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Published on: 01 Jan 2005 - Oncology (S. Karger AG)

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Prognostic Significance of Endogenous Adhesion/Growth-Regulatory Lectins in Lung Cancer

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Key Words

Apoptosis · Galectin · Hyaluronic acid · Lectin · Lung cancer

Abstract

Objective: To determine the expression of endogenous adhesion/growth-regulatory lectins and their binding sites using labeled tissue lectins as well as the binding profile of hyaluronic acid as an approach to define new prognostic markers. **Methods:** Sections of paraffin-embedded histological material of 481 lungs from lung tumor patients following radical lung excision processed by a routine immunohistochemical method (avidin-biotin labeling, DAB chromogen). Specific antibodies against galectins-1 and -3 and the heparin-binding lectin were tested. Staining by labeled galectins and hyaluronic acid was similarly visualized by a routine protocol. After semiquantitative assessment of staining, the results were compared with the pT and pN stages and the histological type. Survival was calculated by univariate and multivariate methods. **Results:** Binding of galectin-1 and its expression tended to increase, whereas the pa-

rameters for galectin-3 decreased in advanced pT and pN stages at a statistically significant level. The number of positive cases was considerably smaller among the cases with small cell lung cancer than in the group with non-small-cell lung cancer, among which adenocarcinomas figured prominently with the exception of galectin-1 expression. Kaplan-Meier computations revealed that the survival rate of patients with galectin-3-binding or galectin-1-expressing tumors was significantly poorer than that of the negative cases. In the multivariate calculations of survival lymph node metastases ($p < 0.0001$), histological type ($p = 0.003$), galectin-3-binding capacity ($p = 0.01$), galectin-3 expression ($p = 0.03$) and pT status ($p = 0.003$) proved to be independent prognostic factors, not correlated with the pN stage. **Conclusion:** The expression and the capacity to bind the adhesion/growth regulatory galectin-3 is defined as an unfavorable prognostic factor not correlated with the pTN stage.

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Introduction

Clinical procedures such as prognostic evaluations can benefit from new insights into the biochemistry of tumor growth and spread. By defining relevant mechanisms at the molecular level it becomes feasible to test the predictive values of newly defined markers in ensuing histopathological studies. Our study is based on the current paradigmatic shift in the way the presence of cell surface carbohydrates and their changes in malignancy are being interpreted [1, 2]. Initially, they were viewed as primarily phenomenological alterations detected by plant lectins and monoclonal antibodies; the emerging concept of the sugar code now ascribes functional significance to them [3]. Explicitly, carbohydrate epitopes of cellular glycoconjugates serve as ligands for endogenous lectins, and this interaction is a key regulator of cell adhesion/migration and proliferation [2, 4]. Especially their spatial accessibility and versatile substitutions confer prominence to β -galactosides at chain termini in this respect [3]. A family of lectins, fittingly termed ‘galectins’, exclusively devoted to read this type of biochemical signal, has developed [2]. Cell biological studies pinpointing members of this family as effectors have given reason to localize these lectins in situ either by neoglycoproteins or galectin-specific antibodies [5–7]. Of note, respective studies have revealed that – in addition to carbohydrate-dependent cell surface

binding which is sensitive to substitutions in the glycan chains [8, 9] – galectins-1 and -3 also execute important malignancy-associated functions in the cytoplasm and nucleus, e.g. in positioning of oncogenic H-Ras [10, 11]. These findings add to the interest of examining the expression of these galectins in tumors and they also suggest to introduce labeled galectins to monitor binding-site expression in parallel. Recombinant expression and labeling without impairing binding activity are instrumental to produce the reagents for this purpose [12, 13]. In addition to monitoring galectins we also assessed features related to extracellular matrix lectins, i.e. receptors for heparin/heparan sulfate and hyaluronic acid. In this retrospective study on tissue sections from 481 patients we ask the questions whether and to what extent these attributes of in vitro growth/adhesion modulation correlate with TNM parameters and survival.

Patients and Methods

Four hundred eighty one patients with lung cancer who underwent potentially curative surgery between January 1, 1990, and December 31, 1995, were included into this study. A total of 246 patients were treated at the thoracic surgical unit of the Thoraxklinik in Heidelberg (Germany) and 235 patients at the Department of Surgery of the University of Szeged (Hungary). Table 1 lists the statistical data of the patients (sex, histology and tumor stage)

Table 1. Patients at the two surgical centers

	Heidelberg	Szeged	Total	p
Men	201	189	390	0.72
Women	45	46	91	
Age, years	60.13 ± 8.85	57.33 ± 8.85	58.7 ± 8.94	0.001
Histological type				
Squamous cell carcinoma	112	114	216	0.786
Adenocarcinoma	92	86	178	
Large cell carcinoma	34	33	67	
Small cell carcinoma	8	12	20	
Stage				
I/A	29	31	60	0.524
I/B	95	83	178	
II/A	4	2	6	
II/B	45	46	91	
III/A	71	66	137	
III/B	2	4	6	
IV	0	3	3	
Survival				
5-year survival, %	41.4	37.5		0.463
Median survival time, months	43	42		

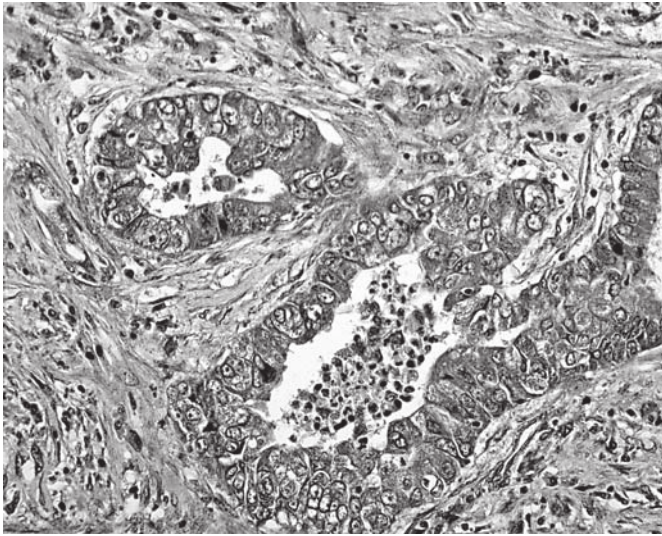


Fig. 1. Lung adenocarcinoma demonstrating intensive galectin-3-binding capacity.

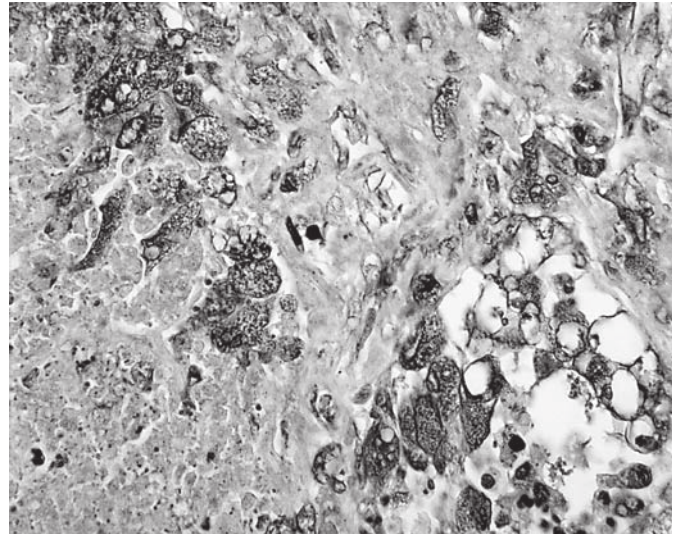


Fig. 2. Lung large cell carcinoma demonstrating intensive galectin-3 expression.

which had to be compared to reliably exclude a center-specific patient bias. With the exception of age, significant differences were not observed between the parameters of the two cohorts of patients and the survival rates at the two centers. The mean duration of the follow-up period was 48.9 months (range 1–125 months).

Sections, 4–6 μm thick, were prepared from the paraffin-embedded tumor tissue and the histochemical staining protocol followed an optimized procedure as reported in detail previously [12, 14]. The biotinylated lectins and hyaluronic acid and the antibodies against lectin were prepared in the Department of Physiological Chemistry at the Faculty of Veterinary Science of the Maximilian Ludwig University. Following routine inhibition of endogenous peroxidase activity and saturation of nonspecific protein-binding sites and biotin-binding sites, the tissue sections were incubated with the probes at room temperature for 60 min. Lectin-binding capacities of the tumor cells were studied with labeled (biotinylated) galectin-1 (Gal-1) and galectin-3 (Gal-3) prepared and checked for purity, extent of label incorporation and activity as described [15–18]. Hyaluronic-acid-binding capacity was investigated with biotinylated hyaluronic acid dissolved in medium which was either Ca^{2+} free (HA) or contained 8 mmol/l Ca^{2+} (HA+ Ca^{2+}) to test Ca^{2+} -independent and Ca^{2+} -dependent binding activities [19]. The final dilution of probes was 10 $\mu\text{g}/\text{ml}$. Visualization of specific binding was performed with the avidin-biotin technique (Vector Laboratories, Burlingame, USA) (fig. 1). In parallel, serial sections were tested with polyclonal antibodies against galectin-1, galectin-3 and the heparin-binding lectin (HBL) which had been examined for target specificity by Western blotting and ELISA assays [12, 20, 21]. The final dilution of probes was 10 $\mu\text{g}/\text{ml}$ as well. Antigen-dependent antibody binding was detected using a commercial monoclonal antibody against rabbit immunoglobulin G, with a streptavidin-biotin method as the staining system (BioGenex, San Ramon, USA) (fig. 2). Routine light-microscopic assessment of the sections was performed by the same pathologist. An automated image-analyzing

system (DIAS, University of Jena, Germany) was routinely applied for bias-free classification of cases into negative or positive on the basis of staining intensity and number of stained cells as described previously [14, 21]. The results were put into relation with the pT and pN status, the histological findings and survival data. The data were subjected to the χ^2 test and an ANOVA. The Kaplan-Meier method and Cox regression analysis were used for the respective calculations: in the former method, significance was established with a log-rank test. The SPSS 11.0 (SPSS Inc., Chicago, Ill., USA) program was applied for the statistical data processing.

Results

Binding of Galectins and Hyaluronic Acid and Presence of Lectins: Relation with Tumor Type and Features

Carbohydrate-dependent binding of these three probes could be detected. As an internal specificity control and evidence for disparate ligand selection, the binding profiles of the two related galectins were found not to be identical. The proportions of tumors with galectin-1- and galectin-3-binding capacities showed a discrete increase of 8% and 3% in relation to progressive lymph node stages, respectively. However, this increase did not reach a statistically significant level. A similar tendency could be observed for the tumors displaying galectin-1 expression (N0: 39.6%, N1: 49.1%, N2: 49.6%, $p = 0.106$), in contrast to galectin-3 expression, which was more frequently seen in N0 tumors (74.7%) than in N1 (64.4%)

Table 2. Distribution (%) of positive cases according to histological type

Cell type	n	Gal-1 BC	Gal-1 EX	Gal-3 BC	Gal-3 EX	HBL EX	HA BC	HA+Ca ²⁺ BC
Squamous cell carcinoma	216	64.81	38.43	35.19	64.35	73.15	31.94	18.98
Adenocarcinoma	179	73.18	50.84	49.72	83.24	80.45	37.99	21.91
Large cell carcinoma	67	61.19	52.24	34.33	65.67	67.16	28.36	17.91
Small cell carcinoma	18	55.56	15.79	21.05	36.84	26.32	21.05	5.26
		p = 0.13	p = 0.003	p = 0.005	p < 0.001	p < 0.001	p = 0.264	p = 0.352

BC = Binding capacity for the biotinylated marker; EX = expression of the lectin.

or N2 (67.3%) tumors ($p = 0.089$). The proportion of the hyaluronic-acid-binding tumors increased with advancing lymph node stages (N0: 31.7%, N1: 34.7%, N2: 36.8%), but the difference was not statistically significant ($p = 0.623$). When the sections were processed in Ca²⁺-containing medium, the proportion of hyaluronic-acid-binding tumors was about 10–16% lower, and the distribution corresponding to the lymph node metastases changed as well (N0: 21.5%, N1: 13.5%, N: 20%, $p = 0.187$).

The proportion of tumors expressing galectin-1 increased with advancing T stages (T1: 42.4%, T2: 43.3%, T3: 46.1%, $p = 0.885$). In contrast, the proportion of tumors with galectin-3-binding capacity (T1: 47.9%, T2: 39% and T3: 36.5%, $p = 0.278$) and galectin-3 expression (T1: 76.7%, T2: 74%, T3: 55%, $p = 0.001$) decreased to a statistically significant extent with increasing pT stage. A similar tendency was observed for hyaluronic-acid-binding capacity (T1: 35.6%, T2: 34%, T3: 30.7%, $p = 0.768$ for the Ca²⁺-free medium and T1: 27.4%, T2: 19.3%, T3: 14.4%, $p = 0.1$ for Ca²⁺-containing medium, respectively). Heparin-binding lectin positivity was found to be maximal in pT2 tumors; the number of positive cases was about 10% lower in pT1 and pT3 tumors ($p = 0.006$). The small number of pT4 (and also pN3) cases precluded extension of the calculations. Galectin-1-binding capacity and galectin-1 expression tended to increase with advancing pT and pN stages. In contrast, the number of galectin-3-positive cases tended to decrease with advancing tumor and lymph node stages.

The proportion of positive cases was significantly lower among the patients with small cell lung cancer for all applied probes compared to those of the non-small-cell lung cancer group. In four of the cohorts (galectin-1, galectin-3 and heparin-binding lectin expression and galectin-3-binding capacity) the difference was statistically significant (table 2).

With respect to cell type within the non-small-cell lung cancer group, the proportion of positive cases was obviously higher among the adenocarcinomas than the squamous cell carcinoma or large-cell lung cancer groups with the exception of galectin-1 expression. The greatest differences were seen for the galectin-3-binding expression (about 20%) (table 2).

Survival Study

Looking at the results of the univariate analysis, the 5-year survival rate of patients with galectin-3-binding tumors was 33.7%, whereas that of the negative cases was 42.6%; the median survival was 35.3 and 45.9 months, respectively ($p = 0.039$) (fig. 3). The survival of patients with galectin-1-expressing tumors was even worse: in case of positive staining, the 5-year survival was 32.5%, and the median survival time was 35 months, the corresponding data for the negative tumors were 44.5% and 48.8 months ($p = 0.027$) (fig. 4). In the event of Gal-1-binding capacity and Gal-3-expression median survival is similarly better for the cases with negative-staining tumors, but the difference between the negative and positive cases is not significant. For Gal-1-binding tumors, mean survival is 40 months, while in the Gal-1-negative cases it is 46 months ($p = 0.368$); for Gal-3-expressing tumors, mean survival is 42 months, while in the non-staining cases it is 44 months ($p = 0.255$). The survival is better for HBL-expressing tumors (44 months) than for the negative cases (29.2 months), but the difference is statistically nonsignificant ($p = 0.508$). The HA-binding capacity itself impairs the chances of survival (33.6 months vs. 44 months), while in the HA + Ca²⁺ medium the survival of cases with HA-binding tumors exceeds that of the negative cases (51 vs. 40.6 months). The difference in survival is not significant.

In the multivariate survival calculations, the classical prognostic factors (pT, pN and cell type) predicted sur-

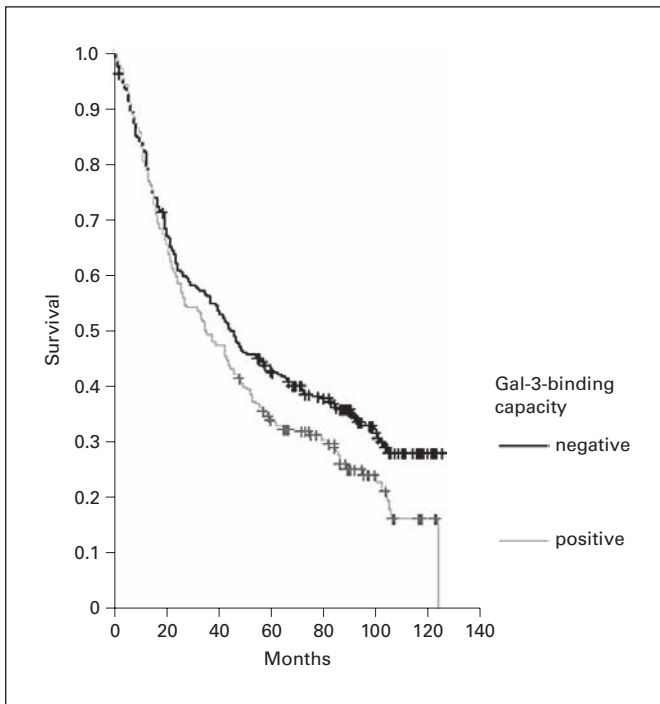


Fig. 3. Cumulative survival of patients with galectin-3 binding and nonbinding lung tumors ($p = 0.039$).

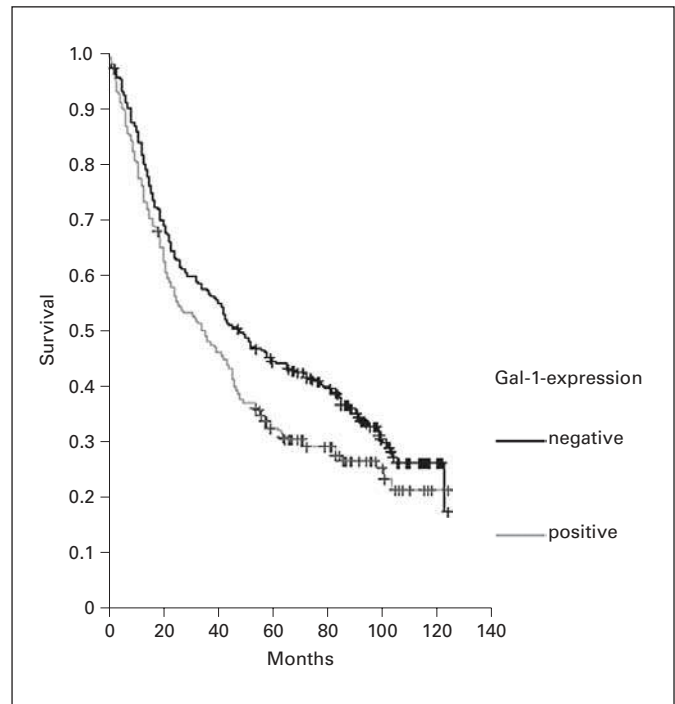


Fig. 4. Cumulative survival of patients with galectin-1-expressing and nonexpressing lung tumors ($p = 0.0274$).

Table 3. Multivariate analysis of survival of radically operated lung cancer patients according to histological type, TNM classification, lectin-binding capacity and lectin expression

	Relative risk of deaths	Significance	95% CI
Histology		0.003	
Squamous cell carcinoma vs. adenocarcinoma	1.277	0.068	0.981–1.661
Squamous cell carcinoma vs. large cell carcinoma	1.387	0.061	0.985–1.953
Squamous cell carcinoma vs. small cell carcinoma	2.549	<0.001	1.494–4.346
pT		0.003	
pT1 vs. pT2	1.674	0.004	1.176–2.381
pT1 vs. pT3	2.091	0.001	1.357–3.224
pN		<0.001	
pN0 vs. pN1	1.362	0.031	1.029–1.828
pN0 vs. pN2	2.195	0.000	1.667–2.961
Galectin-1-binding capacity	1.006	0.963	0.783–1.292
Galectin-3-binding capacity	0.736	0.010	0.582–0.928
Galectin-1 expression	0.897	0.375	0.706–1.140
Galectin-3 expression	0.761	0.030	0.594–0.974
CL-16-binding capacity	0.941	0.643	0.727–1.217
Heparin-binding lectin expression	1.158	0.316	0.869–1.542
Hyaluronic acid binding capacity	0.894	0.379	0.698–1.147
Hyaluronic acid binding capacity (+Ca ²⁺)	1.268	0.119	0.940–1.709

Table 4. Multivariate analysis of survival of radically operated non-small cell lung cancer patients according to histological type, TNM classification, lectin-binding capacity and lectin expression

	Relative risk of death	Significance	95% CI
Histology			
Squamous cell carcinoma vs. adenocarcinoma	1.272	0.074	0.976–1.655
Squamous cell carcinoma vs. large cell carcinoma	1.376	0.068	0.976–1.937
pT			
pT1 vs. pT2	1.651	0.008	1.142–2.387
pT1 vs. pT3	2.047	0.002	1.302–3.218
pN			
pN0 vs. pN1	1.403	0.023	1.047–1.878
pN0 vs. pN2	2.137	<0.001	1.590–2.872
Galectin-1-binding capacity	1.009	0.945	0.779–1.306
Galectin-3-binding capacity	0.734	0.011	0.578–0.932
Galectin-1-expression	0.852	0.201	0.667–1.088
Galectin-3-expression	0.783	0.058	0.607–1.008
CL-16-binding capacity	0.914	0.508	0.699–1.193
Heparin-binding lectin expression	1.172	0.291	0.872–1.574
Hyaluronic-acid binding capacity	0.900	0.417	0.698–1.160
Hyaluronic-acid-binding capacity (+Ca ²⁺)	1.277	0.113	0.943–1.728

vival reliably. From the tested probes, galectin-3-binding capacity and galectin-3 expression proved to be an independent prognostic factor: negative cases displayed an improved survival rate at a statistically significant level (table 3). In the non-small-cell lung cancer group there was no difference in survival between the histological types. According to multivariate analysis, the lymph node status ranked first followed by the galectin-3-binding capacity when estimating the prognostic factors ($p = 0.011$), increases in the status of the T classification likewise negatively influencing survival (table 4).

Discussion

Based on our data galectin-related characteristics appear to be relevant for lung tumor behavior in patient material. These studies are essential to relate galectins to clinical procedures, because cell lines may confound interpretation owing to unique properties of distinct lines. Also, the *in vivo* data are critical to pinpoint cell-type-specific attributes potentially relevant for design of therapeutic approaches. An example of dependence on cell type is provided by previous measurements on metastasis formation from breast and colon cancer to the lung [21]. Regarding thoracic tumors and relating to the results of our study, histological differential diagnosis of epithelial

mesothelioma reached sensitivity and specificity in the case of the hyaluronic-acid-binding capacity of 87 and 91%, respectively [19]. The survival rate for patients whose tumor was positive with labeled hyaluronic acid was better than that for cases which gave negative results on histochemical examination [19]. The introduction of this marker to visualize binding capacity for this glycosaminoglycan which participates in growth regulation and tissue remodeling to lung carcinoma yielded no prognostic information, in contrast to galectin-dependent parameters.

The observation that the survival rate of patients with galectin-3-binding tumors is significantly poorer than that of the negative cases is a salient result of this study. Survival likewise decreases, though not at a statistically significant level, in the event of galectin-3 expression. Galectin-3 plays a role in the progression of tumors at several points. It promotes the angiogenesis of tumor tissue [22] and can block growth-inhibitory effects of galectins-1 and -7 [16]. Unlike homodimeric galectins, galectin-3 can form pentamers besides the monomer, and is thus capable of generating different cross-linked complexes with ligands [15, 23, 27, 28]. Moreover, it has a distinct pattern of intracellular binding. Galectin-3 is a well-known anti-apoptotic effector and enhanced galectin-3 levels are known to inhibit nitrogen-oxide-induced apoptosis in BT549 breast tumor cells [7, 11]. And in-

deed, in a study by Sheikholeslam-Zadeh et al. [24], immunohistochemical monitoring revealed increased galectin-3 expression in cholesteatomas that counteracted apoptosis. The combination of TTF-1 and galectin-3 constitutes an independent prognostic factor indicating that galectin-3 may interfere with tumor progression by enhancing the transcriptional effect of TTF-1 [25]. With most of the examined lectins, the cells of small cell lung cancer displayed a considerably lower binding capacity and expression than those of non-small-cell lung cancer. A similar conclusion was reached by Buttery et al. [26], who investigated the expression of galectin-3 in lung cancer. We assume that the different biological behavior of small cell carcinoma (rapid spread, early metastatization and chemosensitivity) may be connected with these results.

In addition to galectin-3, we also included galectin-1 monitoring to clarify the role of this lectin in lung cancer as well. Our results indicate that galectin-1 expression by tumor cells is associated with a poor survival rate. In the event of more advanced pT and pN stages, the proportion of positive cases tended to be higher, in contrast with the situation for galectin-3. A similar tendency was observed in breast carcinoma, where galectin-1 expression occurs with a higher frequency in cases expressing lymph node metastases than in those without evident lymph node in-

volvement, while colon cancer yielded opposite results, emphasizing cell type specificity [21]. Galectin-1 indeed has a pronounced cell-type-specific reactivity profile. In vitro and in vivo measurements including analysis of biopsies showed growth-inhibitory effects on neuroblastoma and T leukemic cells and tumor-promoting effects in glioblastoma [29, 30]. As binding partner of oncogenic H-Ras, galectin-1 is an effector of cell transformation [10]. It is an important conclusion of our study that galectin-related features are of prognostic significance in lung cancer. The cell type specificity of galectin functionality precludes immediate predictions. The reported results and the emerging network of galectins now pose the challenge to perform galectin fingerprinting in lung cancer in order to delineate the interplay between the different members of this family [31]. Explicitly, monitoring of the presence of galectin and binding capacity beyond galectins-1 and -3 will be a promising subject for future investigations.

Acknowledgement

The financial support of the Verein zur Förderung des biologisch-technologischen Fortschritts in der Medizin e.V. and the International Academy of Telepathology e.V. is gratefully acknowledged.

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