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Galectin-7: will the lectin's activity establish clinical correlations in head and neck squamous cell and basal cell carcinomas?

Z. Čada[1,2], M. Chovanec[1,2,3], K. Smetana Jr[1,3], J. Betka[2], L. Lacina[1,4], J. Plzák[1,2,3], R. Kodet[5], J. Štork[4], M. Lensch[6], H. Kaltner[6], S. André[6] and H-J. Gabius[6]

[1]Charles University in Prague, First Faculty of Medicine, Institute of Anatomy, [2]Charles University in Prague, First Faculty of Medicine and Faculty Hospital Motol, Department of Otorhinolaryngology and Head and Neck Surgery, [3]Center for Cell Therapy and Tissue Repair, Faculty Hospital Motol, [4]Charles University in Prague, First Faculty of Medicine and General Teaching Hospital, Department of Dermatovenerology, [5]Charles University in Prague, Second Faculty of Medicine and Faculty Hospital Motol, Institute of Pathology and Molecular Medicine, Prague, Czech Republic, [6]Ludwig-Maxmillians-University in Munich, Faculty of Veterinary Medicine, Institute for Physiological Chemistry, Munich, Germany

Summary. The human lectin galectin-7 (Gal-7; p53-induced gene-1) has anti- and pro-malignant features in different *in vitro* models. We tried to clarify relation of its expression to cellular and clinical parameters in head and neck squamous and basal cell carcinomas. Using a non-cross-reactive antibody, immunohistochemical staining in squamous cell epithelia (epidermis, epithelium of oropharynx and larynx) (n = 57), squamous cell carcinomas (n = 47) and lymph node metastases (n = 25), as well as basal cell carcinomas (n = 10) were studied. This monitoring was flanked by processing to assess the level of differentiation (cytokeratins 10 and 14), proliferation (Ki67) and basal lamina formation (collagen IV). The results were correlated with clinical and pathological findings (grading, TNM-staging, extracapsular spread, angio- and lymphangioinvasion, perineural invasion, recurrence and survival). Gal-7 resides in all layers of epithelia with cytoplasmic and nuclear localization in normal specimens. Basal cell carcinomas were devoid of the Gal-7 respective signal. Squamous cell carcinomas were positive, presenting different staining profiles. Intense staining was predominantly found in squamous cell cancers with high degrees of differentiation and keratinization. Fittingly, poor level of differentiation (P = 0.0009), absence of keratinization (P = 0.0105) and significant discontinuity or absence of collagen IV expression in the peritumoral basal lamina (P = 0.0024) was found in Gal-7-negative tumors. Gal-7 presence was not related to gender, primary tumor site, T-stage, N-stage, clinical stage, extracapsular spread, angio- and lymphangioinvasion, perineural spread or treatment outcome at a statistically significant level. Immunohistochemical analysis revealed a positive correlation for differentiation and keratinization to Gal-7 presence in squamous cell carcinomas. Absence of Gal-7 expression was detected in basal cell carcinomas. These clinical data delineate Gal-7 influence on differentiation in vivo, without evidence for a role in dissemination reported for lymphoma.

Key words: Carcinoma, Collagen IV, Galectin, Keratinization, Lectin

Offprint requests to: Martin Chovanec, M.D., Ph.D., Charles University, First Faculty of Medicine, Department of Otorhinolaryngology and Head and Neck Surgery, V Úvalu 84, 150 06 Prague 5, Czech Republic. e-mail: martin.chovanec@lf1.cuni.cz

Introduction

The malignant process is known to be associated with aberrant glycosylation. Because the emerging concept of the sugar code ascribes a role as biochemical signals to glycan epitopes of glycoconjugates from normal and tumor cells, these changes may not serve just as phenotypic markers. They also convey new properties to the cells which can be decoded by tissue lectins (Gabius, 1997a, 2006). In fact, these glycan-binding proteins are capable of "reading" even rather subtle modifications in glycan structures, such as the presence of core fucosylation or alterations in epitope density, and translate them into responses, affecting e. g. cell adhesion, growth or migration (Villalobo et al., 2006; Wu et al., 2006; André et al., 2007a). Homing especially

in on spatially accessible branch-end ß-galactosides, the members of the galectin family belong to these endogenous effectors (Kasai and Hirabayashi, 1996; Gabius, 1997b; Cooper, 2002). The recent finding that a tumor suppressor modulates in an orchestrated manner the expression of a galectin and glycan tailoring of its ligands for acquisition of susceptibility to anoikis underscores the effectiveness of such interactions in tumor biology (André et al., 2007b). In addition to sensing changes in glycan profiles, these endogenous lectins are known to exert activities also in the cytoplasm and nucleus by virtue of peptide binding, for example, regulating transcriptional activity, transformation or apoptosis (Rotblat et al., 2004; Wang et al., 2004; Smetana et al., 2005). This background explains our interest in galectins and their presence in tumors.

The homodimeric galectin-7 (Gal-7) was initially detected in studies aimed at identifying markers associated with the normal keratinocyte phenotype, its expression was sensitive to SV40 transformation and linked to p53-related induction of apoptosis in epidermis and human DLD-1 colon carcinoma cells (Madsen et al., 1995; Magnaldo et al., 1995; Polyak et al., 1997; Bernerd et al., 1999; Saussez and Kiss, 2006). *In vitro*, the lectin inhibits growth of neuroblastoma cells and induces apoptosis in activated T cells, but, in stark contrast, is associated with an aggressive phenotype in murine 164T2 lymphoma, characterized by increased matrix metalloproteinase-9 expression (Kopitz et al., 2003; Moisan et al., 2003; Sturm et al., 2004; Demers et al., 2007). Proteomic profiling raised evidence for a relation to differentiation in bladder squamous cell carcinomas and, conversely, tumorigenesis in buccal squamous cell cancer (Østergaard et al., 1997; Chen et al., 2004). Differential display of mRNA populations to chemical carcinogenesis was described in rat mammary gland but not colon (Lu et al., 1997). Thus, Gal-7 activities and relations to disease progression in models appear to indicate contextual functionality, making predictions for clinical correlations on the basis of in vitro data difficult. In view of the same aim, the immunohistochemical analysis of the anti-apoptotic Gal-3 in breast cancer has recently revealed that in vitro activities cannot simply be extrapolated to the clinical situation (Moisa et al., 2007). The question is thus open to define associations between lectin expression and clinical parameters in tumor specimens. Toward this end, we analyzed head and neck squamous cell and basal cell carcinomas, using an antibody preparation non-cross-reactive to other members of the galectin family.

Materials and methods

Tissue processing

Samples of human tissue were obtained with the explicit informed consent of patients according to the Helsinki Declaration during surgical procedures for head and neck squamous cell carcinomas (Department of Otorhinolaryngology and Head and Neck Surgery, First Faculty of Medicine and Faculty Hospital Motol, Charles University in Prague) and basal cell carcinomas (Department of Dermatovenerology, First Faculty of Medicine and General Teaching Hospital, Charles University in Prague). Each sample was divided to two parts. First part was routinely embedded to paraffin and used for histopathologic inspection, second was prepared for preparation of frozen sections. These samples (Table 1) were frozen in liquid nitrogen using Tissue-Tek (Sakura-Finetek Europe B.V., Zoeterwoude, The Netherlands). 7-µm-thick frozen sections were prepared by a Cryocut-E microtome (Reichert-Jung, Vienna, Austria). Tissue-Tek was removed by rinsing in phosphate-buffered saline (pH 7.2; PBS) immediately before starting immunohistochemical processing. Sections were routinely fixed with 2% (w/v) paraformaldehyde in PBS. Carbohydrate-free bovine serum albumin (BSA; Sigma, Prague, Czech Republic) was used to block non-specific protein-protein interactions.

Sections from paraffin embedded tissue were routinely analyzed after the staining by hematoxylin and eosin. Parameters such as differentiation grading, extracapsular spread, angio- and lymphangioinvasion, perineural invasion were evaluated as described (Bryne et al., 1989; Ravasz et al., 1993).

Immunohistochemical processing

After recombinant production Gal-7 was purified using affinity chromatography as a crucial step, and purity was ascertained by one- and two-dimensional gel electrophoresis, gel filtration and mass spectrometry (Kopitz et al., 2003; André et al., 2004). The quality-controlled protein was used as antigen in rabbits, and the resulting polyclonal antibodies were thoroughly checked for any cross-reactivity against other members of the galectin family by Western blotting and ELISA, especially the proto-type proteins, then removing any traces by chromatographic affinity depletion (Kayser et al., 2003; Lohr et al., 2007). Double labeling using commercial antibodies was performed to characterize

Table 1. Number of tissue samples.

Tissue	Number of donors
Human epidermis	10
Basal cell carcinomas	10
Squamous cell epithelia (oral cavity, oropharynx, larynx, hypopharynx)	47
Primary squamous cell cancer (oral cavity, oropharynx, larynx, hypopharynx)	47
Lymph node metastases of squamous cell carcinomas (oral cavity, oropharynx, larynx, hypopharynx)	25

cell characteristics. Cytokeratins 10 and 14 were detected by mouse monoclonal antibodies (Dako, Brno, Czech Republic; SIGMA, Prague, Czech Republic), as were the nuclear Ki67 antigen of proliferating cells (Dako, Prague, Czech Republic) and collagen IV (Sigma, Prague, Czech Republic). Commercially available ExtrAvidin-tetramethylrhodamine isothiocyanate (TRITC) (Sigma, Prague, Czech Republic) and fluorescin isothiocyanate (FITC)-labeled swine-anti mouse and swine-anti rabit immunoglobulins (SwAM-FITC, SwAR-FITC, ALSEVA, Prague, Czech Republic; GoAM-TRITC: Sigma, Prague, Czech Republic) were used as second-step reagents. To exclude a false-positive reaction by non-specific binding of immunoglobulins via Fc receptors, an antibody specific for CD1a (Immunotech, Prague, Czech Republic) not present on epithelial cells was tested in parallel. This reagent replaced the first-step markers during routine processing in a control section. Finally, the specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA, USA.) and then visually inspected and analyzed using an Eclipse 90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with respective filter blocks, a high-resolution CCD camera (Cool-1300Q; Vosskьller, Osnabrьck, Germany) and a computer-assisted image analyser (LUCIA 5.10) (Laboratory Imaging, Prague, Czech Republic). All sections were carefully examined by two independent observers, who were completely blinded with respect to clinical features of the patients. In each case, at least 500 cells within randomly selected and defined area sections on each slide were counted. For statistical analysis, cut-off points were chosen to classify tumors to be intensely or faintly positive for Gal-7 staining. A cut-off point of lower 1/3 of the intensity profile value (arbitrary units) between intensity of the background signal and the intensity profile value in corresponding non-malignant control epithelia within the tumor cell population was arbitrarily set to determine the range of faintly positive cells. Cut-offs were defined prior to relating clinical parameters to results of histochemical staining. Ki67-positive cells were counted per 1000 cells, an in this way percentages of Ki67-positive cells in different samples were determined. Mean average of Ki67-positive cells for tumors with identical Gal-7 cytoplasmatic staining profiles were calculated.

Statistical analysis

The Chi-squared test was used to set Gal-7 parameters in relation to the different clinicopathological parameters, except for the Ki67 status treated with the Mann-Whitney U test. Overall survival and disease-free survival were calculated using the standard method, data sets being analyzed by using the Gehan-generalised Wilcoxon test. Statistica 6.0 software (StatSoft, Prague, Czech Republic) was run in 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.

Results

Normal epithelia in situ

Application of the non-cross-reactive anti-Gal-7 antibody preparation to fixed sections of squamous cell epithelia of the epidermis and mucosal coverings (oral cavity, oropharynx, larynx and hypopharynx) detected lectin presence from the basal region to the most superficial layer (Fig. 1). Cytoplasmic and also nuclear presence, the latter most prominently in nucleoli, were seen in both basal and suprabasal layers. These observations extend the evidence for nuclear presence of galectins from proto-type Gal-1 and chimera-type Gal-3 (Smetana et al., 2005) and, most recently, Gal-2 (Dvořánková et al., 2008). As a measure of cell differentiation, cytokeratin-14 was present in cells of the basal layer in epithelia of all specimens, wheras cytokeratin-10 was encountered in keratinized epithelia of epidermis and tongue only. The typical nuclear expression of the proliferation marker Ki67 was observed in the basal layer and to a restricted extent in the surrounding suprabasal layers. A subpopulation of Ki67-positive cells represented about 5% of the cells in the basal layer. As assessed by monitoring collagen IV presence, the basal lamina was well established and continuous in the studied epithelia.

Basal cell carcinomas

As also shown in Fig. 1, a qualitative difference was seen for negative tumor cells compared to the positive surrounding non-transformed epithelium. Cytokeratin-10 was not detected in studied tumors, and the presence of nuclear Ki67 antigen was observed in about 5-15% of cells, predominantly in peripheral parts of tumor nodules. Continuous collagen IV staining appeared around the studied tumors (not shown).

Squamous cell carcinomas

The staining profile for Gal-7 was not uniform in the different specimens of primary and metastatic squamous cell carcinomas. Four different patterns could be discerned (Fig. 1). Intense staining with homogeneous distribution in all tumor cells (intense and homogeneous) was present in 32% of primary tumors and 12% of regional lymph node metastases. Gal-7 presence confined to the central parts of the tumor, mostly to regions of formation of keratin pearls (intense heterogeneous pattern), was observed in 25.5% of primary tumors and 32% of regional lymph node metastases. Faint but homogeneous staining throughout the entire tumor cell population applied to 25.5% of the primary tumors and 20% of the regional lymph node

metastases. 17% of primary tumors and 36% of regional lymph node metastases did not show immunohistochemical positivity. A clear-cut difference between primary tumors and corresponding lymph node metastases could not reliably be described. Nuclear positivity of tumor cells concerned only cases with intense staining, irrespective of presenting homogeneous or heterogeneous profiles. Regarding the markers for cytodifferentiation, cytokeratin-14 was spotted in all primary carcinomas and regional lymph node metastases, cytokeratin-10 in keratinized carcinomas only (Fig. 2). Gal-7 presence correlated with keratinization (P = 0.0105). Ki67-expressing cells were in the peripheral regions of tumor nodules. Studied tumors differed among each other in the size of the Ki67-positive cell population from 5 to 60% (Fig. 2, Table 2). There was no apparent correlation of the proliferation status with Gal-7 presence (P = 0.1376). In general, peritumoral basement membranes were covered with a continuous layer of collagen IV in the tumors with intense and homogeneous staining for Gal-7. The other Gal-7 staining profiles showed variability for the appearance of the collagen IV layer, ranging from major defects to even complete absence in tumors lacking Gal-7 (P 0.0024) (Fig. 2, Table 2). In contrast, intense and homogeneous Gal-7 staining correlated with the level of differentiation (grading) (P = 0.0009). When Gal-7 staining was set in relation with other factors, i.e. gender (P = 0.3781), primary tumor site (P = 0.2703), T-stage (P = 0.6222), N-stage (P = 0.1065), clinical stage (P = 0.5127), extracapsular spread (P = 0.5998), angioinvasion (P = 0.6443) and lymphangioinvasion (P = 0.3781), perineural spread (P = 0.1306) and treatment outcome, no statistically significant association turned

Table 2. Comparison of clinical and histopathological parameters with Gal-7 presence.

PRIMARY CARCINOMAS		INTENSE SIGNAL 27	FAINT/NO SIGNAL 20	P value
Site	larynx/hypopharynx	13	7	0.2703
	oropharynx/oral cavity	14	13	
Gender	male	24	16	0.3781
	female	3	4	
T-stage	T 1+2	8	9	0.6222
	T 3+4	19	11	
N-stage	N 0	5	5	0.1065
	N 1-3	22	15	
Clinical stage	CS 1+2	1	1	0.5127
	CS 3+4	26	19	
Grading	G1+G2	22	8	0.0009
	G3+G4	5	12	
Keratinization	keratinized	15	4	0.0105
	non-keratinized	12	16	
Extracapsular spread (ECS)	ECS -	21	17	0.5998
	ECS +	6	3	
Lymphangioinvasion	lymphangioinvasion -	22	18	0.3781
	lymphangioinvasion +	5	2	
Angioinvasion	angioinvasion -	22	16	0.6443
	angioinvasion +	5	4	
Perineural spread	perin. spr. -	26	18	0.1306
	perin. spr. +	1	2	
Outcome	local recidive	2	2	
	regional recidive	3	2	
	distant metastases	3	2	
	no evidence of disease	20	16	
		7	4	
Basal lamina (Col IV) formation	well surrounded	19	4	0.0024
	poorly formed	8	16	
Ki 67-positive population	mean	30%	38%	0.1376

Clinical and histopathological parameters (TNM staging, extracapsular spread, grading, keratinization, angio- and lymphangioinvasion, perineural spread), pattern of basal lamina (collagen IV) formation and proliferation (Ki-67) vs. Gal-7 presence in primary head and neck squamous cell carcinomas. Intense signal corresponds to tumors with either intense homogeneous or intense inhomogeneous Gal-7-dependent staining. Faint/No signal corresponds to tumors with either weak homogeneous staining or no detectable Gal-7. Mean average of Ki-67-positive cells counted per 1000 tumor cells for tumors with identical staining pattern were calculated.

up (Table 2). Also, no statistically significant differences of survival among the studied patient groups suffering from squamous cell carcinoma were observed relative to Gal-7 (Fig. 3).

Discussion

Using stratification, a prognostic correlation has been reported for stage IV hypopharyngeal squamous cell carcinoma patients. In this group, Gal-7 therefore has the potential to identify patients at risk of recurrence and with dismal prognosis (Saussez et al., 2006). Of note, staining characteristics for Gal-7 differed from those of Gal-1 belonging to the same subgroup, a strong argument for non-overlapping functionalities in the galectin network (Saussez et al., 2008). Also, associated with a feature of tumor progression, Gal-7 presence was correlated to muscle-infiltrating growth in urothelial cancer (Langbein et al., 2007), whereas Gal-7 monitoring in progression of thyroid cancer appeared to reflect a dual role, with anti- and promalignant features at different stages (Rorive et al., 2002). These results, revealing a tumor-type- and also stage-of-tumorigenesis-related activity profile in the case of Gal-7, have a bearing on considerations to devise new treatment modalities based on modulating endogenous galectin expression.

Absence of the signal for Gal-7 in basal cell carcinoma was also observed earlier (Magnaldo et al., 1998; Chovanec et al., 2005). Explanation of this phenomenon is only hypothetical but it can be related to the low level of differentiation of tumor epithelial cells. They express keratin 19, marker typical for epidermal stem cells and α2,6-linked sialic acid, marker of poorly differentiated epithelial cells (Holíková et al., 2002; Dvořánková et al., 2005). In harmony with these observations, cells of basal cell carcinoma were never recognized by labeled galectin-3, feature typical for suprabasal cells of squamous epithelia (Plzák et al., 2001).

Following its description as a marker associated with the normal keratinocyte phenotype and as p53-induced

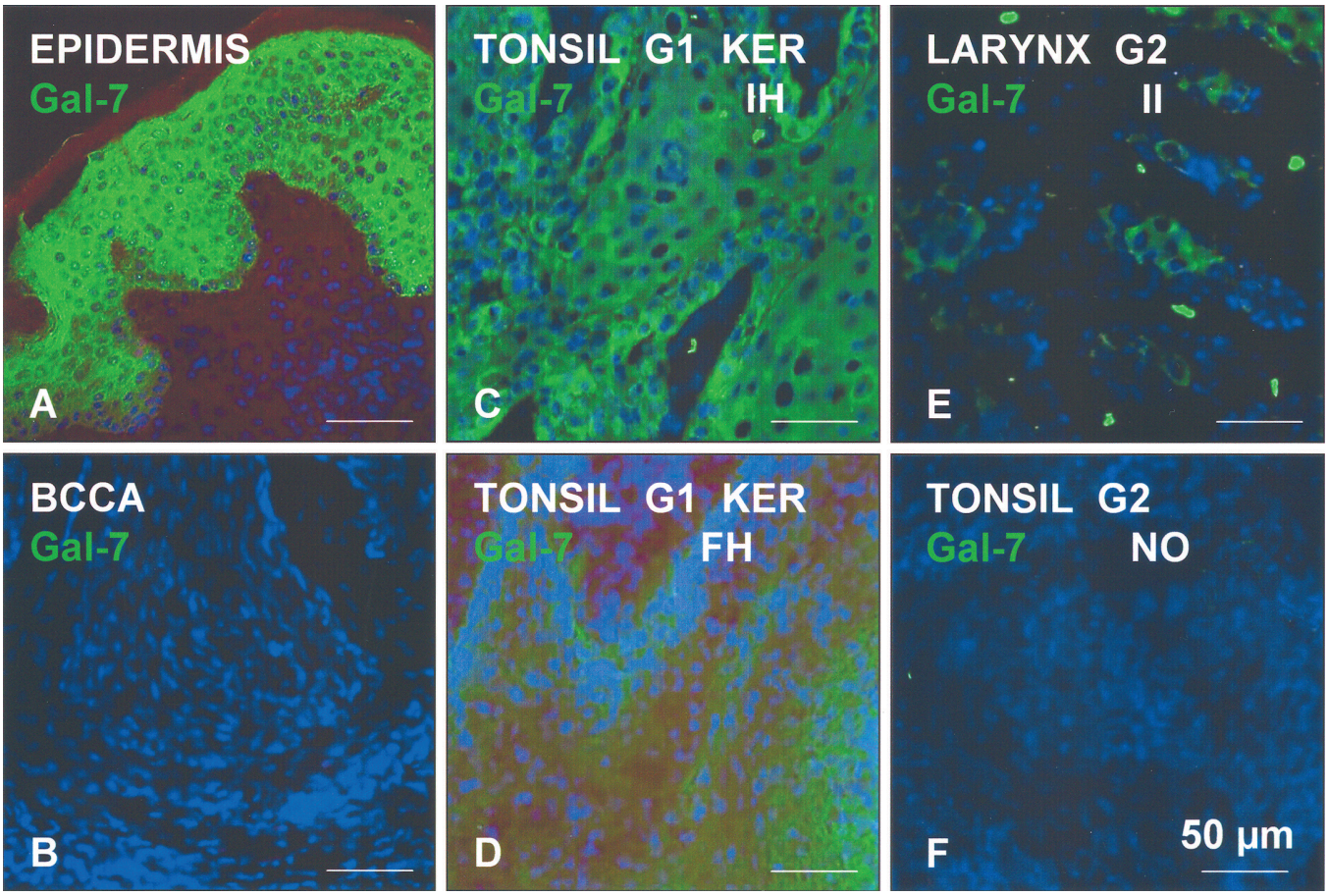

Fig. 1. Immunohistochemical detection of Gal-7 in epidermis **(A),** basal cell carcinoma **(B)** and head and neck squamous cell carcinoma (**C:** intense and homogeneous/IH, **D:** faint and homogeneous/FH, **E:** intense and inhomogeneous/II, **F:** no expression/NO). Grading (G1-G3), keratinized (KER). Nuclei were counterstained by DAPI.

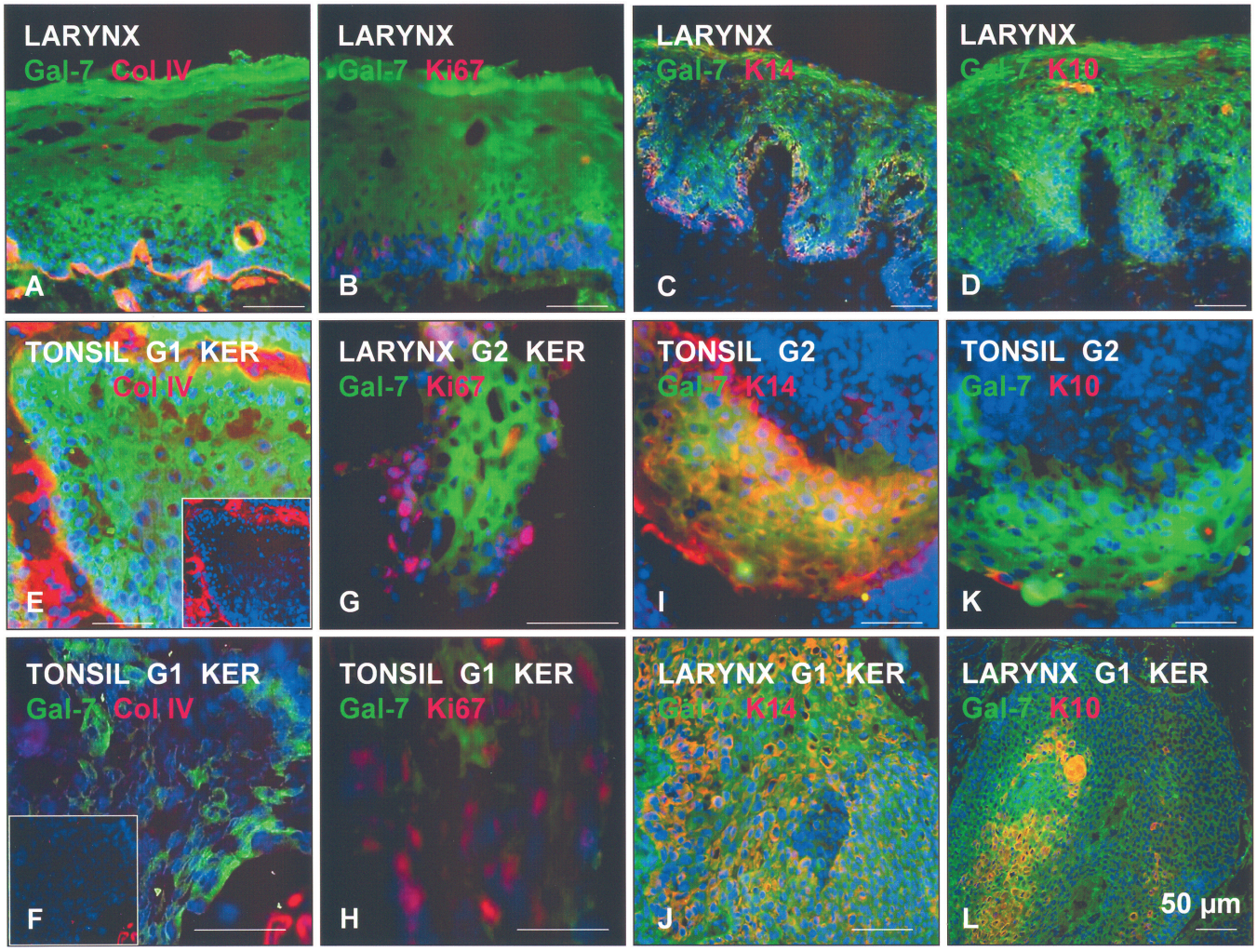

Fig. 2. Immunohistochemcial detection of Gal-7, a marker of proliferation (Ki67), collagen IV (basement membrane) and keratins (K10, K14) in squamous cell epithelia **(A-D)** and head and neck squamous cell carcinomas **(E- L)**. Grading (G1-G3), keratinized (KER). Nuclei were counterstained by DAPI.

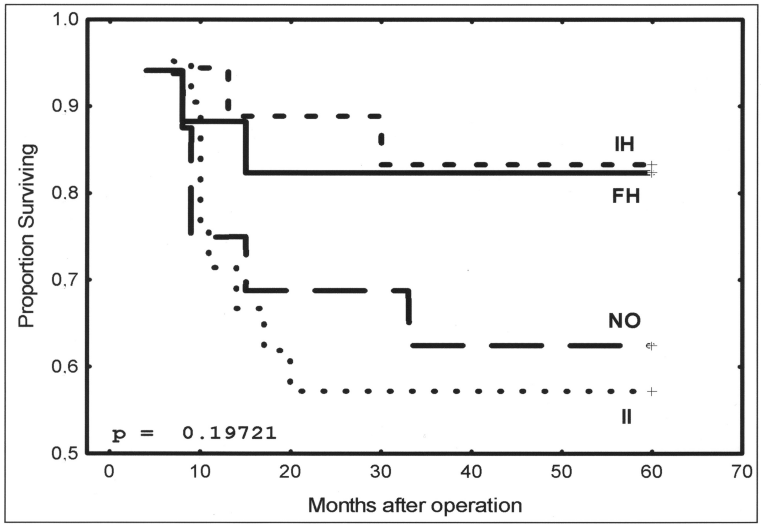

Fig. 3. Kaplan-Meier graph of overall survival of patients suffering from head and neck squamous carcinoma and Gal-7-associated parameters. Phenotype of Gal-7 localization: IH: intense and homogeneous, FH: faint and homogeneous, II: intense and inhomogeneous, NO: no expression.

gene product in DLD-1 colon cancer cells, cell biological data had indicated differential regulation of Gal-7 in squamous cell carcinomas of different origin, a differential response to chemical carcinogenesis in rat models, and anti- or pro-malignancy activities in different human tumor models. To decide on clinical correlations in patient material we studied tissue sections immunohistochemically and disclosed a correlation to increased status of differentiation and keratinization in head and neck squamous cell carcinomas.

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