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Galectin fingerprinting in Warthin's tumors: lectin-based approach to trace its origin?

Sven Saussez[1], Laurence de Leval[2], Christine Decaestecker[3], Nicolas Sirtaine[4], Stéphanie Cludts[1], Anaelle Duray[1], Dominique Chevalier[5], Sabine André[7], Hans-Joachim Gabius[7], Myriam Remmelink[8] and Xavier Leroy[6]

[1]Laboratory of Anatomy, Faculty of Medicine and Pharmacy, University of Mons-Hainaut, Mons, Belgium, [2]Department of Pathology, CHU Sart Tilman, University of Liège, Liège, Belgium, [3]Laboratory of Toxicology, Institute of Pharmacy, Université Libre de Bruxelles, Brussels, Belgium, [4]Department of Pathology, Institut Bordet, Université Libre de Bruxelles, Brussels, Belgium, Departments of [5]Oto-Rhino-Laryngology and [6]Pathology, Faculty of Medicine, Hôpital Claude Huriez and Centre de Biologie-Pathologie – CHRU, Lille, France, [7]Institute of Physiological Chemistry, Faculty of Veterinary Medicine, Ludwig-Maximilians-University, Munich, Germany and [8]Department of Pathology, Université Libre de Bruxelles, Hôpital Erasme, Brussels, Belgium

Summary. Warthin's tumor of the parotid gland is assumed to originate from the proliferation of epithelial inclusions within parotid lymph nodes. In that case, these cells are supposed to retain characteristics similar to common salivary gland ductal cells. Using immunohistochemical fingerprinting with four members of the family of adhesion/growth-regulatory galectins and comparison to intra- and interlobular ducts, marked similarities were noted for presence of galectins-3, -7 and -8. Notably, profiles of lectin binding, determined by applying human lectins as probes, were also similar when testing biotinylated galectins-3 and -8. Besides defining the galectin histochemical parameters in Warthin's tumors this study adds support to the hypothesis of heterotopia.

Key words: Adenoma, Galectin, Growth regulation, Lectin, Salivary gland, Warthin's tumor

Introduction

Warthin's tumors (WTs), also termed papillary cystadenoma lymphomatosum, are benign tumors occuring almost exclusively in the parotid gland, which represent about 4% to 11% of salivary gland tumors (Maiorano et al., 2002). The majority of such cases involves the lower pole of the parotid gland, with about 10% being located in the deep lobe (Seifert, 1996). WT is clinically multicentric in 12-20% of the patients and bilateral in 5-14% (Seifert, 1996; Simpson and Eveson, 2005). The typical histologic presentation is a well-circumscribed and partly cystic mass (Seifert, 1996). WTs are characterized by dual epithelial and lymphoid components. The epithelial part consists of an oncocytic luminal cell layer, composed of large columnar cells with palissading ovoid nuclei, and a basal cell layer made up of flattened or cuboidal cells (Seifert, 1996; Simpson and Eveson, 2005). The stroma encompasses reactive lymphoid tissue typically containing germinal centers (Seifert, 1996; Simpson and Eveson, 2005). The treatment is surgical excision, either superficial parotidectomy or enucleation, the recurrence rate with growth from a new focus is rather low, with about 2-5%, and malignant transformation is exceptional (Simpson and Eveson, 2005).

The etiology of WTs is not well understood and remains controversial, with focus on the question of whether they are true neoplasms or developmental malformations. Two main theories have been put forward to explain WT histogenesis. One is that of heterotopia, assuming a polyclonal origin by proliferation of salivary gland ductal cells entrapped within parotid lymph nodes already during embryogenesis (Chapnik, 1983; Arida et al., 2005; Teymoortash and Werner, 2005). Alternatively, WTs could stem from a monomorphic adenoma with concomitant or secondary lymphocytic infiltration. The lack of evidence for consistent clonal allelic losses had been interpreted to support the first concept (Arida et al., 2005). Evidently, monitoring the presence of key regulators of apoptosis/proliferation, invasion or migration can contribute data relevant to resolve this issue. Toward this end, we here study a family of adhesion/growth-regulatory endogenous lectins, i.e.

Offprint requests to: S. Saussez, M.D., Ph.D., Laboratory of Anatomy, Faculty of Medicine and Pharmacy, University of Mons-Hainaut, Pentagone 1B - Avenue du Champ de Mars, 6, B-7000 Mons, Belgium. e-mail: sven.saussez@umh.ac.be

galectins, which have attracted increasing attention owing to emerging insights into their broad activity profiles. The members of this family are potent cellular effectors by virtue of decoding distinct glycan signals and also by engaging in protein-protein/lipid interactions (Smetana et al., 2006). Galectin expression is subject to alterations upon malignant transformation and can either be connected to characteristics of the malignant phenotype or to the activity of tumor suppressors such as p53 or $p16^{INK4a}$ (Rorive et al., 2001; Nagy et al., 2002; Rappl et al., 2002; Kopitz et al., 2003; Saussez et al., 2006; André et al., 2007). When monitored as a group, the resulting fingerprint has been reported to reveal valuable information for differential diagnosis and prognosis, the data also documenting galectin-type-dependent differences in subcellular localization (Kayer et al., 2003; Nagy et al., 2003; Smetana et al., 2006; Langbein et al., 2007). So far, salivary gland neoplasms have only been studied to a limited extent and only with respect to galectins-1 and -3, indicating a relation to cell differentiation and histogenesis (Xu et al., 2000; Penner et al., 2002; Teymoortash et al., 2006; Ferrazzo et al., 2007). Based on our previous work on head and neck cancer we selected the proto-type galectins-1 and -7, the chimera-type galectin-3 and the tandem-repeat-type galectin-8 to establish the present immunohistochemical fingerprinting by using antibodies rigorously tested to exclude cross-reactivity (Danguy et al., 2001; Saussez et al., 2006a; Saussez et al., 2008a,b; Čada et al., 2009). Due to the individual fine-specificity of each galectin we added application of the labeled tissue lectins as sensors to the immunohistochemical work. The staining was semiquantitatively assessed in sections of WTs and, for comparison, of intra- and interlobular ducts of the parotid gland. These experiments answer the questions on presence and location of the four galectins and their accessible binding sites, as well as on relations between features of WTs and those of cells in tumor-free ducts.

Materials and methods

A total of 42 surgical specimen cases of WT were included in this retrospective study. In 29 specimens, intra- and interlobular ducts in adjacent tumor-free parotid tissue could also be evaluated. The patients underwent superficial parotidectomy with curative intent. Tumor specimens were obtained by retrospective compilation from the records of the Departments of Pathology of the Hôpital Claude Huriez (XL, Lille, France), the Hôpital Erasme (MR, Brussels, Belgium), the CHU Saint Pierre-Institut Bordet (NS, Brussels, Belgium) and the CHU Sart-Tilman (LD, Liège, Belgium). The Institutional Review Boards of these hospitals approved the study.

The H&E sections of the 42 tumors were reviewed by two pathologists to confirm the diagnosis and then processed immunohistochemically under identical conditions to semiquantitatively monitor parameters of presence of galectins-1, -3, -7 and -8 expression. In addition, labeled galectins were applied as probes to determine the patterns of accessible binding sites.

Human galectins obtained by recombinant production were rigorously controlled for purity and used as antigens for raising polyclonal antibodies (Kopitz et al., 2003; Saussez et al., 2006a; André et al., 2007). The resulting immunoglobulin G fractions were checked for cross-reactivity among this lectin family using human galectins-1, -2, -3, -4, -7, -8 and -9 in Western blot and ELISA assays, and chromatographic affinity depletion was performed in any case of positivity, followed by quality controls to ascertain elimination of cross-reactivity (Saussez et al., 2005; Langbein et al., 2007). Labeling by biotinylation was carried out under activity-preserving conditions, its extent measured by a proteomics protocol, and preservation of lectin activity was ascertained by solid-phase and cell assays (Purkrábková et al., 2003; André et al., 2006; Saussez et al., 2006b).

All tumour samples were fixed for 24 h in 10% buffered formaldehyde, dehydrated and routinely embedded in paraffin. Immuno- and galectin histochemistry were performed on 5 μm-thick sections mounted on silane-coated glass slides, as detailed previously (Saussez et al., 2005, 2006b, 2007). To exclude antigen/ligand-independent staining, the incubation step with primary/secondary antibodies or labeled galectin was omitted from the protocol in controls. In all cases, these controls were negative. The biotinylated secondary antibodies and ABC kit reagents came from DakoCytomation (Glostrup, Denmark).

For each microscopic field (15 fields for each WT), we focused our analysis on the epithelial and lymphoid components. The intensity of the immunostaining (Mean Intensity, MI) was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong) and the extent (Labeling Index, LI) was graded into categories of 0 (0% positive cells), 1 (1-25%), 2 (>25% and <75%) or 3 (>75%-100%). The quick score (QS) was calculated by multiplying the score of intensity of reactivity with the percentage of immunopositive cells. Assessment of staining profile was performed by two investigators blinded to the clinical details of the cases. Correlation between these ordinal variables was analyzed by means of the non-parametric Spearman correlation test. Expression data comparison between interlobular ducts and WTs was carried out by means of the non-parametric Wilcoxon Matched Pairs Test. The statistical analyses were carried out using Statistica software (Statsoft, Tulsa, USA).

Results

Results were recorded in the 42 cases of WT and also in adjacent tumor-free tissue (for the study of intra- and interlobular ducts) of 29 cases. All acinar cells were negative for the galectins-1, -3, -7, -8 .

Monitoring of galectin-1 expression in the adjacent

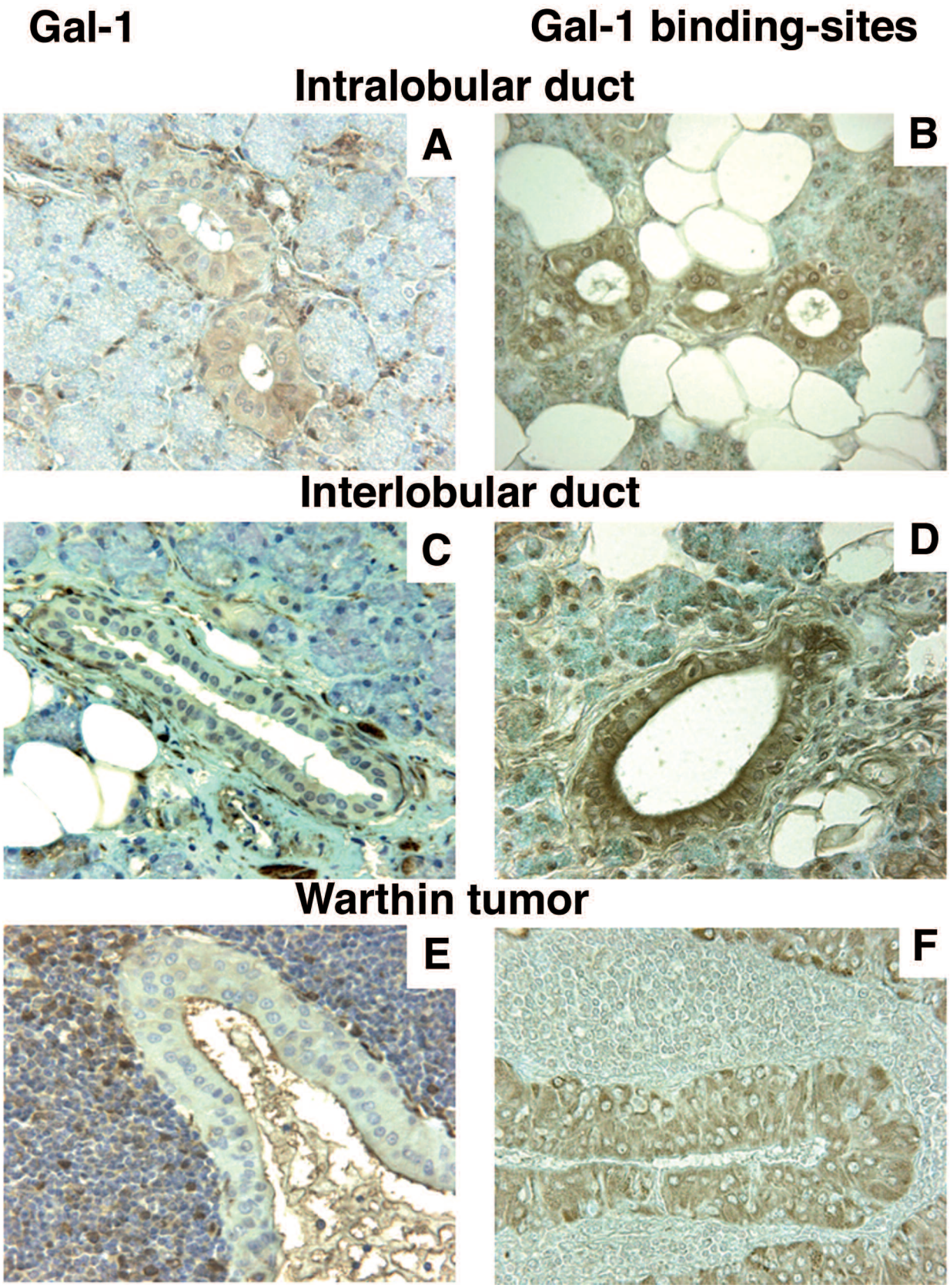

Fig. 1. Immunohistochemical staining profile for galectin-1 in intralobular ducts **(A),** interlobular ducts **(C)** and WT **(E)**. **Panels B, D, F** document profiles of reactivity for labeled galectin-1 in intralobular ducts **(B)**, interlobular ducts **(D)** and WT **(F)**. x 320

normal salivary gland tissues and in WTs revealed similar absence of galectin-1 expression in a small number of cases. In fact, we observed that 14% of intralobular ducts (0 QS in 4/29 cases; Fig. 1A) and 24% of interlobular ducts (0 QS in 7/29 cases; Fig. 1C) were galectin-1 negative as compared to general immunonegativity observed in 100% of WTs (0 QS in 42/42 cases; Fig. 1E). The strong immunopositivity of stromal lymphoid cells excluded false-negative results (mean QS=5; 42/42 cases). A clear increase of galectin-1 expression was observed when comparing interlobular ducts to WTs (Wilcoxon Matched Pairs Test: p=0.00004). Using labeled galectin-1 as a probe for evidencing its binding sites in WTs, as well as intra- and interlobular ducts, no correlation was observed in terms of LI, MI and QS (Fig. 1B,D,F) (Table 1). In the absence of galectin-1 immunopositivity in WTs, no correlation could be computed with duct features for this galectin.

Moving on to the location of galectin-3, positivity was more marked than for galectin-1. Galectin-3 expression based on QS was similar in the three groups (intra-, interlobular ducts and WTs). Of note, the intracellular patterns were different between WT and ducts in adjacent regions. In detail, intralobular ducts showed moderate to strong cytoplasmic staining intensity (mean QS=3.2; Fig. 2A). This distribution of signal was comparable to the uniform and intense labeling of all WTs, yet showing nuclear positivity (mean QS=2.8; Fig. 2C), and in most interlobular ducts (mean QS=3.2; Fig. 2E). Moderate signal intensity for galectin-3 was observed in the stromal compartment. Significant correlations were delineated for each of the three parameters (MI, LI and QS) characterizing galectin-3-immunopositive cell populations between WTs and intralobular ducts (MI: Spearman r=0.62, p=0.0005; LI: Spearman r=0.47, p=0.01; QS: Spearman r=0.56, p=0.002) and between WTs and interlobular ducts (MI: Spearman r=0.73, p=0.00001; LI: Spearman r=0.71, p=0.00002; QS: Spearman r=0.73, p=0.00001). Reactivity for labeled galectin-3 was seen in the cytoplasm of intra- and interlobular ducts and also in WTs (Fig. 2B,D,F) (Table 1). Again, data processing revealed significant correlations for some of the staining features between WTs and intralobular ducts (MI: Spearman r=0.49, p=0.006; LI: Spearman r=0.35, p>0.05; QS: Spearman r=0.39, p=0.04), and between WTs and interlobular ducts (MI: Spearman r=0.49, p=0.006; LI: Spearman r=0.35, p>0.05; QS: Spearman r=0.39, p=0.04) (Table 1).

The profiles of galectin-7 immunopositivity in WTs, intra- and interlobular ducts were remarkably similar (Fig. 3A,C,E). Both types of duct (intra- and interlobular) and WTs presented very strong cytoplasmic and nuclear immunostaining of the basal layer and moderate cytoplasmic expression in the luminal layer (Fig. 3A,C,E). Stromal lymphoid cells showed a moderate signal level. Significant correlations were apparent for each of the three galectin-7 staining features (MI, LI and QS) between WTs and intralobular ducts (MI: Spearman r=0.63, p=0.0004; LI: Spearman r=0.56, p=0.002; QS: Spearman r=0.66, p=0.0002) and between WTs and interlobular ducts (MI: Spearman r=0.68, p=0.0001; LI: Spearman r=0.67, p=0.0001; QS: Spearman r=0.73, p=0.00001) (Table 1). Binding of labeled galectin-7 was detected in the cytoplasm of intra- and interlobular ducts and in WTs (Fig. 3B,D,F) but no evidence for a statistically significant correlation was obtained between the WT data and those characterizing intra- and interlobular ducts (Table 1).

Galectin-8 was distributed in two profiles in the tissue. Moderate cytoplasmic and weak nuclear galectin-8 immunopositivity characterized the intralobular ducts (Fig. 4A). Both layers of interlobular ducts and of WTs presented strong cytoplasmic immunostaining (Fig. 4C,E). Significant correlations were delineated for some of the features characterizing galectin-8-immunopositive cells between WTs and intralobular ducts (MI: Spearman r=0.48, p=0.008; LI: Spearman r=0.25, p > 0.05; QS: Spearman r=0.52, p=0.004) and between WTs and interlobular ducts (MI: Spearman r=0.53, p=0.003; LI: Spearman r=0.37, p=0.05; QS: Spearman r=0.54, p=0.003) (Table 1). Reactivity for galectin-8 in WTs were also significantly correlated to reactivity in intralobular ducts (MI: Spearman r=0.39, p=0.046; LI: Spearman r=0.52, p=0.006; QS: Spearman r=0.39, p=0.045) and to reactivity in interlobular ducts (MI: Spearman r=0.39, p=0.046; LI: Spearman r=0.52, p=0.006; QS: Spearman r=0.39, p=0.045) (Table 1).

Table 1. Correlations for the galectin-dependent parameters (i.e. MI, LI and QS) characterizing galectin expression as well as their binding sites (BS), between WTs and either intralobular ducts or interlobular ducts.

	WTs & intralobular ducts		WTs & interlobular ducts	
	R Spearman	p-level	R Spearman	p-level
Gal-3 MI	0.62	0.0005	0.73	0.00001
Gal-3 LI	0.47	0.01	0.71	0.00002
Gal-3 QS	0.56	0.002	0.72	0.00001
Gal-7 MI	0.63	0.0004	0.68	0.0001
Gal-7 LI	0.56	0.002	0.67	0.0001
Gal-7 QS	0.66	0.0002	0.73	0.00001
Gal-8 MI	0.48	0.008	0.53	0.003
Gal-8 LI	0.25	NS	0.37	0.05
Gal-8 QS	0.52	0.004	0.54	0.003
Gal-1 BS MI	0.18	NS	0.18	NS
Gal-1 BS LI	-0.19	NS	0.34	NS
Gal-1 BS QS	0.16	NS	0.16	NS
Gal-3 BS MI	0.49	0.006	0.49	0.006
Gal-3 BS LI	0.35	NS	0.35	NS
Gal-3 BS QS	0.39	0.04	0.39	0.04
Gal-7 BS MI	0.05	NS	0.05	NS
Gal-7 BS LI	-0.16	NS	-0.16	NS
Gal-7 BS QS	-0.03	NS	-0.03	NS
Gal-8 BS MI	0.39	0.046	0.39	0.046
Gal-8 BS LI	0.52	0.006	0.52	0.006
Gal-8 BS QS	0.39	0.045	0.39	0.045

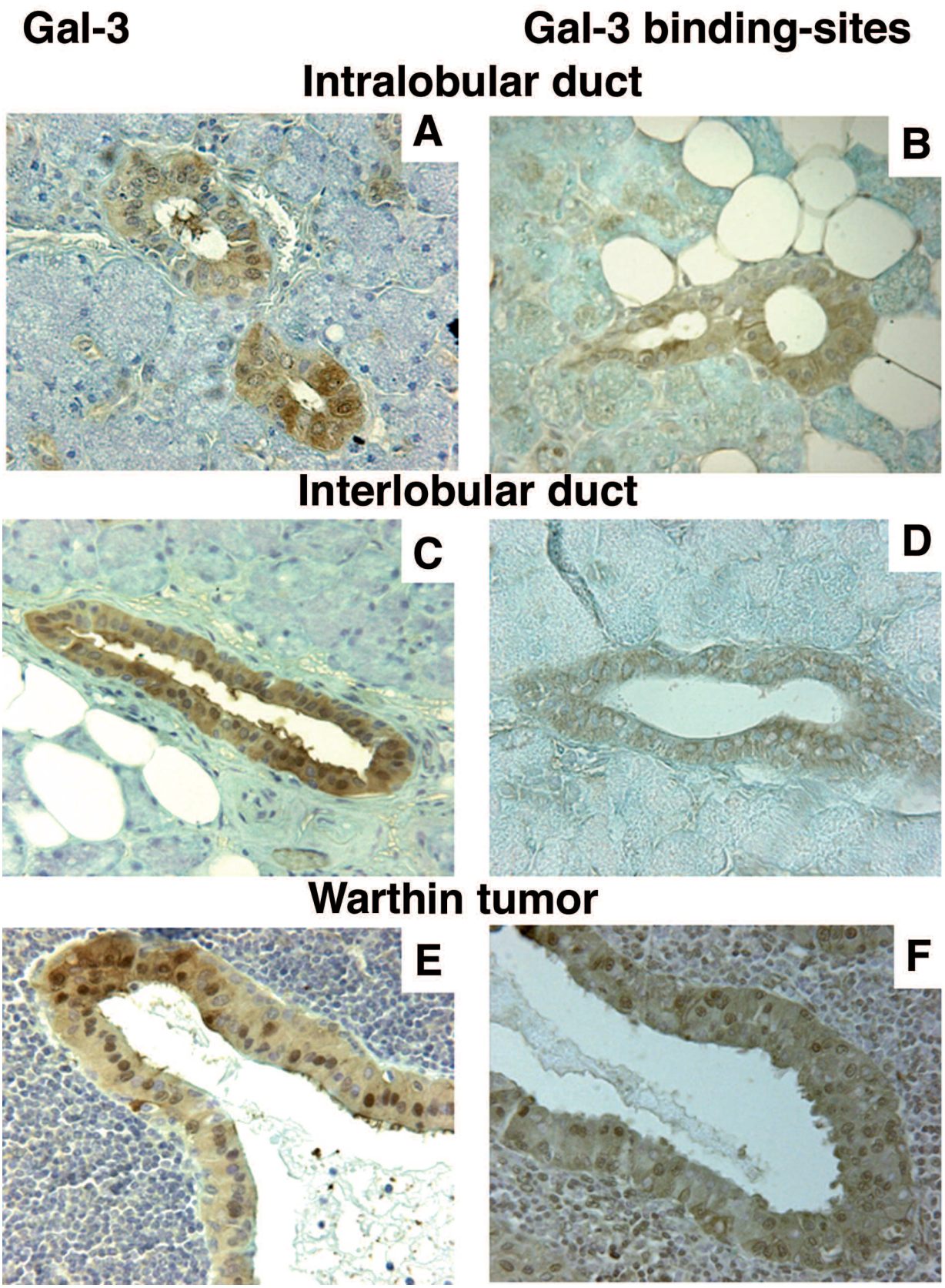

Fig. 2. Immunohistochemical staining profile for galectin-3 in intralobular ducts **(A)**, interlobular ducts **(C)** and WT **(E). Panels B, D, F** document profiles of reactivity for labeled galectin-3 in intralobular ducts **(B)**, interlobular ducts **(D)** and WT **(F)**. x 320

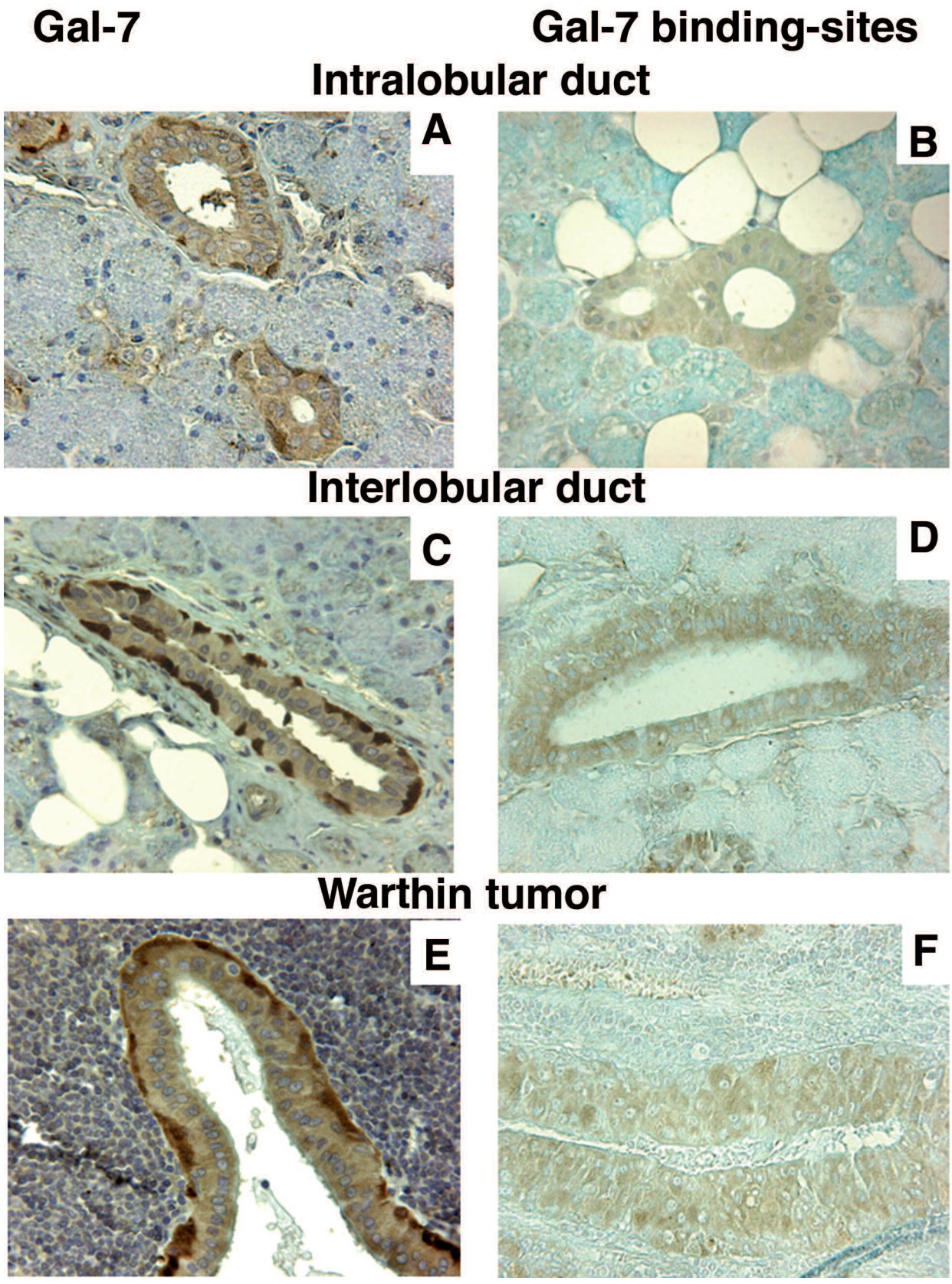

Fig. 3. Immunohistochemical staining profile for galectin-7 in intralobular ducts **(A)**, interlobular ducts **(C)** and WT **(E)**. **Panels B, D, F** document profiles of reactivity for labeled galectin-7 in intralobular ducts **(B)**, interlobular ducts **(D)** and WT **(F)**. x 320

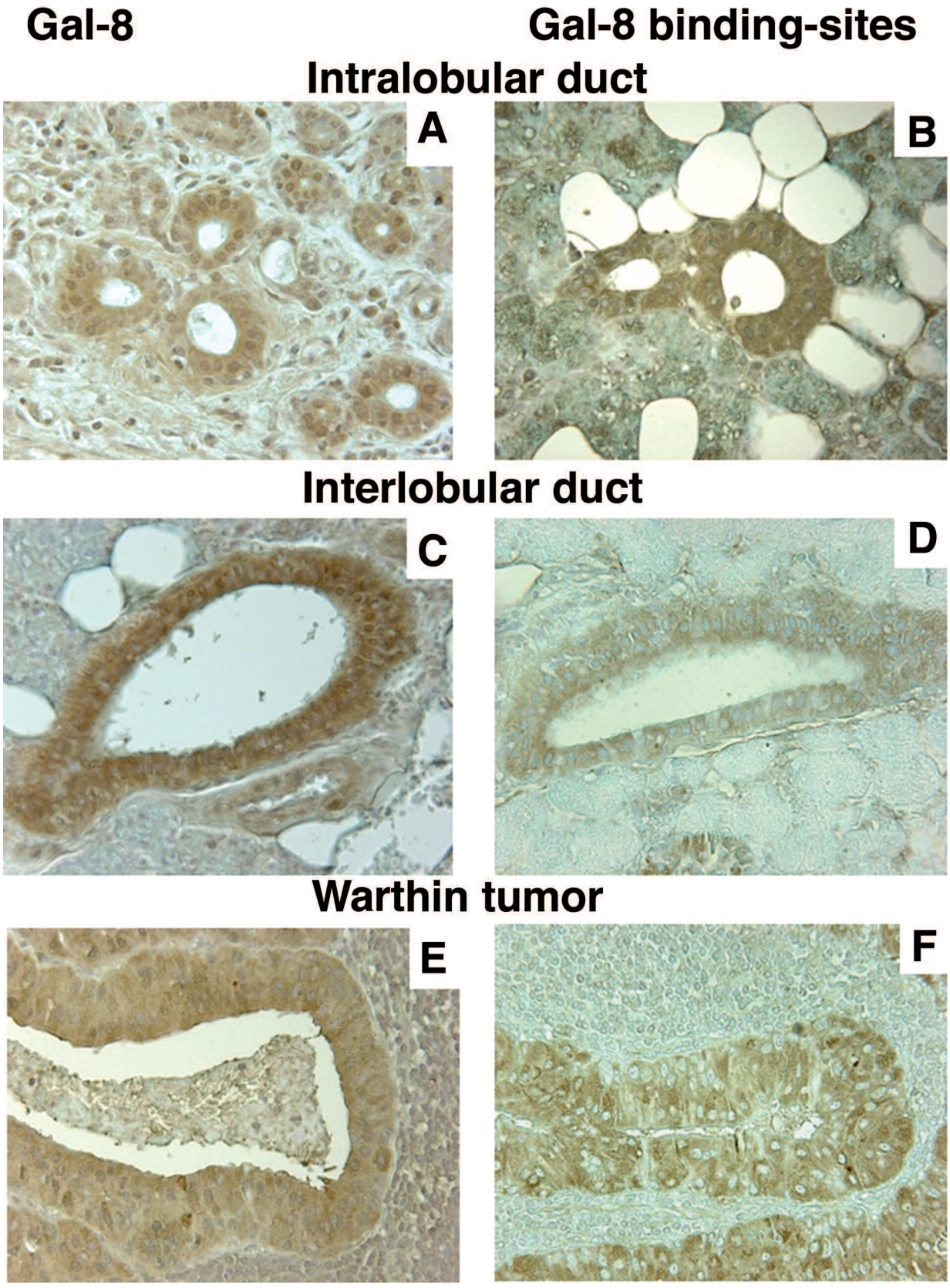

Fig. 4. Immunohistochemical staining profile for galectin-8 in intralobular ducts **(A)**, interlobular ducts **(C)** and WT **(E). Panels B, D, F** document profiles of reactivity for labeled galectin-8 in intralobular ducts **(B)**, interlobular ducts **(D)** and WT **(F)**. x 320.

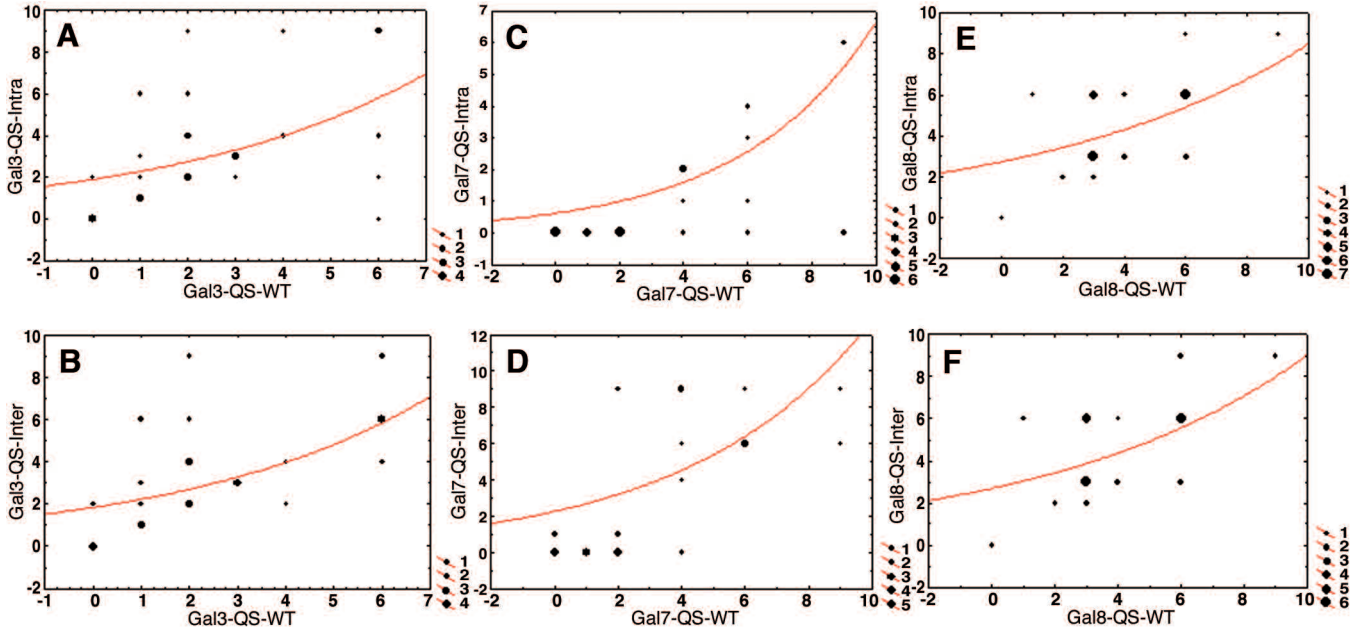

Fig. 5. Graphic representation of the correlation between the galectin-3 QS features evaluated in WTs and intralobular ducts **(A)** or interlobular ducts **(B)**. Identical graphical set-up is given for immunohistochemical galectin-7 **(C, D)** and galectin-8 **(E, F)** data. Curves are only shown as tendency indicators (i.e. positive non-parametric correlations, see Table 1).

Discussion

The cellular signature of glycans has over decades served as a phenomenological marker in histopathology, recently acquiring a functional dimension in the framework of the sugar code (Gabius, 2008, 2009). Since questions on the cellular origin of tumors and tumor-like lesions are commonly addressed by monitoring epitopes with characteristics of a marker, the presence of tissue lectins and the monitoring of their binding profile establish a new modality for this purpose. Thus, our study on WTs not only defines the pattern of presence for four adhesion/growth-regulatory galectins,it also includes data on the reactivity to these effectors, a property indicative of level of differentiation and providing prognostic information in head and neck carcinomas (Delorge et al., 2000; Plzák et al., 2004; Smetana et al., 2006). Of note, the tested family members are known to harbor functional divergence with differences also in subcellular expression patterns (Nagy et al., 2002, 2003; André et al., 2005; Saussez et al., 2008a,b). Moreover, we could also test the hypothesis of maintained similarities between WTs and ductal cells, which may reflect the assumed origin of WTs by ductal inclusions to a lymph node. Admittedly, microenvironmental factors, here from the lymphoid stroma, may well be operative as modulators of gene expression, e. g. known to be exerted by stromal fibroblasts (Lacina et al., 2007). Looking at the statistical calculations to spot correlations (Table 2), frequent presence of similarities to intra- and interlobular ducts at a high level of significance was apparent. On the cellular level, immunonegativity for galectin-1 in ductal epithelial cells was shared, as was the profile of immunopositivity for galectin-7 with its very strong cytoplasmic and nuclear staining of the basal layer, becoming moderate with cytoplasmic localization in the luminal layer. However, the intracellular site of immunoreactivity can constitute differences noted for galectin-3, shifting from cytoplasmic or nuclear in WT in adjacent regions.

Our results strongly support the heterotopia hypothesis, assuming a polyclonal origin of WTs by proliferation of salivary gland ductal cells entrapped within parotid lymph nodes already during embryogenesis (Chapnik, 1983; Arida et al., 2005; Teymoortash and Werner, 2005). As internal control staining profiles reported herein were in line with previous reports, as far as relevant information is available. In the initial study dedicated to galectin presence in salivary gland tumors, ductal positivity for galectins-1 and -3 had been described in normal, benign and malignant salivary gland specimens (Xu et al., 2000). In benign tumors (adenomas), both galectins maintained ductal expression, as certain malignant tumors (polymorphous low-grade adenocarcinomas and carcinoma ex-pleomorphic adenomas) did (Xu et al., 2000). Galectin-3 was later confirmed to be present in normal salivary gland tissue including acinar cells, ductal cells and overlaying epithelium and documented

to be present in adenoid cystic carcinomas (Penner et al., 2002; Teymoortash et al., 2006; Ferrazzo et al., 2007). Beyond relevance for WT our results also encourage to perform galectin fingerprinting systematically on tumors of the salivary gland and test the reactivity to these endogenous effectors. Thus, this report gives further research a clear direction.

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