic marker in advanced head and neck squamous cell cancer with therapeutic potential. Of note, histochemical application of an endogenous effector after its purification and labelling may bear relevance beyond the galectins.

Keywords: Galectin; Head and neck cancer; Lectin histochemistry; Prognosis; Sugar code

1. Introduction

Head and neck squamous cell carcinomas (HNSCC) represent 14% of all new cancer cases in males and 6% in females worldwide. The incidence of tumour occurrence is influenced by life-style param-

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eters, because tobacco smoking and alcohol consumption figure prominently as important risk factors [1]. The clinical course of HNSCC can depend on various tumour and host factors, tumour site, vascularity, lymphatic drainage, differentiation grade, TNM stage, host immune response, patient's age, gender, and nutritional status being on the list of known variables. The search for biochemical markers aims to relate clinical parameters to distinct effectors with potential therapeutic application [2–4]. Ideally, reliable identification of 'high-risk' patients will enable tailored therapeutic regimens, and hopefully, improve the predicted unfavourable prognosis of this subgroup. It is in this context that we focused our study on testing an endogenous protein. Instead of performing immunohistochemical monitoring, we used a tissue protein as a probe to visualise the expression of accessible binding sites in tumour cells. Based on the assumption that a molecular interaction with suitable binding partners will be essential to effect responses on the cellular level, this approach is suitable to measure the extent of *in situ* potential for biorecognition. In general terms, the presented approach, if successful, could find many other applications beyond this test system.

Besides proteins and nucleic acids, glycans of cellular glycoconjugates are gaining attention as a biochemical code system for information storage. In fact, cell–cell/cell–matrix interactions and modulation of cell growth have been delineated to be regulated by glycan determinants serving as ligands for endogenous receptors, such as lectins [5–7]. The presence of these tissue lectins has rekindled interest in lectin histochemistry, because distinct changes in glycan presentation (*glycome*) might become interpretable in functional terms, when evidence for the involvement of tissue lectins as receptors can be provided [8,9]. Prompted by the lectins' various functional roles *in situ*, immunohistochemical staining for the presence of lectins has suggested they may be a potential new class of human markers [10–13]. Focusing on the family of galectins, the only member of the chimera-type subgroup, i.e. galectin-3 (Gal-3), is being increasingly studied due to its role in cancer metastasis, apoptosis regulation and pre-mRNA splicing [14–16]. Following the initial assessment of Gal-3-related parameters in head and neck cancer and the emergence of their correlation with a low degree of differentiation [17–22], it was imperative for us to determine whether the extent of Gal-3 binding might be used as a prognostic marker. To this end, we monitored tumour specimens from 53 patients suffering from advanced squamous cell carcinoma of the larynx and oropharynx, to assess its staining pattern in tumour cell populations in order to test whether it was of prognostic relevance.

2. Materials and Methods

2.1. Patients and histological specimens

Specimens used for the galectin histochemical analysis were obtained from 53 advanced head and neck squamous cell cancer patients who had initially undergone surgery (resection of the primary tumour with neck dissection) and further postoperative radiotherapy, without any prior treatment at the Department of Otorhinolaryngology and Head Neck Surgery, 1st Faculty of Medicine, Charles University, Faculty Hospital Motol, Prague, from October 1998 to March 2000. 31 tumours were of oropharyngeal and 22 were of laryngeal origin. The International Union Against Cancer (UICC) system of TNM classification was used to categorise the patients (45 males and 8 females; median age, 59 years; age range 46–76 years). 30 tumours were graded as well/moderately differentiated (grade 1 or 2) and 23 were graded as poorly differentiated or undifferentiated (grade 3 or 4). Tumour sizes were classified according to the frequency of occurrence as follows: T1 (4 cases), T2 (13 cases), T3 (22 cases) and T4 (14 cases). Regional metastases were found in 44 patients with nodal status N1 (15 cases), N2 (19 cases) and N3 (10 cases). Accordingly, there were 16 stage III patients and 37 stage IV patients. Tumours were also defined as keratinising (35 cases) and non-keratinising (18 cases) based on the presence or absence of keratin, respectively. The median follow-up was 44 months (range 8 and 60 months).

2.2. Sample preparation

Samples of tumour tissue were quickly frozen in liquid nitrogen using Tissue-Tek (Sakura–Finetek Europe B.V., Zoeterwoude, The Netherlands). The 7 μm-thin frozen sections were prepared by employing Cryocut-E (Reichert-Jung, Vienna, Austria). The sections were fixed with 2% (w/v) paraformaldehyde in phosphate-buffered saline (pH 7.2). Carbohydrate-free bovine serum albumin (BSA; Sigma–Aldrich, Prague, Czech Republic) was used to block non-specific protein–protein interactions. Gal-3 was purified to electrophoretic and nano electron-spray ionisation (ESI)-mass spectrometric homogeneity after recombinant production, biotinylated under activity-preserving conditions and checked for activity by solid-phase and cell-binding assays as described in Refs. [23–26]. The degree of biotinylation was determined by a recently established proteomics-based method [27]. Galectin histochemistry with the tissue protein as a marker, including rigorous specificity controls, was performed following an optimised protocol outlined previously in Refs. [20–22]. The presence of cytokeratins (except 1, 8, 19) was detected by the monoclonal antibody Ck1 (lp34) (Dako, Brno, Czech Republic) and cytokeratin 10 was detected by a

suitable monoclonal antibody obtained from the same supplier. Both reactions, i.e. galectin cytochemical and immunocytochemical staining reactions, were performed in parallel as described in Ref. [28]. Commercially available ExtrAvidin-tetramethylrhodamine isothiocyanate (TRITC) (Sigma–Aldrich, Prague, Czech Republic) and flourescent isothiocyanate (FITC)-labelled swine-anti mouse immunoglobulins (SwAM-FITC, ALSEVA, Prague, Czech Republic) were used as second-step reagents. Control reactions to ascertain carbohydrate-dependent specificity were performed using preincubation of biotinylated probe with 5 mM lactose as a competitive inhibitor. To exclude a false-positive reaction by non-specific binding of an antibody predominantly via Fc receptors, an antibody specific for CD1a (Immunotech, Prague, Czech Republic), an epitope, which is not present on epithelial cells, was tested. This reagent replaced the two cytokeratin-specific markers during routine processing in a control section. The specimens were mounted on Vectashield (Vector Laboratories, Burlingame, CA, USA) and inspected by fluorescence microscopy with an Optiphot two microscope (Nikon, Prague, Czech Republic) equipped with a charged coupled device camera Cohu and the computer-assisted image analysis system LUCIA (Laboratory Imaging, Prague, Czech Republic). The percentage of epithelial cells positive for specific binding of Gal-3 was evaluated on a 100× scale of magnification. Sections were examined by two independent observers who were completely blinded with respect to the clinical outcome and clinicopathological features of the patients (reliability of interobserver comparison, $r = 0.95$). At least 300 cells within randomly selected and defined area sections on each slide were counted. For statistical analysis, cut-off points were chosen to classify tumours to be positive or negative for Gal-3-specific binding. A cut-off point of 5% stained tumour cells within the tumour cell population was arbitrarily set on the basis of initial monitoring of the cases to determine the range of positive cells in the slides compared with the results in the controls. Cut-offs were defined prior to relating clinical parameters to results of histochemical staining. The percentage of stained cells in tumours assigned to the positive group was more than 50% in 87% of the cases.

2.3. Statistical analysis

The Mann–Whitney procedure and Chi-squared tests were used to analyse the Gal-3-dependent reactivity status in relation to the different clinicopathological parameters. Overall survival and disease-free interval were calculated using the standard method established by Kaplan and Meier, and differences were analysed by using the Gehan-generalised Wilcoxon test. Multivariate analysis for factors related to disease recurrence was performed by the Cox proportional hazard model. Statistica 6.0 software (StatSoft, Prague, Czech Republic) was used for all statistical analyses. Overall survival was computed from the date of surgery to the documented date of the last follow-up or death, whereas disease-free survival was considered to cover the period from the date of surgery to the date of recurrence.

3. Results

The analysis with a labelled marker depends critically on the lack of impairment of binding activity by the inevitable chemical modification needed to accomplish label incorporation. To ascertain the absence of a negative effect, we first checked the biotinylated lectin for retention of its binding activity by solid-phase assays using the glycoprotein asialofetuin as a matrix and by fluorescent activated cell sorting (FACS) can analysis of colon/ovarian/B lymphoma cell lines using glycoclusters as efficient inhibitors (data not shown). After these rigorous control reactions in two settings revealed carbohydrate-dependent binding activity to ligands presented on plastic and cell surfaces was maintained, we proceeded to systematically assess the binding properties of tumour sections under constant experimental conditions. Lactose, used as a competitive inhibitor in the incubation medium, had a significant inhibitory effect on the binding of Gal-3 to tumour cells as well as to leucocytes (data not shown) supporting the carbohydrate-dependent binding of the probe to cells studied. As an example that we were able to exclude the occurrence of non-specific binding, the lack of reactivity for Gal-3 observed in cells of poorly differentiated tumours from the two studied locations is illustrated (Fig. 1(a)). Gal-3 binding to tumour-infiltrating leucocytes served as an inherent positive control. Underscoring a clear correlation with the degree of differentiation, well-differentiated carcinomas, which frequently expressed cytokeratin 10, had a distinct signal intensity for Gal-3-dependent reactivity at the surface of malignant cells (Fig. 1(b)) in agreement with our previous observation of a colocalisation of Gal-3-reactive epitopes with desmosomal proteins [20,21]. Of the 53 cases examined for Gal-3 binding, sections from 23 (43%) patients were beyond the threshold level of 5% positive tumour cells and were therefore considered to be positive. Conversely, 30 (57%) tumour specimens showed no evidence for staining or only a few positive cells and did not reach the threshold level of 5% of the cell population. Consequently, these cases were considered to be negative. Having collected these data on staining, we set forth to analyse any correlations with the clinicopathological features listed in Table 1.

The computation of the relationship between the experimentally determined parameter (i.e. capacity to bind the tissue protein) and clinicopathological features

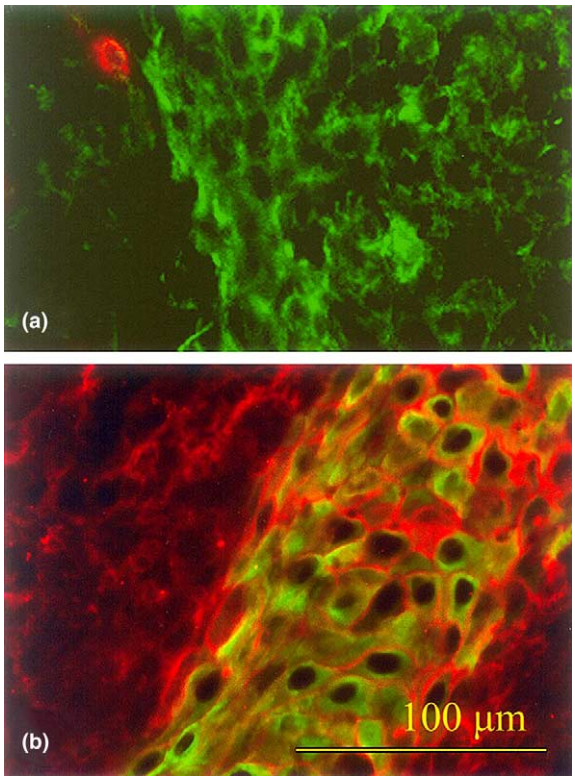

Fig. 1. Expression of galectin-3-binding sites (a, b; red signal), the Ck_1-antibody-defined panel of cytokeratins (a; green signal) and cytokeratin 10 (a; green signal) in sections of tumour specimen from two patients with squamous cell carcinoma of the larynx (a; poorly differentiated squamous cell carcinoma, b; well differentiated squamous cell carcinoma). The signal in leucocytes in panel a, where tumour cells are negative, is an internal positive control for the activity of the marker. Bar is 100 μm.

including age, primary tumour localisation, tumour size, nodal status, tumour stage, tumour differentiation and keratinisation is summarised in Table 1. There were no significant differences between the two groups with respect to age, tumour localisation, tumour size and tumour stage. However, cases with Gal-3 reactivity were associated with histopathological grading ($P = 0.0001$), tumour keratinisation ($P = 0.0007$) and the nodal status ($P = 0.0224$).

During the follow-up period, locoregional recurrences were observed in 23 (43%) cases. At the end of our study, 19 (36%) patients had died of their cancer. No patients in this study died of diseases unrelated to their tumour. Of note, a significant relationship was found between capacity of tumour cells to bind Gal-3 and patient survival. Fig. 2 depicts the Kaplan–Meier plot of the overall and disease-free survival curves stratified by our experimental parameters. At the end of the follow-up period, the estimated relapse-free survival was 63.7% for patients with positive tumours compared with 43.3% for those with negative tumours ($P = 0.0011$). Similarly, the overall survival rate was 79.5% for patients with positive tumours compared with 50% for those with negative tumours ($P = 0.0024$). To determine the independent predictive value of this parameter for patient outcome, a multivariate analysis using the Cox proportional hazard model was carried out. As shown in Table 2, only one parameter, i.e. the galectin-dependent staining, reached the level for independent prognostic significance in both relapse-free and overall survival. Node positivity, the only other factor of statistical significance for this patient group, had an impact on the disease-free interval.

Table 1
Correlation between the galectin-3-dependent parameters and clinicopathological findings

	Low level ($n = 30$)		High level ($n = 23$)		P value
	N.	(%)	*N.*	(%)	
Age (years)					0.3412
< 60	13	(50)	13	(50)	
⩾ 60	17	(63)	10	(37)	
Primary tumour					0.7990
Oropharynx	18	(58)	13	(42)	
larynx	12	(55)	10	(45)	
T classification					0.7116
T1+T2	9	(53)	8	(47)	
T3+T4	21	(58)	15	(42)	
N classification					0.0224
N0	2	(22)	7	(78)	
N1-3	28	(64)	16	(36)	
Stage					0.2144
III	7	(44)	9	(56)	
IV	23	(62)	14	(38)	
Histopathological grade					0.0001
1+2	10	(33)	20	(67)	
3+4	20	(87)	3	(13)	
Tumour keratinisation					0.0007
Keratinising	14	(40)	21	(60)	
Non-keratinising	16	(89)	2	(11)	

4. Discussion

The aims of our study were to test a tissue protein after labelling as a marker (conceptual/technical aim) and to then proceed to answer the question as to whether the reactivity of tumour cells to the endogenous lectin Gal-3, a cell adhesion molecule and growth regulator, might be of prognostic relevance. The results obtained from analysis of data from 53 patients suffering from advanced squamous carcinoma of the larynx and oropharynx revealed that positivity of tumour cells is positively and significantly related to with prognosis. Previous reports which related galectin-3-dependent parameters to differentiation [18,19] had prompted this study. Our results clearly emphasise that Gal-3 should not primarily be viewed as anti-apoptotic molecule and mediator of tumour spread [15,16]. As indicated recently by correlating Gal-3 absence to an unfavourable prognosis in

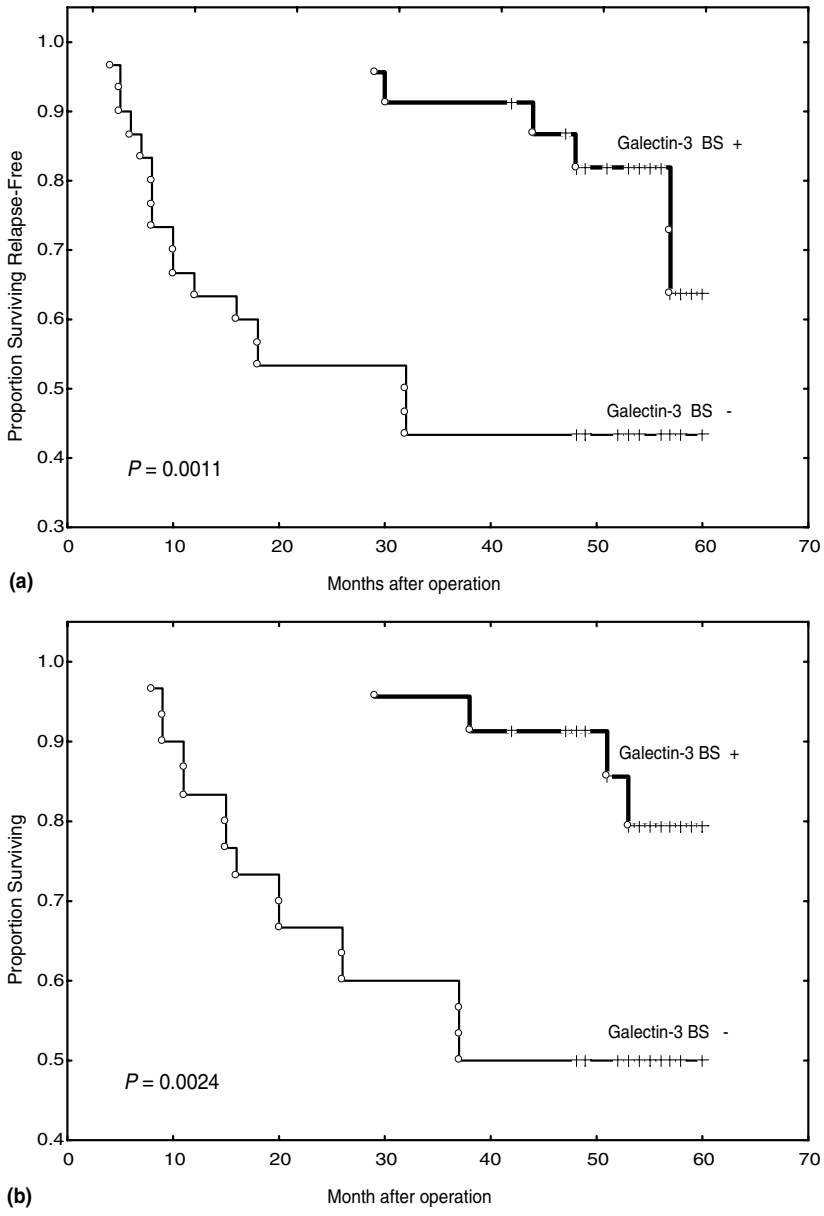

Fig. 2. Survival rate grouped according to the presence of accessible galectin-3-binding sites (= BS) in tumour cells of the 53 specimens. Relapse-free survival (a) and overall survival (b) were computed for the cases with above-threshold positivity of tumour cells (thick line) *vs* those cases in which cells lacked positivity or failed to reach the threshold of 5% positivity (thin line). Circles indicate deceased patients while crosses symbolise living patients. The *P* values were computed using the Gehan-generalised Wilcoxon test.

node-negative patients with laryngeal squamous cell carcinoma [29], Gal-3 might in fact serve to restrict tumour cell spread for this tumour type. Thus, the lesson is clearly taught that the range of galectin activity depends on the cell type. Supporting this notion and arguing against a common role of Gal-3 to further malignancy, it is instructive to add that Gal-3 affects growth and tumorigenicity in the LNCaP prostate cancer cell line negatively and is present in well-differentiated forms of ductal adenocarcinomas of the pancreas, while the presence of Gal-3 ligands points to a favourable prognostic tendency in testicular cancer [30–32]. Interestingly, the lectin shares the pattern of expression of liver-intestine cadherin in pancreatic cancer cases with which it coimmunoprecipitates [32]. After all, Gal-3 (MAC-2) is a mediator of adhesion linking cell surface and matrix glycoproteins, such as laminin, MAC-2-binding protein, β1-integrin or the afore mentioned cadherin [11,23,32,33]. The detection of accessible binding sites intimates that this factor is not limited to a role of ensuring firm cell contact, helping outside–inside signalling, but apparently favourably affects the clinical course in this tumour system. Remarkably, our study suggests this factor may have prognostic relevance as has been reported for distinguishing benign

Table 2
Cox regression model to define prognostic factors for relapse-free and overall survival

	Relapse-free survival			Overall survival		
Parameter	Beta	Exp(beta)	*P* value	Beta	Exp(beta)	*P* value
Primary tumour Oropharynx *vs* larynx	−0.27	0.32	0.5687	0.52	1.69	0.3211
T classification T1-2 *vs* T3-4	−0.85	0.43	0.1033	0.06	1.06	0.9136
N classification N0 *vs* N1-3	−1.53	0.22	0.0327	−0.57	0.56	0.4595
Stage III *vs* IV	0.48	1.61	0.3976	0.28	1.37	0.6457
Histopathological grade 1+2 *vs* 3+4	0.73	2.07	0.2490	−0.43	0.65	0.5396
Tumour keratinisation Keratinising *vs* non-keratinising	−0.99	0.37	0.1676	0.39	1.49	0.5848
Galectin-3-binding sites High *vs* low	1.85	6.33	0.0039	1.58	4.87	0.0259

Beta refers to the coefficient of each variable in the linear combination and exp(beta) to its exponential value. The *P* value denotes the level of significance of the contribution of each variable to the model (thus enabling to conclude that beta is significantly different from zero).

versus malignant uterine smooth muscle tumours [34]. When recalling that α2,6-sialylation, a substitution that blocks Gal-3 binding to the resulting β-galactoside derivative [35], is involved in regulating ligand presentation in squamous epithelia and carcinoma sections [36], our data further intimate that a non-random relation of expression of respective sialyltransferases (e.g. ST6Gal I) and Gal-3, should be a topic for further research. The application of endogenous lectins in future studies will thus be instrumental to delineate new functional aspects of distinct changes of glycosylation. So far, alterations in the glycomic profile during the establishment and progression of malignancy have primarily been discussed phenomenologically [37,38]. Cell surface carbohydrates affect tumour cell interactions with normal cells or with the extracellular matrix during tumour growth and metastatic spread [39]. Our findings of Gal-3 binding sites in advanced HNSCC support the hypothesis that tumour cell surface glycosylation is variable and may be correlated with tumour behaviour. With endogenous lectins at hand as tools, functional correlations can now start to be pinpointed. Emerging results will likely result in innovative therapeutic approaches that exploit endogenous lectins and the regulation of their expression. Our study suggests other cellular effectors should be tested as histochemical probes.

In conclusion, the application of the endogenous lectin Gal-3 as a histochemical marker defines a new prognostic tool in patients with laryngeal and oropharyngeal squamous cell carcinoma. Based on the evidence presented, it is reasonable to assume that a distinct group of HNSCC patients with an unfavourable prognosis might be identified by their Gal-3 status. These patients may benefit from changes in their treatment regimens as a result of their newly-identified high-risk.

Conflict of Interest

None.

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