Original Article

Immunohistochemical Fingerprinting of the Network of Seven Adhesion/Growth-Regulatory Lectins in Human Skin and Detection of Distinct Tumour-Associated Alterations

(apoptosis / galectin / glycohistochemistry / glycoprotein / integrin / stroma / sugar code)

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Abstract. Glycans of natural glycoconjugates are considered as a source of biological information relevant to cell adhesion or growth. Sugar-based messages are decoded and translated into responses by endogenous lectins. This mechanism assigns a functional dimension to tumour-associated changes of glycosylation. Consequently, it calls for mapping the lectin presence in tumours. Such an analysis has so far commonly been performed with the scope to determine expression of a few distinct proteins, e.g. from the effector family of galectins with focus on galectins-1 and -3. Due to the emerging evidence for functional divergence among galectins it is timely to address the challenge to evaluate their presence beyond these few family members. Having raised a panel of non-cross-reactive antibodies against seven human galectins covering all three subfamilies, we describe their expression profiles in human skin. Comparison of normal and malignant tissues enabled us to define galectin-type-dependent alterations, arguing in favour of distinct functionalities. It is concluded that comprehensive monitoring performed to define the different aspects of the galectin network, as documented in this pilot study, is advisable for future histopathologic studies aimed at delineating clinical correlations.

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Introduction

Biological information transfer relevant to malignancy is mainly monitored at the level of nucleic acids and proteins. Of note, a third class of biomolecules is receiving increasing attention based on the emerging concept of the sugar code (Gabius, 2009). Due to the accumulated evidence on aberrations of glycosylation, the most frequent co- and posttranslational modification of proteins, in tumour cells, essential functions of glycans within the course of the disease are rendered likely (Caselitz, 1987; Hakomori, 1996; Gabius et al., 2002, 2004). As a marked conceptual advance from the initial phenomenologic monitoring of structural glycan modifications, a direct link of this aspect to its decoding by tissue receptors (lectins) and ensuing cellular responses has been drawn (Gabius, 2008, 2009). In detail, changes in glycosylation are thus not simply viewed as random events establishing disease-associated parameters, but they are assumed to carry a distinct sugar-encoded message. Its information is then converted by protein (lectin)-carbohydrate interactions to eventually trigger specific signalling and glycan-dependent cell activities (Villalobo et al., 2006). As a consequence, detection of lectins as part of tumour characterization offers the perspective to define relevant molecular pathways of information flow. Toward this aim, we focus here on a family of adhesion/growth-regulatory lectins in a suitable model system.

Squamous epithelium forming the epidermis is morphologically and functionally stratified. Of relevance regarding cell growth, only cells of the basal layer anchored to the basement membrane have the potential to proliferate (Watt, 2002). The cells of the upper layers are terminally differentiated, which makes them prone to desquamation (Kanitakis, 2002). The stratification predestines this type of epithelium to serve as a suitable model for the study of cell parameters at different levels

of cell maturation, as illustrated by previous delineation of finely tuned glycosylation (Holíková et al., 2002). Especially carbohydrate epitopes at branch ends of glycan chains are subject to versatile structural modifications, and these readily accessible β-galactoside determinants can bind to – among others – members of the galectin family. This lectin group is further subdivided into three subclasses (proto-, chimera- and tandem-repeat-type proteins), the individual proteins apparently capable to exert diverse and cell-type-specific roles in the control of cell adhesion, apoptosis, growth and migration via homing on distinct glycans and also peptide motifs (Kasai and Hirabayashi, 1996; Gabius, 2001; Lahm et al., 2004; Smetana et al., 2006). Their remarkable selectivity for cell surface glycans fulfils the requirement for eliciting distinct responses. For example, cross-linking galectins can modulate growth of different cell types by binding either ganglioside GM1 (neuroblastoma cells), $\alpha_5\beta_1$-integrin (colon and pancreatic carcinoma cells) or the T_H1-specific cell surface molecule Tim-3 (galectins-1, -7 and -9), apical membrane trafficking by binding distinct N-glycans and sulphatide with 2'-hydroxylated long-chain fatty acid (galectin-4) or adhesion (HeLa cells), and cell activity (superoxide production of neutrophils) by binding α_M- (of the $\alpha_M\beta_2$-complex) or $\alpha_3\beta_1$-integrins (galectin-8) (Kopitz et al., 2001, 2003; Levy et al., 2001; Nishi et al., 2003; Delacour et al., 2005; Fischer et al., 2005; Zhu et al., 2005; Stechly et al., 2009). As evident from these cases, galectins appear to be capable to fulfil distinct assignments by virtue of non-identical ligand selection. Thus, they will likely not be functionally redundant, as e.g. shown for activated T cells and induction of different routes of caspase-dependent apoptosis or for neuroblastoma cells and a functional divergence toward inhibition of proliferation (Kopitz et al., 2001; Sturm et al., 2004; André et al., 2005a; Stillman et al., 2006). These results intimate to draw an analogy for galectins to the complexities of integrin expression and functionality.

It is therefore a key step on the way to understand the operativity of the assumed galectin network to define the expression profiles of different family members in tumour tissues. The development of non-cross-reactive antibodies will make it possible to move from initial biochemical and RT-PCR profiling of galectin expression in tumour tissues and cell lines (Gabius et al., 1984, 1986; Lahm et al., 2001) to immunohistochemical analysis not restricted to only one or two members of this family, commonly galectins-1 and/or -3. The extended monitoring should ideally comprise members of all three galectin categories. Here we report results of a pilot study on immunohistochemical analysis of expression of human galectins-1, -2, -3, -4, -7, -8 and -9 (for classification into subfamilies, please see Table 1; of note, the presence of genes for galectins-5 and -6 is restricted to the rat or mouse, respectively (Cooper, 2002)). Specimens of normal skin and basal cell carcinomas were processed with a respective panel of non-cross-reactive antibodies under identical conditions.

Table 1. Overview of galectin fingerprinting in normal and transformed squamous epithelium

Galectin type	Epidermis: basal cells	Epidermis: suprabasal cells	BCC
galectin-1 (proto-type)	–	–	–[a]/+*
galectin-2 (proto-type)	++	++	–*
galectin-3 (chimera-type)	++	++	–*
galectin-4 (tandem-repeat)	+++	+++	–*
galectin-7 (proto-type)	+++	+++	–[a]/+*
galectin-8 (tandem-repeat)	+++	+++	–[a]/+*
galectin-9 (tandem-repeat)	+++	+*	+/++[a]*

–: no signal, +: weak but significant positivity, ++: medium positivity, +++: strong positivity, ++++: very strong positivity; [a]predominant category in cases with inter- and intraindividual variability, *statistically significant decrease/increase in comparison between different types of normal cells and between normal and malignant cells, BCC – basal cell carcinoma

Material and Methods

Six specimens of normal skin from breast and 17 samples of basal cell carcinoma of the skin were obtained with informed consent of donors either from the Department of Plastic and Reconstructive Surgery (Charles University in Prague, 3rd Faculty of Medicine, Prague) or from the Department of Dermatovenerology. Tissue samples were immediately protected by Tissue-Tek (Sakkura, Zoeterwoude, The Netherlands), frozen in liquid nitrogen and stored at -80 °C until further processing. Seven-μm-thin sections were obtained using a Cryocut-E microtome (Reicher-Jung, Vienna, Austria). The sections were mounted onto poly-L-lysine (Sigma-Aldrich, Prague, Czech Republic)-coated glass slides, rehydrated with 20 mM phosphate-buffered saline (PBS, pH 7.3) and fixed with 2% (w/v) paraformaldehyde in PBS for 5 min. Washed sections were first processed with albumin-containing solution to block any sites with non-specific protein-binding capacity to minimize protein adsorption during the next steps.

The applied galectin-type-specific polyclonal antibodies had been systematically tested for specificity and lack of cross-reactivity, with affinity depletion being performed by affinity chromatography in each positive case followed by another round of controls by ELISA (Kayser et al., 2003; Nagy et al., 2003; Saal et al., 2005; Lensch et al., 2006; Langbein et al., 2007; Dvoránková et al., 2008). They were used at the constant concentration of 20 μg/ml. After extensive washing with PBS to remove unbound antibody, FITC-labelled swine-anti-rabbit antibody (SwAR-FITC, AlSeVa, Prague, Czech Republic) diluted as recommended by the producer was used as second-step reagent. 4',6'-Diamidino-2-phenylindole (DAPI, Sigma-Aldrich) facilitated staining of nuclei in the sections. To ascertain the absence of antigen-independent binding of the tested immunoglobulin G fractions, e.g. via binding of the F_c part of the antibody to F_c receptors in the tissue, galectin-type-specific antibodies were replaced by a rabbit polyclonal antibody

raised against the tandem-repeat-type mannose receptor, which is not present in this epithelial tissue. Further controls to spot any antigen-independent staining reaction were performed by omitting the incubation with first-step reagent from the protocol. After finishing routine processing under conditions carefully kept constant throughout this study, specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA) to prevent the fluorescent signal from bleaching by UV-light. A fluorescence microscope (Nikon Eclipse 90i, Nikon, Prague, Czech Republic) equipped with filterblocks specific for the optical properties of FITC and DAPI and a high-resolution cooled CCD camera (Cool-1300Q CCD camera, Vosskühler, Osnabrück, Germany) together with a computer-assisted image analyser (LUCIA 5.10, Laboratory Imaging, Prague, Czech Republic) were employed for image analysis and data storage. Data were further processed to visualize quantitative aspects of the immunofluorescence staining (Dubový et al., 2002; Purkrábková et al., 2003; Cada et al., 2009), and calculations using the Student's non-paired *t*-test led to assessment of significance levels for differences between cell types.

Results

Parallel monitoring of frozen sections of human epidermis after processing with the seven antibody preparations was performed to address the issue of the extent of complexity of the galectin presence. The results at-

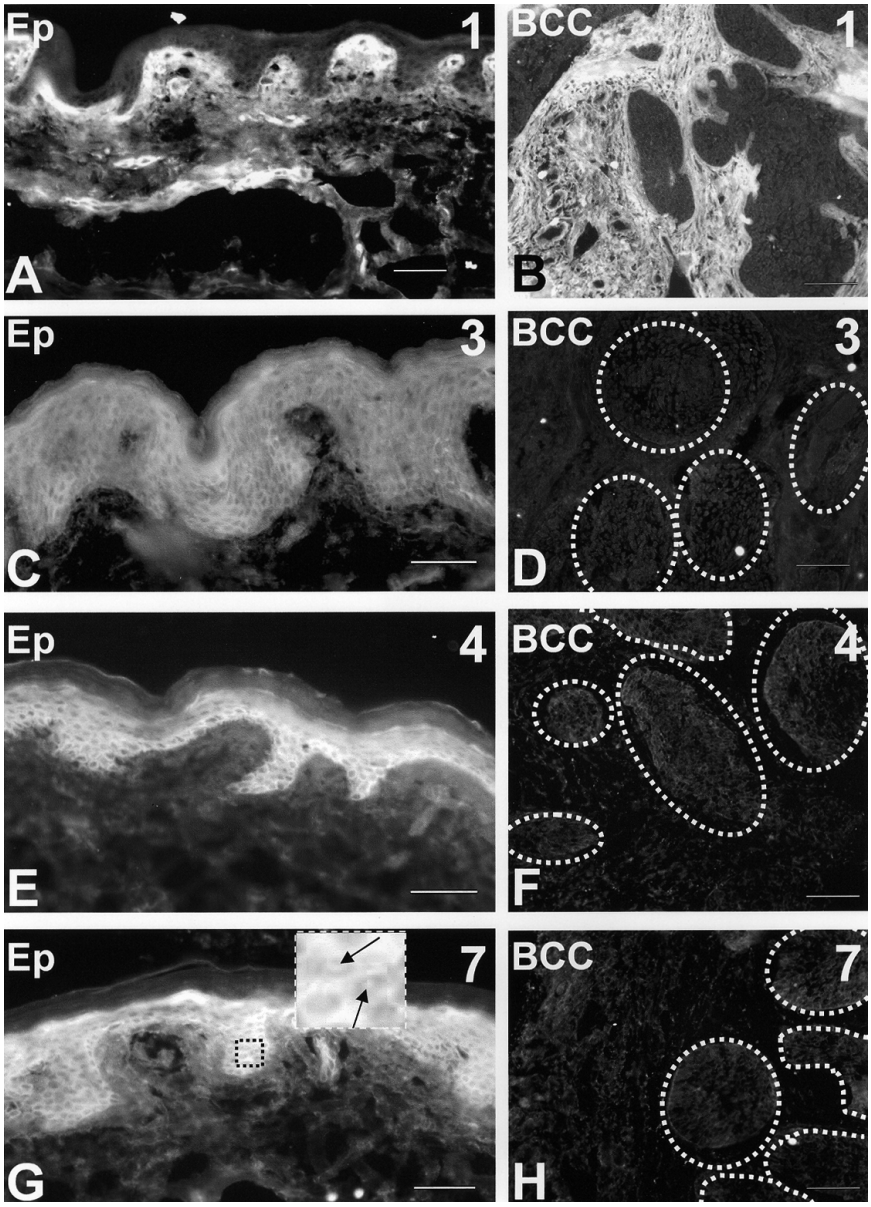

Fig. 1. Immunohistochemical detection of different galectins from the three subfamilies, i.e. galectins-1 (A, B), -3 (C, D), -4 (E, F) and -7 (G, H), in frozen sections of normal human epidermis (Ep; A, C, E, G) and basal cell carcinoma (BCC; B, D, F, H). Insert in G shows nucleolar positivity for galectin-7, nucleoli being indicated by two black arrows. Epithelial knots of basal cell carcinoma in D, F and H are encircled. Bar: 100 μm.

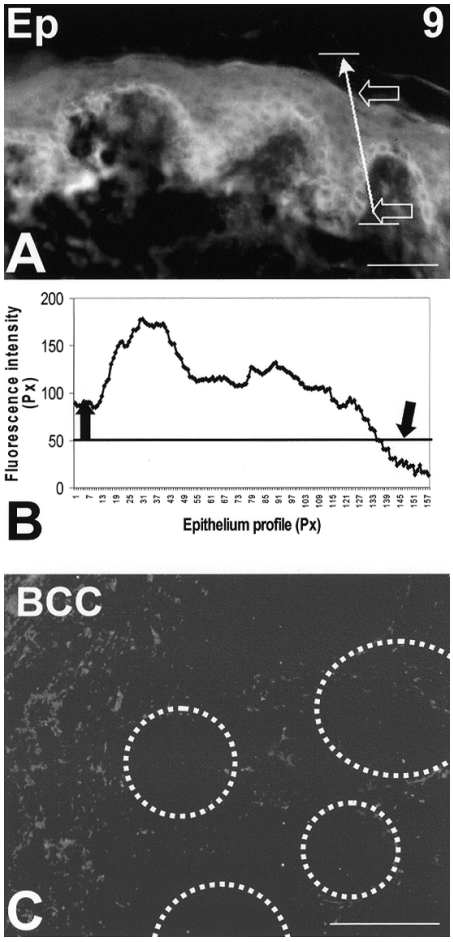

Fig. 2. Immunohistochemical detection of galectin-9 in frozen sections of epidermis (Ep; A, B) and basal cell carcinoma (BCC; C). The white arrows in panels A denote the length of the analysed tissue sector and the direction of quantitative fluorescence profiling illustrated in panel B. For further clarity of the regions under scrutiny, two sets of white (A) and black (C) arrows mark corresponding positions in the sections and the profiles. The background level is depicted by a bold line in panel B. The tumour area is encircled by a dashed line in panel C. Bar: 100 μm.

tested the validity of the concept to extend the range of galectin detection to more than one or two proteins. In fact, our antibody panel enabled us to detect the presence of all seven different galectins tested immunohistochemically, as exemplarily illustrated in Figs. 1–3. Under the given experimental conditions, which were rigorously kept constant throughout this comparative study, reactivity was mostly cytoplasmic and present at cell surfaces, except for nuclear staining of cells of the basal and spinous layers with the anti-galectin-7-specific antibody fraction (Fig. 1G). Monitoring of staining intensity revealed the intensity pattern listed in Table 1, galectin-1 being an exception when examining normal skin epithelium (Fig. 1). In this instance, a strong signal was recorded in the extracellular matrix of the dermis (Fig. 1A). An obvious difference regarding zonal cell positioning was noted in the case of galectin-9 and basal/suprabasal cells (Fig. 2, Table 1). Having hereby provided results on the normal tissue, we proceeded to analyse the malignant counterpart.

The manifestation of basal cell carcinomas led to a conspicuous and rather uniform decrease of the galectin presence, except for galectin-1 (Figs. 1–3, Table 1). However, stromal reactivity was still detectable, and its extent was higher in tumour tissue than in normal skin for galectin-1 (Figs. 1, 2). Underscoring operativity of differential regulatory mechanisms, the opposite situation was measured for galectins-4, -7 and -8 (Fig. 3). Not exceeding background values, monitoring the presence of galectins-2, -3 and -9 practically led to no evidence for stromal expression in this tumour type, further clear evidence for disparate regulation.

Discussion

This study focused on endogenous lectins of the galectin family, because i) they are emerging effectors in the regulation of diverse cell activities, with a range of functions reaching the clinical level, as e.g. documented for clonal selection of $CD4^+CD7^-$ vs. $CD4^+CD7^+$ leukaemic T cells in patients during progression of the Sézary syndrome by galectin-1 and relation of tumour suppressor $p16^{INK4a}$ to this lectin (Rappl et al., 2002; André et al., 2007a), and ii) they can sense changes in glycan structure at branch ends or the core region that may accompany malignancy (Ahmad et al., 2002; Hirabayashi et al., 2002; André et al., 2004; 2005b, 2007b). By using a panel of non-cross-reactive antibodies against seven galectins covering all three subfamilies we moved beyond the scope of previous studies in this area (Smetana et al., 2006). Our panel enabled us to answer the pertinent questions on the extent of complexity of expression profiles and of tumour-associated changes. As a model system, we tested human epidermis and basal cell carcinomas.

It is now clear that the galectin network, at least in these cell types, is not restricted to only few activities. Our strategy therefore makes a strong case for introducing the profiling, as carried out here, to further investigations and hereby gives research in this field a clear direction. Correlation of expression patterns to particular cellular properties may then aid in delineating functional aspects for certain galectins, uncovering new clinical implications. In this respect, the relationship between the galectin-9 presence in basal/suprabasal cells and proliferation may indicate a distinct role of this protein. Interestingly, galectin-9 is strongly expressed in nasopharyngeal carcinomas, down-regulated in oral and cervical squamous cell carcinoma cases/lines and an inducer of apoptosis of MM-RU melanoma cells (Kageshita et al., 2002; Kasamatsu et al., 2005; Pioche-Durieu et al., 2005; Liang et al., 2008). Tumour-associated changes detected in our study pertain to dermal and stromal cells in a galectin-type-dependent manner. These results extend previous observations in the cases of basal and squamous cell carcinomas (Lacina et al., 2007;

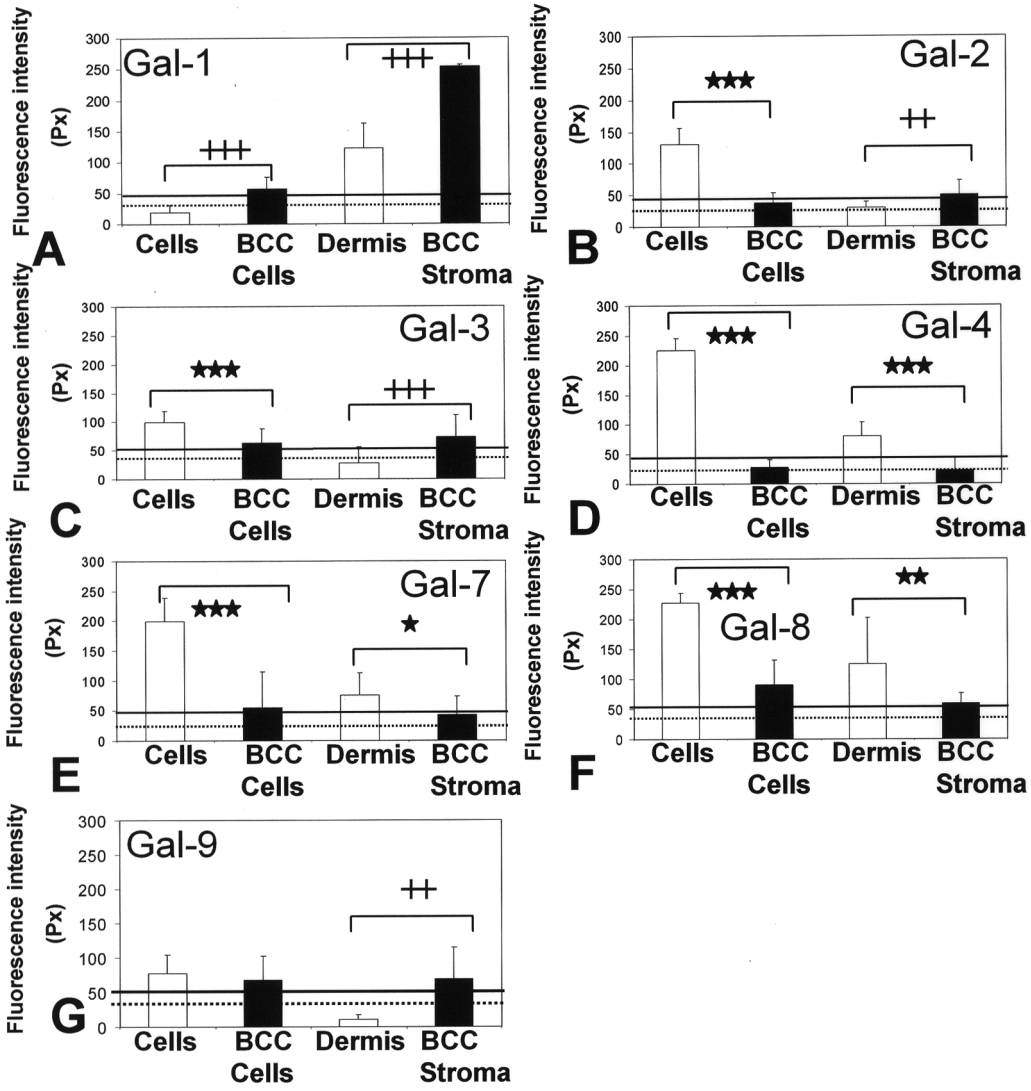

Fig. 3. Quantitation of fluorescence intensity in immunohistochemical detection for the different galectins from the three subfamilies, i.e. galectins-1 (A), -2 (B), -3 (C), -4 (D), -7 (E), -8 (F) and -9 (G), in cells of normal epidermis (termed Cells, white column) and of basal cell carcinomas (termed BCC Cells, black column) as well as in dermis (termed Dermis, white column) and the stroma of tumours (termed BCC Stroma, black column). Statistical significance of differences between signal characteristics of normal and malignant tissues was evaluated by the Student's non-paired *t*-test. Results are given at significance levels of $P < 0.01$, $P < 0.02$ and $P < 0.05$, respectively, a cross denoting an increase and asterisk a decrease of extent of signal intensity. The background level measured in each case is either marked by a bold line (Cells/BCC Cells) or a dashed line (Dermis/BCC Stroma) in each panel.

Saussez et al., 2009a,b). Of note, inverse shifts between nuclear and cytoplasmic localization have even been noted for galectins from the same subgroup upon tumour progression (Saussez et al., 2006, 2008).

These results argue in favour of at least partial functional non-redundancy among galectins. This interpretation is supported by previous immunohistochemical observations on colon carcinomas, especially for the tandem-repeat-type galectins-4 and -8, and monitoring expression of proto- and chimera-type and tandem-repeat-type galectins in murine tissue (Nagy et al., 2002, 2003; Lohr et al., 2007, 2008; Nio-Kobayashi et al., 2009). Evidently, even members of the same subfamily can reveal functional divergence, a finding warranting thorough analysis of tissue sections or arrays. Such studies appear to harbour a noteworthy advantage for figuring out exploitable correlations compared to work with engineered cell systems: the manipulation of levels of galectin expression *in vitro*, a common tool to track down clues for functions, can entail alterations of expression levels of diverse other proteins such as integrins or cadherins, as seen in respective studies with galectins-1 and -3 (Warfield et al., 1997; Matarrese et al., 2000; Camby et al., 2005; Mourad-Zeidan et al., 2008). This detected effect confounds reaching of an unambiguous conclusion on galectin functionality from such models, making immunohistochemical monitoring of clinical specimens indispensable.

Because lectin activity is governed by the protein's binding to ligands (glycoconjugates or peptide motifs), it is tempting to add a methodological aspect, which complements this research line. Explicitly, galectins can not only be detected immunohistochemically, but also be employed as histochemical tools. This study design with a labelled tissue lectin, performed recently in the case of galectin-3 and skin, has already also provided clinical correlations by the analysis of fixed sections of tumours from head and neck cancer patients (Delorge et al., 2000; Plzák et al., 2002, 2004; Chovanec et al., 2005; Szabo et al., 2009). The results presented here, together with this perspective, underscore the potential of comprehensive mapping of lectin-related parameters in the quest to define new molecular aspects relevant for the course of the disease.

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